Advances in
PARASITOLOGY

VOLUME 25

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Editorial Board

Advances in
PARASITOLOGY

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VOLUME 25

1986

ACADEMIC PRESS

Harcourt Brace Jovanovich, Publishers

London Orlando San Diego New York Austin
Boston Montreal Sydney Tokyo

ACADEMIC PRESS INC. (LONDON) LTD
24/28 Oval Road
London NW1

United States Edition published by
ACADEMIC PRESS INC.
Orlando, Florida 32887

ISBN 0-12-031725-7
ISSN 0065-308X

Photoset in Great Britain by
Rowland Phototypesetting Ltd, Bury St Edmunds, Suffolk
and printed by St Edmundsbury Press Ltd
Bury St Edmunds, Suffolk

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PREFACE

This volume includes two papers on purely helminthological topics, by Dr. Kearn and Drs. Bryant and Flockhart, two on more general parasitological subjects, by Dr. Gibbs and Dr. Clark, and one, by Dr. Riley, on that very interesting but too little known group of animals, the pentastomid "worms". Thus we hope that the volume will contain material of interest to a wide range of parasitologists. Dr. Clark's paper on the role of "free" oxygen in pathogenesis complements that by Dr. Thorne and Dr. Blackwell, in volume 22, which dealt, among other things, with the role of oxygen radicals in the intracellular killing of parasites. In volume 24 we printed the first part of a two-part treatment of nematodes as agents of biological control, by Dr. J. J. Petersen; the second part, by Dr. R. Bedding, has been held over and will appear in volume 26.

Readers may note that, in this volume, *Advances in Parasitology* has adopted the common practice of Academic Press by using American spellings in articles originating from North America, and British spellings in other contributions; we point this out in the hope of avoiding unjust accusations of editorial inconsistency from our more astute readers. Apart from this trivial point, we have not made, and do not plan to make, any changes in our editorial policy of attempting to provide an interesting mixture of topics both new and, perhaps like the pentastomids, unjustifiably ignored in recent review publications. We are always pleased to receive suggestions concerning topics that should be included, though we prefer not to receive unsolicited typescripts.

1986

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Oxygen-derived Free Radicals in the Pathogenesis of Parasitic Disease

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I. Introduction

Invasion by parasitic organisms, including forms too small to come within the conventional ambit of parasitology, is a fact of life for most animals and plants, and may result in disease and tissue damage. When these changes do occur their origin and consequences may be self-evident, such as in the anaemia that follows the blood loss caused by hookworms or cattle ticks, but more usually the pathogenesis of parasitic disease is much harder to under-

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ISBN 0-12-031725-7

stand. Urquhart (1978), when opening a meeting on African trypanosomiasis, expressed this problem clearly. "We have been embarrassed for too long", he noted, "by our inability to explain how this parasite, often difficult to find, can be responsible for morbidity and mortality on such a scale."

The wider picture is even more complex. Some host species need many parasites to initiate illness and tissue damage and others very few. *Babesia microti* in mouse and man provides one example, *Plasmodium falciparum* in monkey and man another. The host, not the parasite, appears to determine this relationship. Yet relationships can change with experience: children in holoendemic malarial areas undergo a period of tolerance to the parasite, harbouring high densities without apparent harm, before complete immunity is acquired. Furthermore, tissues around parasites may be less damaged than others anatomically distant. The best approach to understanding many of these complexities appears to be the argument that often the host, through an enthusiastic but misdirected response intended to destroy the parasites, damages its own tissues.

Our main approach in this review will be to discuss phenomena that occur in both parasitic and other diseases, drawing special attention to lessons that the non-parasitological literature contains for those trying to understand how parasites cause tissue damage. Because of our background experience we will draw most heavily on haemoprotozoan-induced diseases for our examples. We will concentrate on assessing the possible role of the highly reactive reduction products of oxygen, generally termed free oxygen radicals, in these processes. The evidence that these forms of oxygen are generated by host cells and that they harm parasites is extensive. Over recent years there has been an increasing awareness that free radicals can be an important cause of cellular injury in many biological systems. Thus if triggered, but not adequately directed or controlled, they may contribute to the tissue damage observed so widely in parasitic and other diseases.

II. Possible Origins of Tissue Damage in Parasitic Diseases

A. DIRECT EFFECTS OF PARASITES

The last few decades have seen great advances in understanding parasite-induced host pathology in terms of a misdirected immune response, but these gains have obscured the fact that this is not the whole story: tissue damage can occur even though the host does not respond to the presence of the parasite. Some examples of these direct effects are given in this section.

1. *Release of toxins from parasites*

Since early this century there have been proposals that toxins released by the invading organism are the direct cause of tissue damage in parasitic diseases. This is not surprising: it seems the obvious choice, and has parallels in staphylococcal and clostridial infections, but difficulties arise in some diseases if the paradigm is followed too closely. Maegraith (1948) summarized this clearly for malaria. The concept that a direct toxin is very important in this disease has encountered newer obstacles, including the T cell dependence of much pathology in experimental infections (Wright, 1968; Roberts and Weidanz, 1978; van Zon *et al.*, 1978). A direct toxin of parasite origin has also been proposed for Chagas' disease, with release by *Trypanosoma cruzi* of a "chagastoxin" (Seneca and Peer, 1966)—likely to be a lipopolysaccharide (Ketteridge, 1978)—being invoked. Similarly, a pathogenic role has been suggested for a low-density lipoprotein generated from host tissue by one of the phospholipases from African trypanosomes (Tizard *et al.*, 1977, 1979). These approaches seem in abeyance at present. However, as discussed in Section V, these observations may warrant renewed examination in the light of work on the toxicity of lipid peroxidation products.

We know of no example of injury to host tissue caused by oxygen radicals secreted by protozoan or helminth parasites, although many of them apparently contain enzymes known to generate superoxide and hydrogen peroxide in mammalian tissues. This area has been well reviewed recently by Docampo and Moreno (1984). Their article contains a report that *Tritrichomonas foetus* can secrete hydrogen peroxide (Ninomiya and Suzuoki, 1952) and reviews much indirect evidence that the malaria parasite may exert oxidant stress on the erythrocyte it inhabits (though not, presumably, further afield). A demonstration that oxygen radicals of parasite origin *can* cause direct tissue damage comes from the literature of plant pathology: Forney *et al.* (1982) have presented evidence that the damage caused to lignin by *Phanerochaete chrysosporium*, one of the Basidiomycetes (white rot fungi), is mediated by hydroxyl radical derived from hydrogen peroxide generated by the fungus itself. Their work invites investigation of these principles in nocardiosis, histoplasmosis and blastomycosis, and other human fungal infections where the pathology includes tissue destruction.

There is evidence that *Trypanosoma brucei gambiense* exerts at least one harmful effect, not involving host cells or the immune response (and presumably not oxygen radicals), on its host. Seed (1980) has shown that this parasite metabolizes aromatic amino acids from host tissue. Not only does this deprive the host of essential nutrients, but it also produces a series of toxic compounds that can induce coma, inhibit gluconeogenesis and reduce mitochondrial activity. Evidence for any product from metazoan parasites,

oxygen metabolite or not, being directly harmful to mammalian host cells is limited. Van den Bossche *et al.* (1983) have demonstrated that material excreted by adult *Fasciola hepatica* is deleterious to hepatocyte mitochondria. There is no information yet of its nature. Another example comes from the work of Boros *et al.* (1983), who showed that *Schistosoma mansoni* eggs can directly stimulate fibroblasts to produce collagen.

2. *Mechanical effects of parasite-altered cells*

Histological descriptions of falciparum malaria typically note venules distended with erythrocytes. Usually more of these red cells are parasitized than are the erythrocytes in larger vessels, with mature stages predominating (Clark and Tomlinson, 1949). This led to the common assumption that erythrocytes containing mature *P. falciparum* are in some way more prone than normal red cells, or those containing merozoites, to adhere to the walls of blood vessels and obstruct the flow, thus causing cerebral malaria. The description by Trager *et al.* (1966) of knobs on the surface of red cells containing mature *P. falciparum*, and the demonstration *in vitro* of their attachment to human endothelial cells (Udeinya *et al.*, 1981), have given a physical basis to this belief. Recently two groups (Green *et al.*, 1985; Langreth and Peterson, 1985) have correlated the presence of these structures, which some cultured strains of *P. falciparum* do not induce, with the pathogenicity of different strains in *Aotus* sp. monkeys. This provides a strong argument that attachment of parasitized red cells to venule endothelial cells has a central role in the pathology of falciparum malaria.

If, however, the explanation of human cerebral malaria involved only adherence of knob-bearing parasitized red cells, we would expect cerebral malaria to develop routinely in every patient with falciparum infection once a certain parasitaemia had been reached. This is apparently not so, although conceivably some wild strains of *P. falciparum* induce more, or more adherent, knobs and it is these strains that cause cerebral symptoms. Furthermore, this approach has not yet answered the questions raised by the phenomenon of malarial tolerance. This is the observation, for which there is much evidence (Kitchen, 1949; McGregor *et al.*, 1956), that in hyperendemic areas children continually exposed to malaria are often apparently healthy despite high parasitaemias. It needs investigating: factors other than adherence to endothelial cells by knob-bearing red cells will need to be invoked, and understanding this puzzle could increase our comprehension of the disease as a whole. Antibody to the histidine-rich parasite protein in the knobs (Langreth and Reese, 1979) or the antibody described by David *et*

al. (1983) conceivably could prevent sequestration, but neither would explain lack of other aspects of illness (for example, fever) in tolerant individuals. Since fever depends on release of interleukin-1 (IL-1), we need to consider that these children are tolerant to at least one macrophage-mediated event, and so may be to others as well.

A similar picture is emerging to explain differences in pathogenicity to cattle of different strains of *Babesia bovis*. The disease caused by this parasite is strikingly similar to falciparum malaria, including the tendency for venules to clog with parasitized red cells and cause cerebral symptoms. In 1972 Wright reported that such parasitized cells were often covered with stellate projections that attached them to the capillary endothelium. This work was recently extended by Aikawa *et al.* (1985), who described "elliptical protrusions" on the surface of red cells containing this parasite. Protrusion density was associated with virulence, both being reduced by passage through splenectomized calves. It will be instructive to see if *B. bigemina*, which is less pathogenic and does not cause cerebral symptoms, induces these structures on red cells it has invaded. Cerebral malaria and babesiosis are discussed more thoroughly in Section VI.

B. OXYGEN RADICALS AND PROTEASES OF HOST ORIGIN

1. *Oxygen radicals*

The ubiquity and high reactivity of oxygen has forced a choice on all living organisms: either they must devote much of their chemistry to systems for dealing with its toxicity or they must find some oxygen-free niche, such as the bile duct or intestinal tract, where they can avoid it altogether. Aerobes would have no special problem if all the oxygen they metabolize went through a single tetravalent reduction step in the cytochrome oxidase system to form water, as was formerly supposed. Their difficulties arise because a small proportion of their oxygen acquires these four electrons sequentially, forming several very toxic intermediates in the process. Several of these (see below) are free radicals, which are atoms or molecules with one or more unpaired electrons, represented by a dot in their formula, in their outer shell. This gives them a virtually indiscriminate reactivity with biological molecules, since their highest priority is to acquire an extra electron in their outer shell, and they ignore the normal chemical conventions followed by most molecules—those with paired outer electrons—in order to achieve this. One consequence is that they behave as strong "hydrogen magnets", removing an electron, typically in the form of a hydrogen atom, and thus altering the structure and therefore function of adjacent molecules. These

altered molecules, being one electron short, are themselves now free radicals and are destructive in their turn. This process continues, affecting lipid, protein and carbohydrate molecules, until a free radical scavenger (a molecule that after reacting with a radical shares the energy of its remaining single outer electron between several sites, so that it has "resonance stability" and is not reactive) is encountered, breaking the chain reaction. Most biologically generated free radicals stem from oxygen, but many other sources, typically formed during metabolism of pharmacological agents, occur (reviewed by Mason and Chignell (1982)).

Aerobes have developed a complex series of defences against these radical forms of oxygen, and remain healthy only for as long as these ramparts are intact. Thus the combined antioxidant activity of the glutathione system, superoxide dismutase (SOD), catalase, and the chain-breaking radical scavengers (mainly vitamin E) are adequate, in a normal individual, to cope with the oxidant stress imposed by superoxide ($O_2^{\cdot-}$) and the hydroxyl radical ($\cdot OH$, which is particularly reactive) generated by the univalent reduction of oxygen (Fig. 1). It appears that hydrogen peroxide (H_2O_2) is most toxic in the presence of a catalytic transition metal (in practice usually iron) or myeloperoxidase and a halide. These concepts have been reviewed extensively in recent years (e.g. Fridovich, 1978; Dormandy, 1983; Halliwell and Gutteridge, 1984a), and their role in biology and medicine is the subject of a new monograph (Halliwell and Gutteridge, 1985).

As well as being an inevitable consequence of aerobic metabolism, oxygen radicals are generated inside leucocytes, enabling them to kill phagocytosed microorganisms (Babior *et al.*, 1973). Unfortunately for the host, these leucocytes can also secrete $O_2^{\cdot-}$, along with other mediators, from their outer membrane into the surroundings (Nathan and Root, 1977). This indiscriminate and self-inflicted process contributes to the tissue damage of inflammation (see Fantone and Ward, 1982; Weiss and LoBuglio, 1982; Freeman and Crapo, 1982), and clearly has the capacity to cause tissue damage in parasite-induced disease. As summarized by Johnson and Ward (1982), these events occur much more vigorously when certain receptors on the surface of leucocytes, such as those responsive to antigen–antibody complexes (Fc receptors) and to complement components, are activated. Thus endothelial damage in the rat, initiated by either immune complexes or C5 activation, can be prevented by depleting animals of neutrophils (Till *et al.*, 1982), infusing SOD or catalase (Johnson and Ward, 1981; McCormick *et al.*, 1981), radical scavengers (Ward *et al.*, 1983a; Fligiel *et al.*, 1984a; Fox, 1984) and iron chelators (Ward *et al.*, 1983a; Fligiel *et al.*, 1984a). Macrophages are also implicated (Ward *et al.*, 1983b). These papers bring to mind the earlier work of H. W. Cox (Soni and Cox, 1975) and Neva

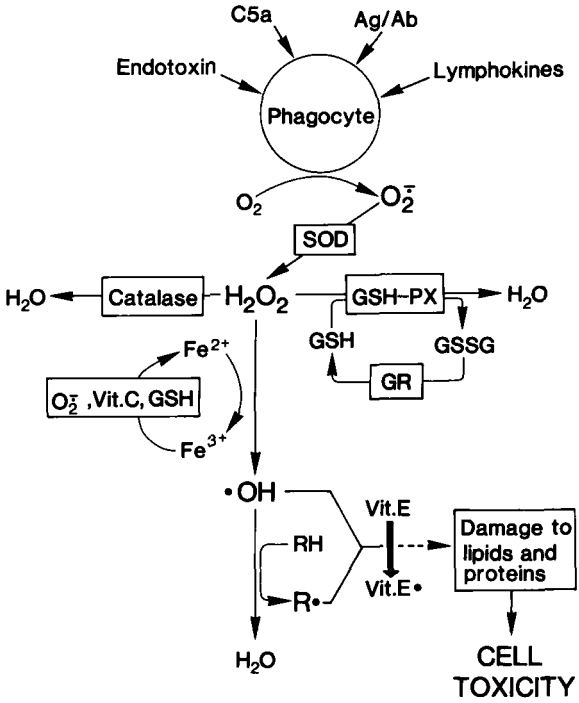

FIG. 1. Formation and detoxification of reactive oxygen species in biological systems. O_2^-, superoxide; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH-PX, glutathione peroxidase; GR, glutathione reductase; ·OH, hydroxyl radical; R·, secondary radical.

et al. (1974), who suggested a role for immune complexes and complement activation respectively in the pathogenesis of malaria.

Lymphokines, particularly γ-interferon released from antigen-stimulated memory T cells, also induce phagocytes to release O_2^- (Nathan *et al.*, 1983). The results of Ockenhouse *et al.* (1984), who found that both catalase and antibody to γ-interferon would inhibit non-phagocytic monocyte-induced killing of *P. falciparum*, are in keeping with both this concept and the proposal that free radicals of macrophage origin could damage intra-erythrocytic malaria parasites during the immune response (Clark and Hunt, 1983). Recent experiments *in vivo* (McCabe *et al.*, 1984) argue that γ-interferon is the lymphokine that induces killing of intra-macrophage protozoan parasites (Noguiera and Cohn, 1978; Murray *et al.*, 1982). As discussed in Section III, there is much evidence that free radicals are important in this process.

In addition to the oxygen radicals themselves, certain products of radical-induced lipid peroxidation, including a series of aldehydes, may be toxic to invading organisms (Gutteridge *et al.*, 1974) and host cells (Morel *et al.*, 1983). Being much more stable than free radicals, these toxic compounds can cause injury some distance from the site of radical generation. This is discussed further in Section V.

It is appropriate to recall here that, as well as being destructive at higher concentrations, lipid peroxides also appear to have an essential enzyme-activating role in the arachidonate metabolizing pathways (Hemler and Lands, 1980; Lands and Hanel, 1982; reviewed by Cleland, 1984). Through this indirect pathway these peroxides gain much potential, in very small concentrations, to influence systemic events in parasitic infections through the multifarious inflammatory and regulatory actions of prostanoids.

2. *Proteases*

Selective release of lysosomal proteases from leucocytes can be induced by triggers such as immune complexes (Cardella *et al.*, 1974), and there is much literature on the possible importance of these enzymes in the tissue damage seen in inflammation (reviewed by Davies and Bonney (1980)). It became evident that they were less important in some *in vivo* systems than had been previously supposed when reports emerged that vascular damage after immune complex deposition was as high as ever in mouse strains genetically deficient in leucocytic protease activity (Johnson *et al.*, 1979), and that antiproteases had little suppressive effect in neutrophil-induced lung injury (Fantone and Ward, 1982). We are concerned with proteases in this review not merely because they may be a somewhat superseded explanation of leucocyte-induced tissue damage, complementary to oxygen radicals, but because there is ample evidence that the two mechanisms can act synergistically.

3. *Synergy between oxygen radicals and proteases*

The net lytic activity of proteases on tissue depends on the balance between these enzymes and the antiproteases present in body fluids. The main antiprotease is α-1-proteinase inhibitor, and several workers (Cohen, 1979; Dooley and Pryor, 1982) have shown that its methionine residue makes it very vulnerable to inactivation by oxygen radicals. This probably explains why the activity of α-1-proteinase inhibitor in lung fluid from patients with adult respiratory distress syndrome (ARDS, a type of pulmonary oedema strongly associated with oxygen radicals; see Section V A) is reduced, allowing greater activity of proteolytic enzymes (Lee *et al.*, 1981; McGuire *et*

al., 1982). Another mechanism for synergy has been documented by Speer *et al.* (1984), who reported that human monocyte-derived macrophages could be primed for enhanced release of oxygen radicals by prior exposure to elastase or cathepsin G, two proteolytic enzymes released by neutrophils. There is also recent evidence that protein pretreated with H_2O_2 is more susceptible to destruction by purified trypsin, chymotrypsin, elastase or plasmin (Fligiel *et al.*, 1984b). In addition, neutrophil collagenase apparently needs activating, after its release, by a process involving reactive oxygen species (Weiss *et al.*, 1985). This may prove true for other enzymes as well.

III. Susceptibility of Parasites to Oxygen Toxicity

In a review that explores the relevance of oxygen radicals in the tissue injury of parasitic disease it is useful to keep in mind their most widely acknowledged role in this context—that of killing the parasites themselves. At present these radicals are thought to be the main mediator of immunity to intra-macrophage protozoa (Nathan *et al.*, 1979). A particularly telling experiment (Murray, 1982) showed that activated macrophages, depleted for a short time of the capacity to generate $O_2^{\cdot-}$ and H_2O_2, retained their viability and subsequent ability to generate $O_2^{\cdot-}$ and H_2O_2. Such cells were temporarily unable to kill three diverse protozoa, *Toxoplasma gondii*, *Leishmania donovani* and *Trypanosoma cruzi*. Recent reviews of this subject are by Nathan (1983) and Docampo and Moreno (1984).

The susceptibility of different parasites to these processes depends, as with host tissues, on their antioxidant defences, and can usually be rationalized in these terms. This also applies to non-protozoa: *Fasciola hepatica* lacks catalase and glutathione peroxidase (Barrett, 1980), and all developmental stages of *Trichinella spiralis* lack catalase, whereas adult worms and the dormant muscle stage contain about five times the SOD and glutathione peroxidase found in newly hatched larvae and are correspondingly more tolerant of H_2O_2 (Kazura and Meshnick, 1984). Blood forms of African trypanosomes cannot synthesize haem (Meshnick *et al.*, 1977; Editorial, 1978), and so lack haemoproteins such as catalase. Likewise, *T. cruzi* lacks catalase and glutathione peroxidase, but may possess a functional ascorbate peroxidase (Docampo *et al.*, 1976; Boveris *et al.*, 1980). This could confer the capacity to detoxify H_2O_2 through reaction with ascorbate, as happens in higher plants (Halliwell and Gutteridge, 1985). In addition, red cells parasitized by *P. berghei* (Fairfield *et al.*, 1983) and *P. vinckei* (Stocker *et al.*, 1985) contain less SOD than normal. Fairfield *et al.* (1983) also showed that *P. berghei* has negligible endogenous SOD, but adopts it from the red cell.

P. vinckei-infected red cells also contain much less catalase than normal (Picard-Maureau *et al.*, 1975; Stocker *et al.*, 1985). One can assume that these low levels of antioxidant enzymes contribute to the profound sensitivity of *P. vinckei* to H_2O_2 generators such as alloxan (Clark and Hunt, 1983) and divicine (Clark *et al.*, 1984a). It is noteworthy that *P. vinckei* is more sensitive to alloxan than are pancreatic islet β cells, its traditional experimental target. *In vivo* experiments with the iron chelator desferrioxamine (Clark and Hunt, 1983; Clark *et al.*, in preparation) and the radical scavenger butylated hydroxyanisole (Clark and Cowden, 1985; Cowden *et al.*, 1985) argue that ·OH is the likely mediator in each case.

As discussed in subsequent sections, we visualize that the host brings much of the pathology of malaria and babesiosis on itself when it tries to kill the parasites by free radical-initiated mechanisms, but does so too enthusiastically and without a close enough focus on the target. The host suffers accordingly.

IV. Implications for Oxygen Radicals of Experiments with Endotoxin

Attempts to understand the nature of endotoxicity, the complex array of pathology that occurs when animals receive injections of bacterial endotoxin (lipopolysaccharide; LPS), have a long and venerable history. It is a worthy subject to explore, not least because it still causes many deaths during sepsis in even the most medically advanced countries. We became interested in the topic in 1977, when we first realized that it seemed to be telling us something about our experiments with malaria. We still do not know what its message is, but increasingly think that it will prove to involve oxygen radicals.

Fifty years ago Taliaferro and Taliaferro (1934, 1944) reported degenerate intra-erythrocytic *Plasmodium brasilianum*, arguing that they resulted from the immune response, and called them "crisis forms" because they saw them in monkeys undergoing the crisis of malarial illness. Forty years later we rediscovered the phenomenon in rodents (Clark *et al.*, 1976a, 1977a) and found that it could be reproduced at low parasitaemias by pretreating animals with a range of macrophage activators: BCG (Clark *et al.*, 1976b), *Corynebacterium parvum* (Clark *et al.*, 1977b), *Brucella abortus* (Herod *et al.*, 1978), extract of *Coxiella burneti* (Clark, 1979a), and cord factor, chlorite oxidized oxyamylose, zymosan, glucan and *Salmonella typhimurium* (Clark, 1979b). These agents were selected because they had a history of protecting animals against tumours or intra-macrophage protozoa (see references given by Clark (1978)). They also proved to share the capacity to make mice very susceptible to injected endotoxin from *Escherichia coli*. As

part of this pattern we found that the parasites against which we could protect mice with this array of agents *themselves* made mice very sensitive to endotoxin, as Loose *et al.* (1971) had found in *P. berghei*-infected rats in another context. Further, we noted that the pathology routinely observed in mice dying of undisturbed *P. vinckei* infection was also seen in healthy, but infected, mice that died soon after receiving a small injection of endotoxin (Clark, 1978; Clark and Clouston, 1980). In mice of both groups one sees coagulopathy and hypoglycaemia, as well as identical liver and lymphoid damage. Other groups subsequently noted the phenomenon of increased sensitivity to endotoxin of rodents infected with *Schistosoma mansoni* (Ferluga *et al.*, 1979) and *T. lewisi* or *T. musculi* (Ferrante *et al.*, 1984).

Since injected BCG, *C. parvum* or glucan cause systemic macrophage activation, we proposed from these results that this phenomenon also occurred in malaria, and that macrophage-derived mediators, as in endotoxicity (Michalek *et al.*, 1980), might be important in the pathogenesis of haemoprotozoan diseases (Clark, 1978; Clark *et al.*, 1981). We also drew attention to the correlation between the susceptibility of host species to malaria or *Babesia* infection and to endotoxin (Clark, 1982). As Peavey *et al.* (1979) had found with BCG-infected mice, we then showed that the sensitivity of parasitized mice to endotoxin appeared to reside in their macrophages, as these cells were much more readily induced to release monokines than would normally be expected (Wood and Clark, 1984). Thus sensitivity to endotoxin, as seen in malaria and other systemic parasite infections, implies the potential for macrophage-driven tissue damage. Activated macrophages readily secrete $O_2^{\cdot-}$, particularly when they come from mice previously treated with BCG (Johnston *et al.*, 1978). It is therefore notable that we could induce, by injecting H_2O_2 (Clark and Hunt, 1983) or chemical generation of $O_2^{\cdot-}$ (Clark and Hunt, 1983; Clark *et al.*, 1984a), similar intra-erythrocytic killing of parasites to that seen in mice pretreated with BCG; in both groups of mice, we saw much more haemolysis than occurred in uninfected animals given the same agents.

The effects *in vivo* of endotoxin are multifactorial and interlocked, and are mediated through various cells (reviewed by Bradley (1979)). Much effort has gone into establishing where macrophages fit into the picture, and there are reports that endotoxin can instruct them to release enzymes (Wahl *et al.*, 1974), IL-1(Snell and Atkins, 1967), prostaglandins (Fischer *et al.*, 1977), leukotrienes (Lüderitz *et al.*, 1983), procoagulant activity (Niemetz, 1971) and tumour necrosis factor (Matthews, 1979). Most of these monokines have been proposed to contribute to endotoxicity. Endotoxin does not induce phagocytes to secrete $O_2^{\cdot-}$ directly, but makes them very sensitive to the action of stimulators of $O_2^{\cdot-}$ release (Pabst and Johnston, 1980; Pabst *et*

al., 1982). This has recently been given a biochemical basis by a report (Guthrie *et al.*, 1984) that NADPH oxidase, the enzyme that governs reduction of O_2 to O_2^- on the membrane surface of phagocytes, is more active when cells are exposed to endotoxin.

Consistent with this, *in vivo* evidence that endotoxin can set radical-mediated events in train is now accumulating. Experimental pulmonary oedema provides an example. In one well characterized model, damage to endothelial cells appears to be caused by oxygen radicals released from C_{5a}-triggered neutrophils. Cobra venom factor (Till *et al.*, 1982) or skin burn (Till *et al.*, 1983) can stimulate this process, and in both cases the antioxidant enzyme catalase lessens damage. Recently Bernard *et al.* (1984) showed that *N*-acetylcysteine, a recognized radical scavenger, significantly attenuated all of a series of pathophysiologic changes in the lungs of endotoxin-injected sheep. Likewise Flick and Hoeffel (1985) reported that the pulmonary oedema in this model (Demling *et al.*, 1981) can be prevented by catalase, implying an important role for H_2O_2 in this system also. In keeping with this, Wong *et al.* (1984) have documented increased lung lipid peroxidation in such sheep. These results correlate with the results of experiments in which SOD and catalase, given together, inhibited endotoxin-triggered, neutrophil-mediated damage to endothelial cells in culture (Yamada *et al.*, 1981). In addition, small doses of endotoxin protect rat lungs from oxygen toxicity, most likely by inducing the antioxidant enzymes SOD, glutathione peroxidase, and catalase (Frank *et al.*, 1978, 1980), and there is a report that vitamin E reduced mortality at 72h in rats infused with endotoxin (McKechnie *et al.*, 1985).

Other evidence linking endotoxin and radical species *in vivo* comes from experiments designed to understand endotoxin-induced disseminated intravascular coagulation (DIC). DIC is worse in rats deficient in vitamin E (Yoshikawa *et al.*, 1984) and is largely prevented by prior injections of this vitamin (Yoshikawa *et al.*, 1982). Furthermore, lipid peroxidation, an indicator of radical activity, is increased in serum, aortic wall and gut mucosa in animals infused with endotoxin (Yoshikawa *et al.*, 1983). This is consistent with an earlier report (Stamler, 1959) that the generalized Schwartzman reaction, an experimental model of DIC usually produced by two spaced injections of endotoxin and characteristic of eclampsia, can be induced in pregnant rats by feeding a diet deficient in vitamin E. Recent experience with the human disease agrees with these concepts: plasma concentrations of oxidation products (diene conjugates) induced by free radicals are significantly higher in human pregnancy complicated by pre-eclampsia (Wickens *et al.*, 1981), and the measurement of these conjugates has been found to be a useful predictor of the clinical course of the condition (Erskine *et al.*, 1985).

V. Endothelial Cell Damage in Malaria and Babesiosis

For convenience it is common practice to speak of oxygen radicals injuring endothelial cells, but, as discussed in Section II A 2, the intervening steps are complex, with the oxygen radical being a very transient species that merely sets a destructive chain reaction in motion. When lipids react with radicals they undergo a series of molecular rearrangements termed peroxidation and form a series of oxidation derivatives, including lipid peroxides, lipid hydroperoxides and aldehydes (see Esterbauer, 1982). Some of these products of lipid peroxidation are toxic to various cells, including endothelial cells (Sasaguri *et al.*, 1984; Peng *et al.*, 1985). Being much more stable than radicals, these compounds can mediate damage distant from where they were formed.

This concept has clarified much work in which serum lipoproteins have been argued to be toxic to endothelial cells (reviewed recently by Van Hinsbergh (1984)). Several groups have now shown that, provided care is taken to include radical scavengers or antioxidant enzymes during isolation of these lipoproteins to avoid peroxidation induced by free radicals, virtually all toxicity is abolished (Evensen *et al.*, 1983; Morel *et al.*, 1983). It seems possible, in the light of these studies, that some of the toxic effects attributed in the past to free fatty acids (references collected by Tizard *et al.* (1977)) could have been caused by products of lipid peroxidation that accumulated during handling *in vitro*. If this material indeed produced effects very much like the pathology of African trypanosomiasis, as these authors reported, this itself has important implications, in terms of secondary effects of free radical generation, for understanding this disease. Tizard *et al.* (1977, 1979) could produce similar effects with free fatty acids prepared from *T. congolense*. There are now reports that free radicals secreted by human monocytes, neutrophils or umbilical vein endothelial cells can render normal low-density lipoproteins cytotoxic by these processes (Chisholm *et al.*, 1983; Cathcart *et al.*, 1984). Oxidation of low-density lipoproteins by oxygen radicals secreted by endothelial cells apparently alters their lipid and protein components (Steinbrecher *et al.*, 1985). Evidently, therefore, this concept has to be taken into account when considering the cellular damage associated with inflammatory processes, including damage to endothelial cells mediated by neutrophils and monocytes. It implies that processes induced by free radicals cannot be dismissed simply because no phagocytes are nearby.

A. LUNGS

The combination of severe pulmonary oedema and haemorrhage that routinely accompanies terminal *Babesia bovis* infections in cattle (Dalgliesh *et al.*, 1976; Wright *et al.*, 1979) is a dramatic manifestation of endothelial damage in a parasitic disease. It can also occur in severe falciparum malaria, notably in patients who are not fluid overloaded (Deaton, 1970; Martell *et al.*, 1979; Duarte *et al.*, 1985). Since pulmonary wedge pressure is low (Fein *et al.*, 1978; Martell *et al.*, 1979; James, 1985), the fault is likely to be an increased capillary permeability rather than some obstruction further downstream, such as adherent parasitized red cells (Green *et al.*, 1985; Langreth and Peterson, 1985) or a failing left ventricle. Changes in serum colloidal osmotic pressure consistent with endothelial damage have been recorded (Fein *et al.*, 1978). Thus the pulmonary oedema sometimes seen in acute falciparum malaria qualifies as an example of what has, for 20 years, been referred to as adult respiratory distress syndrome or ARDS (Ashbaugh *et al.*, 1967). Less severe respiratory symptoms are common in malaria and presumably have the same pathogenesis.

ARDS, sometimes termed a post-traumatic, wet, or shock lung, is best defined as a noncardiogenic pulmonary oedema occurring in patients with previously normal lungs. Functional (Anderson *et al.*, 1979) and ultrastructural (Tomashefski *et al.*, 1983) studies agree that pulmonary endothelial cells are indeed injured in human ARDS. The condition is a common sequel to shock, burns, systemic sepsis or traumatic injury, and is fatal in about a half of the estimated 150 000 cases in the USA each year (quoted by Rinaldo and Rogers (1982)). Therefore, much research effort has been invested in establishing the cause of this endothelial damage (Lind and McDonald, 1981; Rinaldo and Rogers, 1982; Lloyd *et al.*, 1984; Editorial, 1984). We think that those interested in understanding the lung pathology of malaria and babesiosis can learn from this broader base of knowledge, which is summarized below, and will conclude from these parallels that oxygen radicals are probably implicated in pulmonary oedema induced by infection with haemoprotozoa.

As well as being induced by direct radical generators such as alloxan, ARDS can be reproduced experimentally with endotoxin, cobra venom factor (an activator of C_5), or immune complexes. Sheep have commonly been used in the endotoxin studies: it may be no accident that ruminants, which are very prone to endotoxin-induced pulmonary oedema, are also very prone to pulmonary oedema during haemoprotozoan infections. In 1979 Brigham *et al.* demonstrated an increased lung vascular permeability in sheep after infusion with *E. coli* endotoxin. They implicated neutrophils by showing that infusing endotoxin made these cells accumulate and marginate

in the pulmonary microcirculation (Meyrick and Brigham, 1983), and that their depletion prevented endotoxin-induced vascular permeability (Heflin and Brigham, 1981). Destruction of cultured endothelial cells *in vitro* by neutrophils has now been extensively studied, and shown to depend largely on toxic species of oxygen (Weiss *et al.*, 1981; Martin, 1984). Neutrophils isolated from patients with chronic granulomatous disease lack the ability to reduce O_2 to $O_2^{\cdot-}$, and are said to be unable to destroy endothelial cells in culture (Weiss *et al.*, 1981). A more recent report, however, describes a non-lytic, neutrophil-mediated mechanism of increased permeability of endothelial cells that functions with neutrophils from patients with chronic granulomatous disease as well as from normal individuals, and which is independent of oxygen radicals (Harlan *et al.*, 1985). This system has not yet been explored *in vivo*. Several groups have noted that the protein content of lung lymph of sheep injected with endotoxin approaches that of plasma, denoting damage to the endothelial cells (Demling *et al.*, 1981; Meyrick and Brigham, 1983). There are now two sources of evidence (Bernard *et al.*, 1984; Flick and Hoeffel, 1985) that endothelial injury in this model is mediated by oxygen radicals.

Exposure to activated complement components can induce neutrophils to secrete $O_2^{\cdot-}$ and kill endothelial cells (Sachs *et al.*, 1978). Elevated levels of C_{5a} seem particularly important, and have been closely correlated with ARDS in patients (Hammerschmidt *et al.*, 1980). One method of generating C_{5a} *in vivo* is to inject cobra venom factor, a C_{3b} analogue. As does endotoxin in the sheep lung, C_{5a} produces neutrophil-dependent endothelial cell injury that can be attributed to oxygen radicals, since it can be blocked by SOD or catalase (Till *et al.*, 1982). This group have taken their rat model further, arguing a role for $\cdot OH$ by showing that a radical scavenger and iron chelators also prevent injury *in vivo*, and that iron salts exacerbate it (Ward *et al.*, 1983a). The pulmonary oedema caused by skin burns, and also studied in this model, is another example of the same phenomenon (Till *et al.*, 1983, 1984). Diene conjugates, which are reliable indicators of lipid peroxidation, were monitored in this work (Till *et al.*, 1984), and mirrored the degree of pulmonary oedema. Likewise, complement activation in the sheep produces a pulmonary oedema that can be greatly attenuated by infusing SOD, with both lymph lung flow and protein clearance being decreased (Perkowski *et al.*, 1983).

Since immune complexes activate complement it is not surprising, in view of the above observations, that they also injure pulmonary endothelial cells *in vivo*, causing pulmonary oedema and haemorrhage (Scherzer and Ward, 1978). When IgG is involved, this damage is dependent on neutrophils or macrophages (Ward *et al.*, 1983b) and is mediated by oxygen radicals, since SOD will suppress damage (McCormick *et al.*, 1981). IgA complexes do not

need leucocytes to cause this same injury (Ward *et al.*, 1984), and this could be linked with the capacity of endothelial cells themselves to generate O_2^- (Johansson and Björkman, 1983; Rosen and Freeman, 1984). The only work so far published on the role of iron as a catalyst for H_2O_2 reduction in this system has been done on immune complex-induced endothelial cell damage in skin vessels (Fligiel *et al.*, 1984a). These authors reported that, as with injury to small pulmonary vessels induced by C_{5a} (Ward *et al.*, 1983a) or skin burn (Till *et al.*, 1984), iron chelators inhibited damage and Fe^{3+} salts potentiated it. These results incriminate ·OH or other species of activated oxygen. One possibility is the perferryl ion ($Fe^{2+}O_2$), which has been proposed as an important mediator (Aust and Svingen, 1982) to accommodate the observation that, in systems such as this, Fe^{3+} is more active than Fe^{2+} (Ward *et al.*, 1983a; Fligiel *et al.*, 1984a).

As noted earlier, pulmonary wedge pressure is not increased in patients infected with *P. falciparum* and showing respiratory distress (Fein *et al.*, 1978; Martell *et al.*, 1979), and recently (Duarte *et al.*, 1985) ultrastructural evidence of endothelial injury, as well as vessel occlusion with monocytes, has been documented. Endothelial damage has also been observed by electron microscopy in *B. bovis* pulmonary oedema (Wright *et al.*, 1979). Although we can find no record of wedge pressure studies in this disease, several groups (Rodgers, 1971; Dalgliesh *et al.*, 1976; Wright *et al.*, 1979) have drawn attention to small vessels in affected lungs containing numerous neutrophils or monocytes. These cellular accumulations are seen in the small pulmonary vessels in all experimental ARDS models, whether induced by endotoxin (Heflin and Brigham, 1981), C_{5a} (Ward *et al.*, 1983a) or immune complexes (Till *et al.*, 1982). Cells are thought to be drawn to the area by chemoattractants generated by the first wave of oxygen radical released by resident cells (Turner *et al.*, 1975; Harada *et al.*, 1984). As discussed earlier, there is now ample evidence that active oxygen secreted by these cells is instrumental in the observed endothelial damage. It may act in collaboration with enzymes also released by these cells (Section II B 3), and be assisted by other, as yet undefined, agents (Harlan *et al.*, 1985), but the results achieved *in vivo* with SOD, catalase, antioxidants and iron chelators imply that the contribution from active oxygen is vital. Therefore we think that there is sufficient evidence to argue that the pulmonary oedema seen in infection with *P. falciparum* or *B. bovis* qualifies as ARDS, and predict that it will prove to be mediated by the mechanisms elucidated by the ARDS experimental model.

If we assume that this system is operating in the pulmonary oedema of malaria and babesiosis, there is as yet no indication of which of the experimental models most closely resembles the trigger for mediator release in the real disease. The essential element, be it endotoxin-like, antigen to

form immune complexes, or some other activator of complement, is likely to be released from schizonts as they mature to release new merozoites, since in synchronous infections this governs the onset of cyclic illness. In this context any of the three could fairly be described as functionally like endotoxin, a quality we have suggested (Clark, 1978; Clark *et al.*, 1981) is necessary to trigger cell-mediated tissue damage in malaria and babesiosis. Rather than concentrating on defining this trigger, it could be more productive, in our view, to establish the nature of the link between the cellular infiltrate and the endothelial injury, and see if oxygen radicals or some other system (Harlan *et al.*, 1985) can be implicated. Studies *in vivo* on malarial pulmonary oedema that could settle this question are handicapped by the lack of an animal model. However, vitamin E is commonly administered to human beings, and the iron chelator desferrioxamine is beginning to be used, with caution, in non-iron overload states as an anti-inflammatory agent (Editorial, 1985). Desferrioxamine is, as a bonus, an antimalarial drug (Raventos-Suarez *et al.*, 1982). This drug, and antioxidants such as the phenolic radical scavenger butylated hydroxyanisole, could be used immediately in experimental *B. bovis* infections. Butylated hydroxyanisole has already been reported to have been used successfully in experimental fog fever (Gregory *et al.*, 1984), a known radical-induced pulmonary oedema in ruminants (Kubow *et al.*, 1984). The pathology of falciparum malaria is so similar that success with *B. bovis* would tell us much about both diseases.

B. KIDNEY

Parasitic infections can cause polyclonal activation of B cells, and since parasites are complex organisms they provide a varied source of antigenic material. Thus circulating immune complexes are commonly formed in these diseases, and the likelihood that they might cause tissue injury, particularly in the kidneys, is perennially a topic of discussion (see D'Amelio, 1980). The requirement of complement, neutrophils and oxygen radicals for much of this type of damage has recently been reviewed (Johnson and Ward, 1982). There is now evidence that complexes containing IgA can injure endothelial cells without help from neutrophils (Ward *et al.*, 1984) and that endothelial cells can secrete $O_2^{\cdot-}$ (Johansson and Björkman, 1983), so this lesion may not be so strictly cell-dependent as was previously thought.

As well as in the pulmonary and dermal contexts discussed above (McCormick *et al.*, 1981; Fligiel *et al.*, 1984a), the immune complex system has now been investigated in a renal model by Rehan *et al.* (1984). These authors injected into rats antibody raised in sheep against rat glomerular basement

membrane, producing an acute proteinuria, which could be inhibited by up to 75% by injecting catalase. Animals treated with catalase or depleted of neutrophils did not develop proteinuria, and SOD was ineffective. The positive catalase but negative SOD results indicate that the oxygen reduction pathway could cause proteinuria without O_2^- taking an active part beyond being a source of H_2O_2. This is not surprising: O_2^- itself is not thought nowadays to be very destructive directly, but to contribute to tissue damage mainly by helping the cyclic reduction of ferric iron to the ferrous state, allowing it, in turn, to continue to reduce H_2O_2 to more active metabolites. If adequate amounts of other relevant reducing agents (such as ascorbate) are available the system will run without O_2^- (Winterbourn, 1981; Rowley and Halliwell, 1983). It was proposed that the O_2^- dependency of this system, and hence its inhibition by SOD *in vivo*, is largely governed by ascorbate availability. There is no information yet on whether the increased permeability of these neutrophil-damaged glomeruli (Rehan *et al.*, 1984), like that of dermal vessels in similar circumstances (Fligiel *et al.*, 1984a), is iron-dependent. Immune complexes and complement components have been described in the glomerulonephritis of human malarial infections (Ward and Kibukamusoke, 1969) and studied experimentally in a rodent model (Annable and Ward, 1974). The way is now open to investigate kidney lesions produced by haemoprotozoa further in the light of these newly defined mechanisms.

VI. Cerebral Malaria and Babesiosis

A. Nature of Primary Lesion

Were immune complex-induced vasculitis to occur in malaria and babesiosis the brain would be vulnerable, and this could contribute to cerebral malaria and babesiosis; indeed Adam *et al.* (1981) have produced evidence suggesting that the presence of circulating immune complexes in patients with falciparum malaria greatly increases the chance that cerebral complications will develop. We recall that the *P. berghei* ANKA rodent model of cerebral malaria (which may have a different pathogenesis to the human disease) is T cell-dependent (Finley *et al.*, 1982). A requirement for helper T cells to produce antibodies that contribute to immune complexes is one possible explanation for this. Apart from this one rodent model, however, cerebral symptoms appear to be limited to infections with *P. falciparum* or *B. bovis*. These are the only two parasites known to induce, on the surface of the red cell they inhabit, structures able to adhere to endothelial cells (Green *et al.*,

1985; Aikawa *et al.*, 1985). While restriction of clinical cerebral involvement to these two species implies that attachment is necessary for this manifestation of malaria, attachment alone, for reasons discussed in Section II A 2, may not always be sufficient. In summing up their studies on the histopathology of 43 *Aotus* monkeys infected with *P. falciparum*, Jervis *et al.* (1972) expressed surprise that the tissue damage typical of human infections was absent, since the small blood vessels in their monkeys were as packed with marginated red cells containing parasites as were those in sections from human cases. They therefore suggested that factors other than plugging of capillaries, absent from the *Aotus* model, could be contributing to the tissue damage of human falciparum malaria. One such factor that may help tip the balance (for we have to remember that cerebral symptoms are not common in either disease) could be primary endothelial damage, as in ARDS. Another could be phagocyte-mediated reduced deformability of red cells, as discussed in Section VIII A. Either of these would accommodate (i) the results of Adam *et al.* (1981), (ii) reports of a close correlation between the degree of cerebral involvement and the onset of pulmonary oedema (Brooks *et al.*, 1968; Neva *et al.*, 1970; Punyagupta *et al.*, 1974), and (iii) the phenomenon of tolerance (Kitchen, 1949; McGregor *et al.*, 1956; Neva *et al.*, 1970), discussed in Section II A 2. There is evidence for ultrastructural endothelial cell injury in *P. berghei* cerebral malaria in hamsters (Rest and Wright, 1979) and mice (Rest, 1982) and cattle infected with *B. bovis* (Wright, 1972), but no one has determined whether it is a primary event or secondary to occlusion by parasitized red cells.

B. CEREBRAL ISCHAEMIA

Whatever the precise primary lesion in cerebral malaria is, it is not likely to be directly harmful: what causes clinical damage is very probably the subsequent hypoxia (White *et al.*, 1985), brought about by a reduced flow of blood to the brain, or cerebral ischaemia. Ischaemia is not a static condition in a living animal; it is a dynamic state with intervals of relatively increased flow, or reperfusion. There is now impressive evidence, recently reviewed by White *et al.* (1984) and McCord (1985), that the tissue injury in ischaemic reperfusion is caused by reduction products of oxygen generated in a reaction between hypoxanthine, which accumulates in ischaemia, and xanthine oxidase. Reperfusion episodes supply the oxygen to be reduced, by this reaction, to $O_2^{\cdot-}$. Evidence *in vivo* from various organs that antioxidant enzymes, radical scavengers, iron chelators and allopurinol (an inhibitor of xanthine oxidase) inhibit damage from this source is growing rapidly (e.g., Paller *et al.*, 1984; Guaduel and Duvelleroy, 1984; Manning *et al.*,

1984). It seems a reasonable prediction that this line of enquiry will soon have something practical to tell us about the ischaemia of cerebral malaria.

C. TOXICITY OF EXTRAVASATED HAEMOGLOBIN

Whether as a primary or secondary event, the vascular endothelium is usually damaged in severe cerebral malaria, so much so that diffuse petechial haemorrhage is often present at autopsy. Toro and Roman (1978) observed this lesion in 15 out of 19 fatal cases of cerebral malaria. The normal fate of the haemoglobin from these extravasated red cells is to be bound to haptoglobin and then cleared by the reticulo-endothelial system. The high reactivity of haemoglobin and similar molecules with H_2O_2, producing harmful species, has been appreciated for some time (George and Irvine, 1951; Keilin and Hartree, 1954). Recently Sadrzadeh *et al.* (1984) have proposed, on the basis of experiments monitoring the reaction of dimethylsulphoxide to yield methane and formaldehyde, that free haemoglobin catalyses the reduction of H_2O_2 to $\cdot OH$. Although this interpretation is yet to be confirmed in other detection systems, their observations serve to stress the potentially hazardous consequences *in vivo* of unbound haemoglobin, particularly where H_2O_2 is being generated, as in an ischaemic area undergoing periods of reperfusion. The brain, with its high content of unsaturated fatty acids, is particularly vulnerable. Once the supply of free haptoglobin is exhausted, damage could occur by this pathway. In keeping with these concepts, Panter *et al.* (1985) have shown that haemoglobin is cleared from the brain more slowly than normal in hypohaptoglobinaemic mice, and that both lysed red cells and purified haemoglobin induce dose-related lipid peroxidation in homogenates of mouse brain.

Evidently epileptiform seizures can be common in cerebral malaria; Schmutzhard and Gerstenbrand (1984) observed them in 42 of 66 cases in Tanzanian children, as did White *et al.* (1985) in 50% of their Thai patients. Several lines of evidence implicate the type of radical-induced event described in the previous paragraph in the pathogenesis of these seizures. They tend to occur after head trauma, when haemorrhage is to be expected (Loiseau and Jallon, 1981), and iron injections induce epileptiform EEG discharges in rats (Willmore *et al.*, 1978; Willmore and Rubin, 1981). In addition, focal epileptogenesis follows intracortical injection of haemoglobin (Rosen and Frumin, 1979). Furthermore, Panter *et al.* (1985) have shown an association between familial idiopathic epilepsy and hypohaptoglobinaemia, arguing that the low haemoglobin-binding capacity in these individuals predisposes them to haemoglobin-promoted oxidation of brain

lipids, and thus allows epileptiform seizures to be precipitated by brain haemorrhages normally too small to be of consequence.

Thus we suggest that the epileptiform seizures seen in cerebral malaria could be, at least in part, another example of pathology induced by oxygen radicals. This could contribute to coma in these patients, since a comatose phase usually follows an epileptic attack (Bannister, 1978). The proposed sequence is that small vascular leaks provide the haemoglobin that, once the binding capacity of haptoglobin is exceeded, catalyses reduction of H_2O_2 to more reactive species that exert oxidant stress on brain tissue. The inactivity of haptoglobin-bound haemoglobin (Sadrzadeh *et al.*, 1984) implies that availability of haptoglobin is another influence controlling this source of oxidant stress. Haemoglobin liberated intravascularly at schizogony reduces haptoglobin levels during malaria (Blumberg *et al.*, 1963), and this has been suggested to be the main reason why ahaptoglobinaemia is common in certain populations in Papua New Guinea (Curtain *et al.*, 1965) and The Gambia (Boreham *et al.*, 1981). Ahaptoglobinaemic individuals would be expected to be more susceptible to haemoglobin-driven pathology, and it would be interesting to know whether such people show a distinctive clinical spectrum, tending to include epileptiform seizures, during cerebral malaria.

VII. Fibrin Formation in Malaria and Babesiosis

Some degree of intravascular coagulation is frequently noted in severe falciparum malaria (Devakul *et al.*, 1966; Dennis *et al.*, 1967; Punyagupta *et al.*, 1974) and *B. bovis* infection (Mahoney and Goodger, 1969; Dalgliesh *et al.*, 1976) and conflicting views have been expressed on whether it is an essential part of the pathogenesis of the disease (Maegraith and Fletcher, 1972; Wright *et al.*, 1979; Jerusalem *et al.*, 1983).

A wider view, encompassing a range of conditions argued to be associated with radical generation *in vivo*, is most revealing: fibrin formation is part of most of the types of tissue damage we have described in this review. The simplest example of lung damage induced by oxygen radicals occurs in animals breathing oxygen at higher than normal concentrations for extended periods, and fibrin deposition is well documented here (Kistler *et al.*, 1967; Kapanci *et al.*, 1969). It is also present in clinical ARDS, apparently irrespective of its origin (Tomashefski *et al.*, 1983), and in experimental ARDS triggered by endotoxin (Meyrick and Brigham, 1983), cobra venom factor (Till *et al.*, 1982; Ward *et al.*, 1983a) and skin burn (Till *et al.*, 1983). In both the C_{5a} activation model of ARDS (Ward *et al.*, 1983a) and the cutaneous model of immune complex-induced vasculitis (Fligiel *et al.*,

1984a), fibrin deposition is one of the changes prevented by injecting a radical scavenger (dimethylsulphoxide) or iron chelators. In addition, fibrin is formed intravascularly in eclampsia, a condition argued in Section IV to have free radical generation somewhere vital, as yet undefined, in its pathogenesis.

Various mechanisms could contribute to this activation of the coagulation pathway, including platelet activity, procoagulant activity originating from monocytes (recently reviewed by Shands (1984)), and tissue factor from perturbed endothelial cells (Stern *et al.*, 1985). Since free radical generation provides a common thread in the above examples of tissue damage, it also seems possible that fibrin formation *in vivo* is helped by thrombin generated when lipid peroxides react with plasma lipoproteins, as occurs *in vitro* (Barrowcliffe *et al.*, 1984). As these authors suggest, the process is perhaps inevitable whenever lipid peroxidation occurs in the presence of plasma, and would tend to form fibrin nearby. This concept implies that fibrin formation in malaria and babesiosis is an epiphenomenon, and not crucial to tissue damage.

VIII. Effect of Oxygen Radicals on Red Blood Cells

A. POOR DEFORMABILITY

Red cells have a reputation for behaving oddly in malaria and babesiosis—agglutinating, flowing poorly and tending to clog up small vessels. Explanations have included poor viscosity caused by fluid loss through damaged endothelium (Maegraith, 1948; Maegraith and Fletcher, 1972) and involvement of fibrin (Knisely *et al.*, 1945; Punyagupta *et al.*, 1974). The free radical literature demands that another factor, the poor deformability of the red cells themselves, be taken into consideration.

Oxygen presents red cells with a particular problem, more than that faced by most other cells: they are exposed to high oxygen concentrations in the lungs, their membranes contain unusually high concentrations of vulnerable unsaturated fatty acids, and they lack the cytochrome respiratory pathway that allows most of the oxygen in other cells to avoid sequential acquisition of electrons. Thus reactive forms of oxygen are generated at a high rate in red cells, and the balance between this stress and their antioxidant defences determines their life span (see Carrell *et al.* (1975) for a review). Since the mature red cell cannot synthesize proteins the antioxidant enzymes cannot be maintained, and lipid peroxidation eventually occurs. Pfafferott *et al.* (1982) and Jain *et al.* (1983) have shown that a product of this peroxidation

cross-links proteins in the red cell membrane, reducing their deformability. Thus they cannot survive passage through the intermediate circulation of the spleen, and are removed from the circulation (Weiss, 1963).

If circulating phagocytes were triggered to secrete O_2^- during malarial illness, erythrocytes would be among the first cells to experience increased oxidative stress and, given their vulnerability, their deformability and survival (Howard and Mitchell, 1979; Weatherall *et al.*, 1983) would be prematurely decreased. Reduced deformability has been reported in a study in which blood from a group of eight patients with active malaria (one *P. vivax*, the rest *P. falciparum*; parasitaemias ranging from 0.02% to 0.17%) took, on average, three times longer than control bloods to pass through 5 μm pores (Lee *et al.*, 1982). As these authors note, this low filtration rate in blood containing so few parasites cannot easily be attributed to parasitized cells alone, as it can be in monkeys infected with *P. coatneyi* (Miller *et al.*, 1972). Another clinical study (Punyagupta *et al.*, 1974) recorded increased numbers of distinctively deformed red cells (known as burr cells, or echinocytes) in nine out of 12 falciparum malaria patients exhibiting both cerebral symptoms and pulmonary oedema. These altered red cells are seen in rats breathing low concentrations of ozone (O_3), a source of O_2^- (Larkin *et al.*, 1978), and they take their distinctive shape from cross-linking of proteins in their membranes, dependent on malonyldialdehyde (Pfafferott *et al.*, 1982) and calcium (Smith *et al.*, 1981). After incubation for 2 hours, red cells from mice infected with *P. vinckei* accumulate some ten times more malonyldialdehyde than do normal mouse red cells (Clark *et al.*, 1984b), and lipid peroxidation products, including malonyldialdehyde, damage the membrane's calcium transport system (Kagan *et al.*, 1983), increasing intracellular calcium. This has been observed in unparasitized red cells from monkeys infected with *P. knowlesi*, such cells containing four times the amount of calcium found in control erythrocytes (Gupta *et al.*, 1982). Aminophospholipids usually present only on the cytoplasmic side of red cell membranes are present in the outer layer in these cells (Gupta *et al.*, 1982) and in cells exposed to H_2O_2 (Jain, 1984). The involvement of free radicals in changes in transbilayer fluidity induced by peroxidation have been documented by electron spin resonance (Bruch and Thayer, 1983).

Vitamin E appears to be moved around the body to wherever its antioxidant properties are in demand, since exposure of lungs to oxidant stress increases their vitamin E content (Sevanian *et al.*, 1982; Elsayed and Mustafa, 1982). Our group has recently demonstrated a 4–5 fold increase in vitamin E levels in all red cells, particularly in a largely unparasitized fraction, from mice ill with *P. vinckei* infection (Stocker *et al.*, 1985). This may be indirect evidence that these red cells are responding to oxidant stress.

B. DYSERYTHROPOIESIS

Erythropoietic depression is observed in human malaria (Srichaikul *et al.*, 1969; Weatherall *et al.*, 1983), and includes the presence in bone marrow of multinucleate erythroid precursors (Abdalla *et al.*, 1980; Weatherall *et al.*, 1983). These changes, particularly the distinctive multinucleate cells, occur in vitamin E deficient monkeys (Porter *et al.*, 1962) and pigs (Lynch *et al.*, 1977), and in rabbits attempting to regenerate bone marrow after gamma irradiation (Bloom, 1948). Thus the voluminous literature attributing the pathology of both vitamin E deficiency and gamma irradiation to radical-initiated processes seems an obvious starting point to attempt to gain insight into the dyserythropoiesis of malaria. In addition, one does not need to invoke immunology to explain erythrophagocytosis in malaria (Greenwood *et al.*, 1978), since it also occurs in vitamin E deficiency (Lynch *et al.*, 1977).

C. HAEMOLYSIS

There is evidently a marked haemolytic component to the anaemia of human malaria, affecting non-parasitized red cells as well as those containing parasites (see Weatherall *et al.* (1983) for a summary). Many authors have proposed that a conventional immune mechanism is an adequate explanation, but a contribution from haemolysis induced by free oxygen radicals warrants consideration. This concept is best illustrated by injecting alloxan, an oxygen radical generator, into vitamin E deficient rats (Rose and György, 1950, 1952): severe haemolysis very rapidly ensues. Mice infected with malaria are particularly susceptible to this type of haemolysis (Clark and Hunt, 1983), but further analysis of this model is handicapped by the fact that non-parasitized red cells provide a much smaller contribution to the total haemolysis in mice than they do in human malaria. As with pulmonary oedema, cattle infected with *B. bovis* seem a good model on which to try the antioxidant enzymes, radical scavengers and iron chelators that are required to test this hypothesis. The need to use this model raises the question why man and cattle, which both tend to experience pulmonary endothelial damage with these infections, should also share the characteristic of a tendency to much higher haemolysis than the degree of parasitaemia would indicate. We recall that both pulmonary endothelial cells (Kistler *et al.*, 1967) and red cell membranes (Mengel and Kann, 1966) are outstandingly susceptible to oxygen toxicity.

IX. Schistosome Granulomas

The main lesion in schistosomiasis, and the chief clinical concern, is the granuloma induced by schistosome eggs that lodge in tissue. In murine infections with *Schistosoma mansoni*, the most studied model, this reaction relies on T cells (Domingo and Warren, 1967) and appears to be a form of delayed hypersensitivity (Warren *et al.*, 1967). Macrophages dominate, and the cells in acute lesions are apparently kept in a continuous state of activation by lymphokines (Boros, 1981). Thus they secrete $O_2^{\cdot-}$, prostaglandins and products of the lipoxygenase pathway without exogenous stimulus (Chensue *et al.*, 1983). Much less $O_2^{\cdot-}$ comes from macrophages from granulomas not dependent on T cells (Chensue *et al.*, 1984).

These processes can be expected to increase the oxidant stress on the tissue surrounding a T cell-dependent granuloma, and perhaps cause injury. Evidently the oxygen radicals are also necessary for the granuloma to keep growing *in vivo*, since treatment with vitamin E, SOD or catalase suppresses its development (Chensue *et al.*, 1984). These concepts will probably not prove to be unique to schistosome granulomas: they may well imply a role for $O_2^{\cdot-}$ or its reduction products in the growth of similar manifestations of chronic local hypersensitivity, such as the granulomas of sarcoidosis and berylliosis, and of fungal and mycobacterial infections.

Obviously the process *in vivo* is complex, involving, among other monokines, the oxidative metabolites of arachidonate (Kunkel *et al.*, 1984). The reduction of schistosome granuloma size achieved with BW775 and nordihydroguaiaretic acid (NDGA) was attributed to the inhibitory activity of these agents against the oxidative pathways of arachidonic acid. Both of these agents are, however, potent radical scavengers (Duniec *et al.*, 1983; Levine, 1983; Morisaki *et al.*, 1984), whereas indomethacin, which Kunkel *et al.* (1984) found actually enhanced granulomas, had negligible antioxidant properties under the conditions tested (Duniec *et al.*, 1983). Thus this experience with BW775, NDGA and indomethacin in the mouse schistosome granuloma model (Kunkel *et al.*, 1984), while informing us about arachidonate metabolites, also strengthens the arguments that granuloma growth requires $O_2^{\cdot-}$ generation by its macrophages (Chensue *et al.*, 1984).

Release of interleukin-1 (IL-1) by alveolar macrophages correlates with disease activity in pulmonary sarcoidosis (Hunninghake, 1984) and has been identified in tissue extracts from experimental granulomas (Kobayashi *et al.*, 1985). IL-1 is probably a family of similar molecules (Murphy *et al.*, 1981; Van Damme *et al.*, 1985; Wood *et al.*, 1985), and induces many activities as well as T cell proliferation. Three of these activities, collagenase release (Dayer *et al.*, 1979), collagen synthesis (Matsushima *et al.*, 1985) and

fibroblast proliferation (Schmidt *et al.*, 1982), clearly could influence granuloma growth. Fibroblast activity is high in murine schistosome granulomas (see Rennard *et al.* (1984) for a summary) and both collagen synthesis (Dunn *et al.*, 1981) and collagenase activity (Tagahashi *et al.*, 1980) are increased.

In various ways, not yet fully elucidated, the functioning of IL-1 seems to be linked with the generation of radicals. This concept could rationalize a series of apparently disparate observations. IL-1 induces synovial cells to secrete collagenase (Dayer *et al.*, 1979). When this enzyme is secreted by neutrophils it must be activated by a process requiring generation of a reactive oxygen metabolite before it can act on collagen (Weiss *et al.*, 1985). Conceivably collagenase released by synovial cells requires this same radical-mediated activation. If this were so, this arm at least of the multifactorial activity of IL-1 would require radical generation before it could go to completion. Furthermore, IL-1 and $O_2^{\cdot-}$ are secreted by macrophages under the influence of the same lymphokine, γ-interferon (Nathan *et al.*, 1983; Boraschi *et al.*, 1984), and bleomycin, a radical generator (Sugiura and Kikuchi, 1978), causes release of endogenous pyrogen (Dinarello *et al.*, 1973), a molecule closely related, if not identical, to IL-1 (see Dinarello (1984)). The endotoxin-induced temperature rise in rabbits, which is mediated by endogenous pyrogen, is inhibited by injecting the iron chelator desferrioxamine (N. Bowern, unpublished results). In our other systems *in vivo* desferrioxamine appears to act by preventing H_2O_2 reduction to ·OH (Clark and Hunt, 1983; Clark *et al.*, 1984a), and conceivably it acts similarly here. Bleomycin also enhances a delayed-type hypersensitivity (DTH) reaction (Parker and Turk, 1984), and our laboratory now has evidence, based on DTH being inhibited by desferrioxamine and butylated hydroxyanisole (Section III), for involvement of radicals in this reaction (W. B. Cowden and N. Bowern, in preparation). We recall that the schistosome granuloma is a delayed hypersensitivity reaction (Warren *et al.*, 1967).

Fibrin deposition is an integral part of delayed hypersensitivity reactions (see Geczy, 1983), including the schistosome granuloma. Its origin is unknown, but lymphokine-induced macrophage procoagulant activity (Geczy and Hopper, 1981) is a distinct possibility, since migration inhibition factor (MIF), the lymphokine implicated in this phenomenon, has been identified in experimental immune granulomas (Kobayashi *et al.*, 1985). The work of Chensue *et al.* (1983, 1984), in which cells from a fibrinous granuloma were noted to generate $O_2^{\cdot-}$, admits the possibility that thrombin formation, and hence fibrin deposition, is secondary to radical-induced lipid peroxidation. This process has recently been demonstrated in a cell-free system (Barrowcliffe *et al.*, 1984).

At present it is possible only to speculate on how oxygen radicals might contribute to the growth and maintenance of the schistosome granuloma.

One potential mechanism is generation of fibrinopeptide B (which is chemotactic for monocytes; Richardson *et al.* (1976)), when thrombin, formed in the presence of lipid peroxides (Barrowcliffe *et al.*, 1984) acts on fibrinogen. Another possible mechanism is based on evidence that fibronectin is chemotactic for fibroblasts (Postlethwaite *et al.*, 1981; Rennard *et al.*, 1981), and that hyperoxia, a process mediated by oxygen radicals, increases synthesis of fibronectin (Glass *et al.*, 1984). Thus fibroblasts could be attracted to the area by high concentrations of fibronectin. Alveolar macrophages from patients with various granulomatous lung diseases produce fibronectin at a faster rate than normal (Rennard *et al.*, 1981). So far as we are aware, this is yet to be explored in schistosome granulomas.

X. Conclusions

Processes induced by free radicals are now appreciated to contribute to tissue damage in a diverse array of pathological states. The exact details of the underlying chemistry are still debated, mainly because the high reactivity of these mediators makes them very difficult to study. In addition, enthusiasm can lead to over-extrapolation; indeed Halliwell and Gutteridge (1984b) have recently cautioned against assuming that all lipid peroxidation is a part of primary cellular damage rather than a consequence of injury. This does not, however, detract from the practical influence of well documented examples of these processes. This literature is growing rapidly, encompassing what would previously have been thought to be unconnected aspects of medical research. As we have stressed, net oxidant balance in a tissue is what determines the outcome of free radical generation. Schirmer *et al.* (1984) refer to the OSVAC (oxidant stress versus antioxidant capacity) state of a biological system when describing this concept.

As is often noted, there is nothing unique about most of the tissue damage of parasitic disease. While this can create diagnostic confusion, it is an advantage for those interested in mechanisms of injury, since the numbers of direct parallels from which lessons can be learnt is correspondingly large. Insight gained when exploring these overlaps can then be put to good use when concentrating on the parasite-induced disease itself. We think that processes induced by free radicals are an example of this.

Acknowledgements

The authors are grateful to Wendy Lees for expert secretarial assistance, and to the National Health and Medical Research Council of Australia and the

malaria component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases for salary and maintenance support.

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The Biology of Pentastomids

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ADVANCES IN PARASITOLOGY VOL. 25
ISBN 0-12-031725-7

I. Introduction

Pentastomids, otherwise known as linguatulids or tongueworms, are a relatively neglected and poorly understood class of endoparasites which occupy a unique position among invertebrates in that, as adults, they are entirely restricted to the respiratory tract of vertebrates: the majority of species grow to maturity in the lungs (an outline classification of pentastomids is provided in Table 1).

Baer (1952) commented that about 90% of species infect reptiles and it is probable that they have been associated with these hosts since the Mesozoic. Despite this long period for potential adaptive radiation, the basic body design is remarkably conservative, and pentastomids comprise a homogeneous and distinctive systematic assemblage of about 100 species (Table 1). All possess a vermiform, often conspicuously annulated abdomen, usually strongly united with a rounded cephalothorax which bears, on its ventral surface, a small sucking mouth flanked by two pairs of hooks. Most zoologists, and indeed parasitologists, have never seen a pentastomid, a fact not altogether surprising when one considers the nature of their hosts: many species are found in crocodilians, monitor lizards, and many of the more notorious constricting and venomous snakes. To employ these hosts as vehicles for pentastomids in long-term studies is a particularly esoteric branch of parasitological research with relatively few (one?) adherents! Nonetheless, as Self (1969) pointed out, pentastomids have received the attentions of many more biological investigators than is usually realized (as evidenced by the bibliography accompanying his review) and since 1969 a number of important papers and specialist reviews have appeared. However, there has been no recent attempt to present a balanced and comprehensive conspectus covering all aspects of pentastomid biology: this review seeks to redress this situation.

Pentastomids, in common with other parasites, are regulators of host populations, and many of the species I receive for identification have been recovered from zoo autopsies. In some cases, host death is directly or indirectly attributable to a pentastomid infection (Section VII). Since many pentastomids infect rare and often endangered host species, it is important, if only from the standpoint of conservation, that we should attempt to understand the role of pentastomids in these regulatory processes. Another reason why these parasites should not be ignored is that in certain regions of the world, particularly the Middle East, Africa and South-East Asia, man is commonly implicated in the life cycles of four species with occasional fatal consequences: this subject is reviewed in Section VIII.

Most modern textbooks of general invertebrate zoology accord pentastomids the status of an independent phylum (Russell-Hunter (1979) and

TABLE 1 *An outline classification of the class Pentastomida (slightly modified from Riley, 1983)*

Order	Family	Genus	No. of species	Definitive host	Intermediate host	References[a]
Cephalobaenida	Cephalobaenidae	*Cephalobaena*	1	Snakes	?	von Haffner and Rack (1971)
		Raillietiella	c34	Snakes, lizards amphisbaenians amphibians	Direct (?), insects amphibians, lizards	Ali *et al.* (1985)
	Reighardiidae	*Reighardia*	2	Marine birds	Direct (?)	Banaja *et al.* (1975, 1976)
Porocephalida	Sebekidae	*Sebekia*	6	Crocodilians (Chelonians)	Fish (snakes, lizards ?)	Heymons (1935)
		Alofia	5	Crocodilians		Giglioli (1922b)
		Leiperia	2	Crocodilians	Fish	Fain (1961)
	Subtriquetridae	*Subtriquetra*	3(?)	Crocodilians	Fish	Vargas (1971, 1975)
	Sambonidae	*Sambonia*	4	Monitor lizards	Direct (?)	Fain and Mortelmans (1960)
		Elenia	2	Monitor lizards	?	Heymons (1939)
		Waddycephalus	10	Snakes	Mammals	Riley and Self (1981b)
		Parasambonia	2	Snakes	?	Riley and Self (1982)
	Diesingidae	*Diesingia*	2(?)	Chelonians	?	Fonseca and Ruiz (1956)
	Porocephalidae	*Porocephalus*	8	Snakes	Snakes and/or mammals	Riley and Self (1979)
		Kiricephalus	5	Snakes	Amphibians or lizards or mammals and snakes	Riley and Self (1980)
	Armilliferidae	*Armillifer*	7	Snakes	Mammals	Fain (1961); Riley and Self (1981a)
		Cubirea	2	Snakes	?	Heymons (1935)
		Gigliolella	1	Snakes	Mammals	Chabaud and Choquet (1954)
	Linguatulidae	*Linguatula*	6	Mammals	Mammals, direct (?)	von Haffner *et al.* (1969)

[a]The references offer the most complete description of a genus (or taxon) and/or contain information on life cycles.

Barnes (1980) being typical in this respect): however, recent evidence convincingly points to a rather close affiliation to the arthropods—this evidence is critically reviewed in Section II. Many aspects of pentastomid structure and function (Sections IV and V), reproduction (Section VI), and immunology (Section IX) seem to have no counterpart in other parasites, and it is interesting to observe the ways in which the basic arthropod ground plan has been modified in response to the selective pressures inherent in an extremely specialized endoparasitic niche.

Much remains to be learned about the biology of pentastomids. Some of the more important deficiencies in our knowledge will be highlighted in this review, which it is hoped might serve as a platform for future research.

II. Phylogeny

Baer (1952) observed that pentastomids occupy a relatively isolated place among invertebrates, and that the combination of a non-segmented body cavity, metamerically arranged muscles, and a chitinous tegument with numerous glands, made it difficult to assign them to any certain position among what might eventually be considered related groups. Furthermore, pentastomids, being soft-bodied, have no palaeontological record and necessarily, therefore, any thoughts concerning their evolutionary history must remain speculative. Notwithstanding this, the subject of pentastomid phylogeny is much debated: useful reviews of the field, providing a background to the following arguments, were provided by Osche (1963), Doucet (1965), Legendre (1967), Self (1969), von Haffner (1971, 1977), Wingstrand (1972) and Riley *et al.* (1978).

Self (1969) and Riley *et al.* (1978) pointed out that the major stumbling block to an understanding of pentastomid relationships arises from the fact that nearly all species are without a free-living stage (there is one known exception; see Section VI F and Fig. 1b). The primary larva, which hatches from the egg when ingested by a suitable host, is highly specialized for tissue migration (Self, 1969)—the "limbs" are reduced to two pairs of double-hooked stumpy appendages and the cephalothorax carries penetration spines (Fig. 1a). Even this, the earliest stage of post-embryonic development, can bear little resemblance to similar stages in free-living relatives and this must be equally true of the later stages of embryogenesis (see, for example, Böckeler (1984b)). This has important and far-reaching consequences. Riley *et al.* (1978) emphasised that, wherever free-living and parasitic forms occur within the same group of animals, it is usually possible to see what kind of morphological adaptations the latter have undergone relative to the free-living forms. This is manifestly not so in certain parasitic

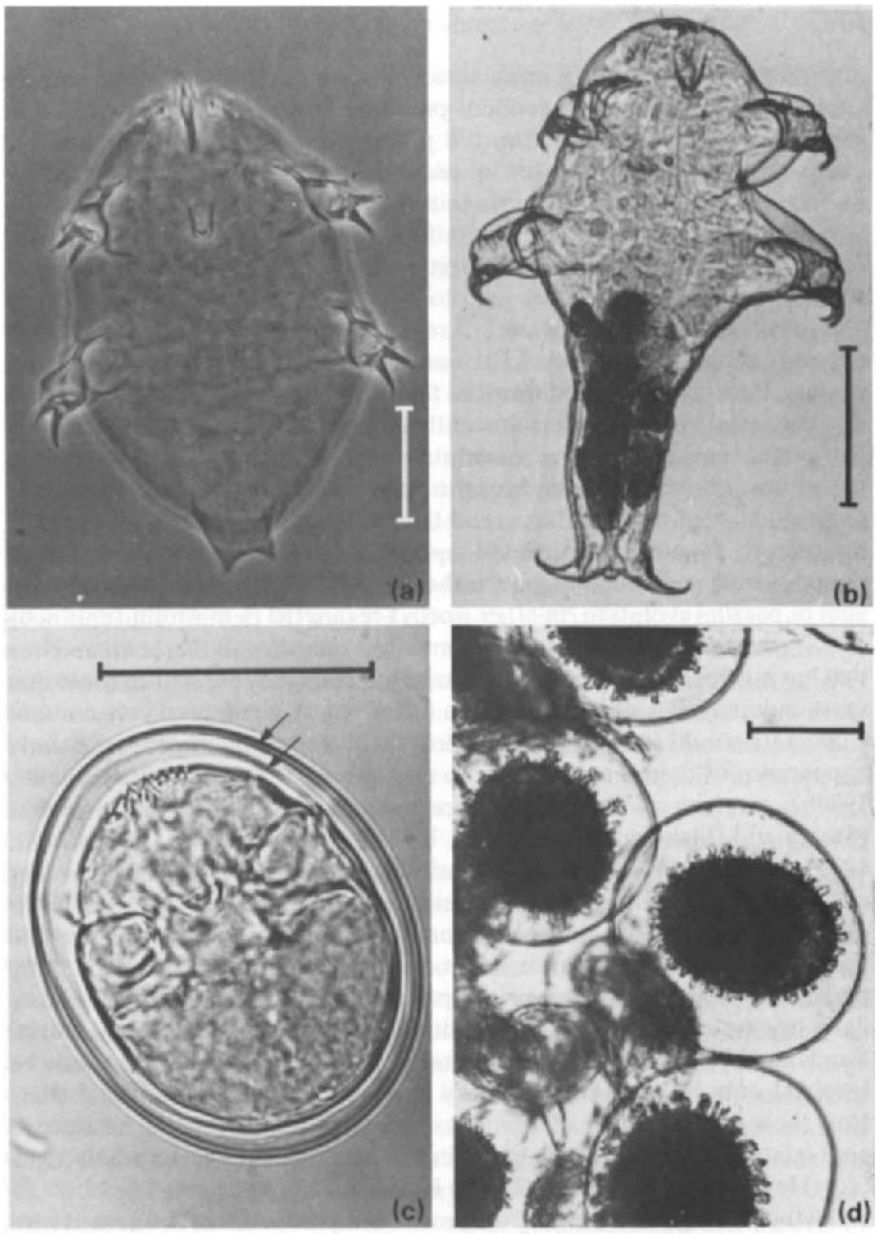

FIG. 1. (a) Primary larva of *Porocephalus crotali*. The median penetration stylet is uppermost (scale = 50 μm). (b) Primary larva of *Subtriquetra subtriquetra* showing the unusually long, strongly-hooked tail (scale = 100 μm). (c) Fully-mature egg of *Raillietiella agcoi*: the outer and inner eggshells are arrowed (scale = 50 μm). (d) Mature eggs of *Parasambonia minor*: the outer eggshell is spiny and the hyaline outer capsule is obvious (scale = 50 μm).

arthropod groups, notably isopods, copepods and cirripedes, where many adults are so profoundly modified, particularly with regard to the loss of jointed appendages, that, from the point of view of gross morphology, comparison with similar stages in free-living relatives has little feasibility. Many extreme forms can be recognized as arthropods and classified with certainty only because they possess free-swimming, typically crustacean, early larvae, and because the various stages in the metamorphosis from larva to adult are known.

So where does this leave us? Are pentastomids so modified that all ancestral traits have been lost? Fortunately, this seems not to be so because, despite these profound modifications for an endoparasitic existence, several diverse items of evidence have recently emerged which combine to suggest that pentastomids are indeed euarthropods. Although it must be conceded that some are the consequence of the possession of a chitinous cuticle, the sheer number of these features, and the precise structural correspondence between them and the arthropod equivalent, makes it sensible to accept them as being truly homologous, rather than simply by-products of convergent or parallel evolution. In other words I regard the pentastomid chitinous cuticle as a primitive character, present in the euarthropod forbear, and one that has not been determined or excessively changed by parasitism (note that most endoparasites do not have such a cuticle). A vignette of concomitant characters would include periodic ecdysis during development, the early appearance of paired appendages in the embryo (Osche, 1963; Böckeler, 1984b), the lack of free cilia—there are modified cilia in sense organs (Storch and Böckeler, 1979; Storch, 1984) and spermatozoa (Wingstrand, 1972)—and the possession of a haemocoel. The many derived or secondary characteristics, resulting from adaptations to life in the vertebrate respiratory tract, can also be readily enumerated: limbs eventually reduced to hooks; an elongate, tubular or flattened aerodynamic body; movement by peristalsis; parietal musculature composed mainly of circular and longitudinally orientated fibres; larval adaptations to tissue migration, and so on. Fundamentally, the question of pentastomid phyletic relationships can be resolved only by separating primary characters reflecting ancestral traits from the many secondary adaptations imposed by parasitism: it is a failure to appreciate this distinction that has, in the past, bedevilled the whole issue (e.g. Heymons, 1935; Self, 1969; von Haffner, 1964, 1971, 1977).

All the features that are truly diagnostic of arthropods are connected with the exoskeleton (Russell-Hunter, 1979). However, because pentastomids rely upon differential contractions of the tegument to achieve locomotion (von Haffner *et al.*, 1969; von Haffner, 1972; Riley *et al.*, 1978), the cuticle remains thin and untanned throughout life (Riley and Banaja, 1975); nevertheless its overall morphology is unmistakably arthropodan (Riley and

Banaja, 1975; Trainer *et al.*, 1975) (see also Section IV A and Fig. 6). However, Karuppaswamy (1977) reported the occurrence of ß-chitin in the cuticle of a raillietiellid: apparently the arthropod cuticle contains only α-chitin. This anomalous finding demands further study.

The dorsal organ of the pentastomid egg, which in *Reighardia* and all other pentastomids secretes a mucous layer around the inner eggshell, has its counterpart in many other groups of arthropods, and the inner eggshell, a chitinous secretion of the epidermis of the early embryo, termed a blastoderm cuticle, is also highly characteristic of that phylum (Osche, 1963).

The elucidation of other basic features of pentastomid development from a comparative viewpoint is extremely difficult; the eggs are small, critical small-scale events are obscured by yolk, and, since most larval appendages are suppressed, post-embryonic development is likely to be unusual. Even within a particular arthropod group there are numerous variations on the "basic" developmental theme (Anderson, 1973) and, not surprisingly, evidence generated from such studies, mainly of the embryo and early development of cephalobaenids, is at first sight equivocal on the subject of arthropod affinities (Osche, 1963; Doucet, 1965; Böckeler, 1984b). The early embryo of *Reighardia* carries four pairs of limb buds, arbitrarily designated antennae, mandibles and maxillae, each associated with ganglia and coelomic pouches (Osche, 1963). Osche (1963) concluded from these (and other) observations, made on embryos at essentially the same stage of development, that pentastomids were primitive uniramian arthropods. Later Böckeler (1984b), studying a complete range of embryonic stages, was able to show that the first two "limbs" eventually reduce to sense organs in late larvae and adults. When larvae hatch they contain just seven pairs of somites; no indications of other segments were found, and further elongation was thought to occur by "pseudosegmentation". Böckeler (1984b) concluded, mainly out of deference to this last point, that pentastomids had originated from the base of the arthropod stem and that any arthropod-related characteristics were the result of convergent evolution. But, as mentioned above, because most thoracic and abdominal appendages never appear, post-embryonic development is likely to be atypical. I do not seek to overinterpret these results, but it nevertheless seems that the pentastomid embryo shares many of the characteristics of mandibulate arthropods. To which of the two mandibulate subphyla they belong (Crustacea or Uniramia) may be inferred from the following, because two further aspects of the early development of pentastomids have, potentially, great phyletic significance. Doucet (1965) noted that cleavage in *Raillietiella boulengeri* eggs was initially equal but, at the eight blastomere stage, four cells were offset from the others by 45° so that the possibility of spiral cleavage could not be excluded. Cleavage in crustaceans has a spiral basis and this sets them

apart from other arthropods (Anderson, 1973). Also Anderson (1973) commented that Crustacea have small eggs and total cleavage as a primary feature; recently Böckeler (1984b) gave details of precisely this phenomenon in the eggs of *Reighardia*.

The development (von Haffner, 1924b; Osche, 1963; Böckeler, 1982, 1984b) and functional anatomy (Doucet, 1965; von Haffner, 1971; Hollis, 1979) of the nervous system has also been used to argue phylogenetic relationships, but again there is no consensus. The arthropod brain consists of three major regions, an anterior protocerebrum, a median deutocerebrum and a posterior tritocerebrum, serving respectively the eyes, the antennae (that is, the first antennae in crustaceans: in chelicerates, where antennae are lacking, so is the deutocerebrum) and finally the labium, digestive tract and the second antennae of crustaceans (Barnes, 1980). Since pentastomids lack eyes, and appendages are much reduced or absent, it follows that associated ganglia will be similarly reduced. Thus Osche (1963) argued that the protocerebrum is absent simply because there are no eyes, whereas von Haffner (1971) attempted to homologize the supraoesophageal ganglion with the annelid condition. If Osche was correct, and the protocerebrum is missing, then Doucet (1965) has described a nerve trunk connecting the deutocerebrum to the dorsal papilla (and the anterior ventral papilla) and another uniting the tritocerebrum with the posterior frontal papilla and the mouth. These papillae (dorsal and frontal) are known to be vestiges of the first two embryonic limb pairs (Böckeler, 1984b) and they may be homologues of the antennules and antennae of crustaceans, the mandibles being the third limb pair. Nonetheless, the final relationship between many of the seven ganglia present in the cephalothorax of larval *Reighardia* (Böckeler, 1984b) and the associated coelomic pouches is still far from clear.

It seems unlikely that neuroanatomical studies of pentastomids will yield any further significant insights into their phyletic relationships, because the modifications shown by larvae and adults render studies of the innervation of particular appendages, as clues to their homology, extremely difficult to interpret.

Wingstrand (1972), in a detailed analysis of sperm structure and development in the cephalobaenid *Raillietiella hemidactyli*, found a whole catalogue of specialized features which had counterparts in only one other animal, the branchiuran fish-louse, *Argulus foliaceus*. These features are too detailed to enumerate here, but their fundamental nature, coupled with a precise correspondence in detail, led Wingstrand to conclude that pentastomids and branchiurans were closely related. The alternative explanation, that each sperm type had undergone independent specialization and that the end products are convergent phenomena, was rejected on the basis of statistical

probability. But there are some similarities in reproductive behaviour: sperms of most branchiuran species are transferred directly to the female and stored in spermathecae (Fryer, 1982); in the genus *Dolops* the spermathecal duct is very narrow and the sperms are filiform (Fryer, 1960); and, in *Argulus*, a single mating is sufficient to fertilize all the eggs that are ever laid (Kollatsch, 1959) (compare with Section VI F). Therefore some convergence might be expected, but the many refinements of sperm structure reported by Wingstrand seem to preclude this as a complete explanation. It would be imprudent at this stage to attach too much phyletic significance to sperm morphology—convergence can produce remarkably similar endpoints—but if it is subsequently found that all pentastomids and all branchiurans produce essentially the same kind of sperm this would constitute powerful evidence of common ancestry.

In conclusion, the available evidence overwhelmingly indicates that pentastomids are euarthropods and, more specifically, that their affinities are closer to crustaceans than uniramians. Riley *et al.* (1978) could find little evidence to refute this suggestion, and the additional evidence discussed above (apart from that of Karuppaswamy (1977)) endorses this view. These authors speculated about the evolution of parasitism within the group and advanced two alternative theories, one of which can now be discounted. It was suggested that the life cycle of pentastomids belonging to the genus *Subtriquetra* may be primitive because the primary larva is free-living. We have since acquired crocodilians infected with *Subtriquetra* and our studies of the primary larva (Fig. 1b) have shown that it is virtually identical to equivalent parasitic stages (Fig. 1a). Its one modification, a strongly hooked tail, is designed to hook mechanically on to fish intermediate hosts (Vargas, 1975; Riley, 1983; Winch and Riley, in press b). There are no other obvious modifications for a free-living existence and it seems that it has become secondarily adapted to an aqueous environment and, therefore, little of phyletic significance can be inferred from it.

III. Classification and Taxonomy

A. General

Pentastomids comprise a highly modified, entirely parasitic group of organisms, with very obscure origins, and consequently their classification poses singular problems. A concise historical perspective of the present scheme, which was founded by Sambon (1922), elaborated by Heymons (1935) and Heymons and Vitzthum (1936) and subsequently revised by Fain (1961),

Nicoli (1963) and Self (1969), was given by Stunkard and Gandal (1968), who pointed out some of the more important nomenclatural anomalies which had become incorporated within it.

Fundamental disagreement concerning the taxonomic ranking of the group persists; for example, Fain (1961) accorded the assemblage class status, whereas Nicoli (1963), Self (1969) and most modern textbooks, opt for that of phylum. For the reasons outlined in the preceding Section, I regard pentastomids as a class within the Arthropoda, but to which of the constituent subphyla they belong may never be resolved unless Wingstrand's (1972) work is extended to include a representative selection of pentastomid and branchiuran species.

All of the above authors endorsed the original scheme of Heymons (1935), dividing the taxon into two orders, the Cephalobaenida, generally held to be the more primitive, and the Porocephalida, which possess advanced features; the most important characteristics by which these can be differentiated are listed in Table 2. Recent amendments and additions to the classification (embodied in Table 1) involve both orders; some of these changes will be discussed later and further revisions will inevitably be necessary.

B. DIAGNOSTIC CHARACTERS

In taxonomic studies of pentastomids the problems associated with identifying suitable anatomical criteria are well known; representative species, of both orders (Fig. 2), serve to illustrate the extent of morphological diversity, and it should be apparent that pentastomids can possess relatively few external characters upon which to base an identity. Those traditionally employed, body shape, hook morphology, annulus number and the position of the female gonopore, are mostly adequate for establishing broad generic differences. In specific diagnosis, however, small differences in the number, shape and size of non-rigid parts of the body have often been used, but there is considerable scope for error unless fixation and other handling procedures are standardized. Even then, many of these characters are, to a greater or lesser extent, variable. Another problem is that the majority of species' descriptions are based on relatively few specimens, so that little is known of the range of this variation and its impact on taxonomy. Indeed, the application of biometrical techniques to pentastomid taxonomy is largely handicapped by lack of data. Recently, considerable emphasis has been placed on rigid parts of the anatomy such as hooks and copulatory spicules, as these are less susceptible to artifacts (Riley and Self, 1979, 1980, 1981a,b, 1982; Ali *et al.*, 1981, 1982a,b, 1984a,b, 1985) and it is propitious to review briefly the

TABLE 2 *The major anatomical differences between the two pentastomid orders (much modified after Fain, 1961 and Nicoli, 1963)*

Character	Cephalobaenida	Porocephalida
Mouth and hooks	Mouth almost terminal and anterior to hooks which are disposed in a trapezium. Posterior hooks lie outside the anterior pair and are usually[a] much larger.	Mouth distinctly subterminal, situated between inner hook pair. Hooks disposed in straight line or arc. Size differences between hooks not pronounced.[b]
Hook structure	Hooks simple, ensheathed by a median podial lobe[c] and flanked by parapodial lobes. Hooks suspended by muscles in a U-shaped fulcrum.	Hooks complex, sometimes with accessory spines, more or less completely retractable into grooves. Hooks articulating directly onto a boat- or gutter-shaped fulcrum.
Caudal extremity	Bifid,[d] anus opens between diverging caudal lobes.	Pointed, or rounded, and/or flattened.
Glands	Cephalothorax contains loosely aggregated masses of gland cells. Many thin efferent ductules terminate at frontal papillae, buccal cavity, hook pits and anterior edge of cephalothorax.	Cephalothorax contains compact glandular mass with four efferent ducts leading to hook pits. Intestine flanked by compact glandular masses with paired ducts leading to frontal papillae.
Cuticle	Cuticle devoid of spines.[e]	Spines often present on posterior edge of abdominal segments.
Female genitalia	Uterus saccate, vagina opens anteriorly on ventral abdomen close to junction with cephalothorax.	Uterus elongate and tubular, tightly coiled in haemocoel, vagina opens towards caudal extremity, either in a common pit with the anus or separated from it by one or more annuli.
Male genitalia	Cirrus threads relatively short, cirrus tips lodged in hollow shafts of paired, club-shaped copulatory spicules. Seminal vesicle is a median alongate sac.	Cirrus threads long and coiled within paired cirrus pouches. Cirrus tip thickened, ornamented and free. Cirrus base passes through a copulatory spicule which is not club-shaped. Seminal vesicle Y-shaped, encircling intestine.
Eggs	Eggs with three layers, outer shell hard.[f]	Eggs with three layers,[g] mucous envelope around inner egg shell.
Embryo	Primary larva with double hooks in an oval fulcrum.	Primary larva with double hooks in a Y-shaped fulcrum.

TABLE 2 (*continued*)

Character	Cephalobaenida	Porocephalida
	Penetration apparatus consists of a median bifid stylet, flanked by 2–3[h] pairs of simple accessory spines.	Penetration apparatus consists of a simple median stylet, flanked by a pair of bifid accessory spines.
Nymphs	Early nymphal hooks usually doubled with hook barbs lying side by side.	Early nymphal hooks doubled, hook barbs lying one on top of the other.
Cerebral ganglia	Sub-oesophageal ganglion composed of three fused ganglia connected to at least four more or less separate ganglia by a doubled ventral nerve chord.	Ganglia fused into a compact, sub-oesophageal mass.

[a] In *Reighardia* and *Cephalobaena* hook pairs are similar in size.
[b] Except in certain *Sambonia* species (Self and Kuntz, 1957, 1966).
[c] Podial lobes much elongated in *Cephalobaena*, parapodial lobes absent.
[d] Except in *Reighardia* where it is rounded (Dyck, 1975).
[e] Tubercles present in *Reighardia* where cuticle has a rough surface.
[f] Except in *Reighardia* and [g] *Subtriquetra* (see text).
[h] Up to seven pairs in *Reighardia*, in one or two rows (Dyck, 1975).

relative usefulness and limitations of these various taxonomic criteria. Much of what follows concerns females: males tend to be comparatively short-lived, so that mature infections are often composed of mostly females; they are smaller and intergeneric differences are much less pronounced. Only in raillietiellids do males become important in specific diagnosis (see Ali *et al.*, 1985).

1. *Body shape*

The pentastomid body wall is thin and elastic (Section IV A), with the consequence that body shape is markedly affected by fixation. Giglioli (1927) commented on this from observations of living *Porocephalus* species; the obvious swollen anterior region of females, an important generic characteristic (Fain, 1961; Riley and Self, 1979), is a permanent property of alcohol-fixed material only (Fig. 3). Gravid female *Porocephalus crotali* and *Raillietiella frenatus* removed from the lungs of freshly killed hosts, and fixed immediately in 70% ethanol, increase in length by 30–50%; full extension is apparent after 15–30 min (Fig. 3). A less dramatic effect is seen with 10% formalin, and expansion is absent in specimens fixed post-mortem (Riley and Self, 1981a; Ali *et al.*, 1981). Live pentastomids are exceedingly

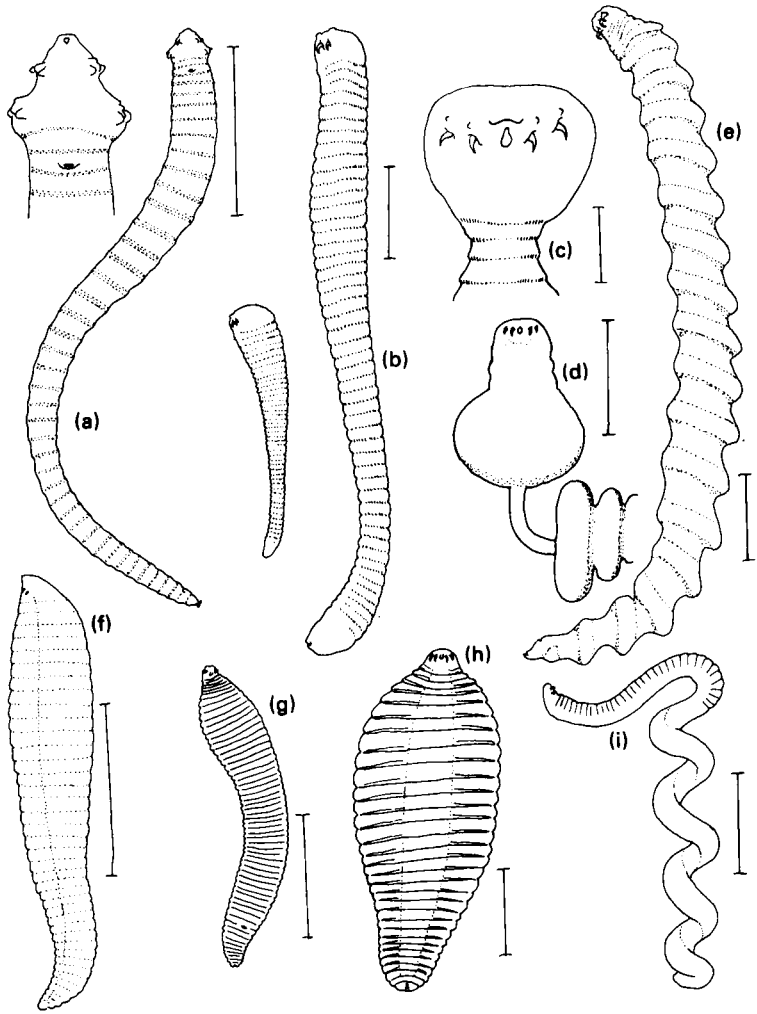

FIG. 2. Female (except (b)) pentastomids representative of nine genera (seven families). (a) *Raillietiella orientalis* with detail of cephalothorax: ventral aspect (scale = 10 mm). (b) Adult male and female of *Porocephalus stilesi*: lateral aspect (scale = 10 mm). (c) Detail of ventral cephalothorax of *Kiricephalus pattoni* showing globular cephalothorax and constricted neck (scale = 2 mm). (d) Ventral cephalothorax of *Cubirea pomeroyi* adapted for permanent attachment to lung of cobra (scale = 5 mm). (e) Lateral view of adult *Armillifer armillatus* (scale = 10 mm). (f) Lateral view of adult *Sambonia lohrmanni* (scale = 5 mm). (g) *Elenia travassosi*; ventral aspect (scale = 10 mm). (h) *Subtriquetra subtriquetra*; ventral aspect (scale = 5 mm). (i) *Leiperia cincinnalis* (lateral); uncoiled anterior region is buried in bronchial wall of a crocodilian (scale = 20 mm). ((a) Redrawn from Ali *et al.*, 1982b; (b) redrawn from Riley and Self, 1979; (c) redrawn from Riley and Self, 1980; (e) redrawn from Fain, 1961; (f) redrawn from Fain and Mortelmans, 1960; (g) redrawn from Heymons, 1932a).

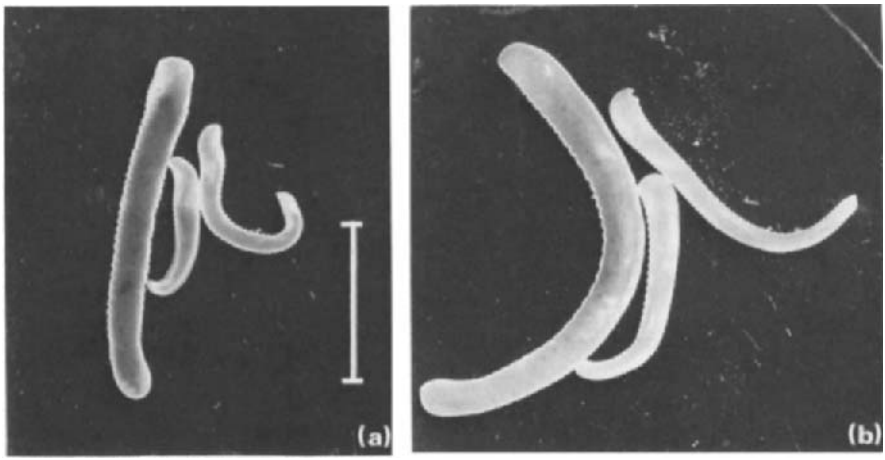

FIG. 3. (a) Living males and a female (left) of *Porocephalus crotali* in 0.9% saline immediately after removal from rattlesnake lung (scale = 2 cm). (b) The same specimens 30 minutes after fixation in 70% ethanol at the same magnification as (a).

delicate, and even the slightest puncture causes deflation and contraction, which among other things can produce spurious annulus counts. The taxonomist, working with already preserved material, should be acquainted with these problems. Seventy per cent ethanol is nonetheless recommended as a fixative and preservative because, somewhat artificial though the end-product is, ethanol does provide the most consistent results.

A further complication is that body size (at least) is possibly influenced by the host. For example, Giglioli (1927) observed that the largest specimens of *Armillifer armillatus* invariably came from puff adders (*Bitis* spp.), whereas those from much larger African pythons (*Python* spp.) were considerably smaller. These conclusions were to some extent supported by the results of Fain (1961), who showed that the opposite was true of hook dimensions. This manifestation of the host–parasite relationship is clearly an important factor which merits further study.

2. *Annulus number*

The number of abdominal annuli is a frequently used and important diagnostic character in certain porocephalid genera where differences are marked (for example, *Linguatula* (Sachs *et al.*, 1973); *Kiricephalus* (Riley and Self, 1980); *Armillifer* (Fain, 1961; Riley and Self, 1981a); and *Parasambonia* (Riley and Self, 1982)), but it has long been recognized that this character is not rigidly determined, and that in certain taxa, most notably

large raillietiellids, pronounced intraspecific variation is evident even within infections in a single host animal (Ali *et al.*, 1982b, 1984b). In these circumstances its value seems highly questionable. In other taxa, particularly the genus *Porocephalus* (Riley and Self, 1979) and the blunt-hooked raillietiellids (Ali *et al.*, 1981, 1984a), annulus counts are sometimes too close and overlapping to be of diagnostic value. However, with care, for example in the interpretation of so-called incomplete annuli (Heymons, 1932a; Fain, 1961; Riley and Self, 1981a), interannular distances (Ali *et al.*, 1985) and, with protocols for the counting procedure (see Ali *et al.* (1981) 1982a,b for raillietiellids and Riley and Self (1979, 1980, 1981a,b) for porocephalids), annulus counts, can be of value.

Self (1969) commented that the number of annuli is variable and probably not consistent until after the last moult. While the first part of this statement is undoubtedly true, evidence from three genera, *Kiricephalus* (Riley and Self, 1981b), *Armillifer* (Fain, 1961; Riley *et al.*, 1985) and *Sebekia* (Winch and Riley, in press a), has established that a definitive number of annuli is acquired by the infective (to the final host) nymphal stage, and thus it is possible to identify specifically at least these nymphal porocephalids using this sole criterion.

3. *Hook dimensions*

Gross hook morphology can reliably separate certain otherwise closely related porocephalid genera (for example, *Sebekia* and *Alofia*—see Heymons (1941c)) and hook size has recently been used extensively in specific diagnosis (e.g. *Armillifer* (Fain, 1961; Riley and Self, 1981a); *Porocephalus* (Riley and Self, 1979); *Waddycephalus* (Riley and Self, 1981b) and *Raillietiella* (Ali *et al.*, 1981, 1982b, 1984a,b)). Hooks are tough, heavily sclerotized structures that are unaffected by fixation and which are easily removed and measured (see the above references for protocols). Hooks increase in size at each moult (Fain, 1964), and measurement of different components of the developing hooks of three pentastomid species in the final host has shown that the growth rate of each to a finite size was linear throughout the final five or so instars (Riley, 1981; Ali and Riley, 1983). In practice hook data can only be meaningfully compared between fully adult specimens, and a major obstacle arises in deciding what constitutes the adult stage. Ali and Riley (1983) showed experimentally that the percentage of fully developed eggs in the uterus of raillietiellids is a reliable indicator of maturity (see also Sections IV F and VI E), but with porocephalids the criterion is rather less objective and involves gauging whether the elongate uterus is at a late stage of development (that is, distended with eggs, some of which should contain primary larvae, and occupying most of the

available haemocoel). Adult males of both orders are readily diagnosed as only they carry fully elaborated copulatory spicules.

A simple, practicable method of separating closely related species on the basis of hook dimensions was given by Riley and Self (1979, 1981a,b, 1982) and Ali *et al.* (1981, 1982a,b, 1984a,b), in which two dimensions (e.g. overall length and barb length or shank length) were plotted against each other to produce cluster groups of values. When these are discrete and non-overlapping, as is true with the female raillietiellids in Fig. 4a, separation is easy, but males of the same species (Fig. 4b) show intergrading and distinctions are blurred.

These cluster groups are natural, in an empirical sense, and thus, in appropriate circumstances, are of high predictive value. It should be emphasized that it is the cluster group that is the taxonomic character; little can

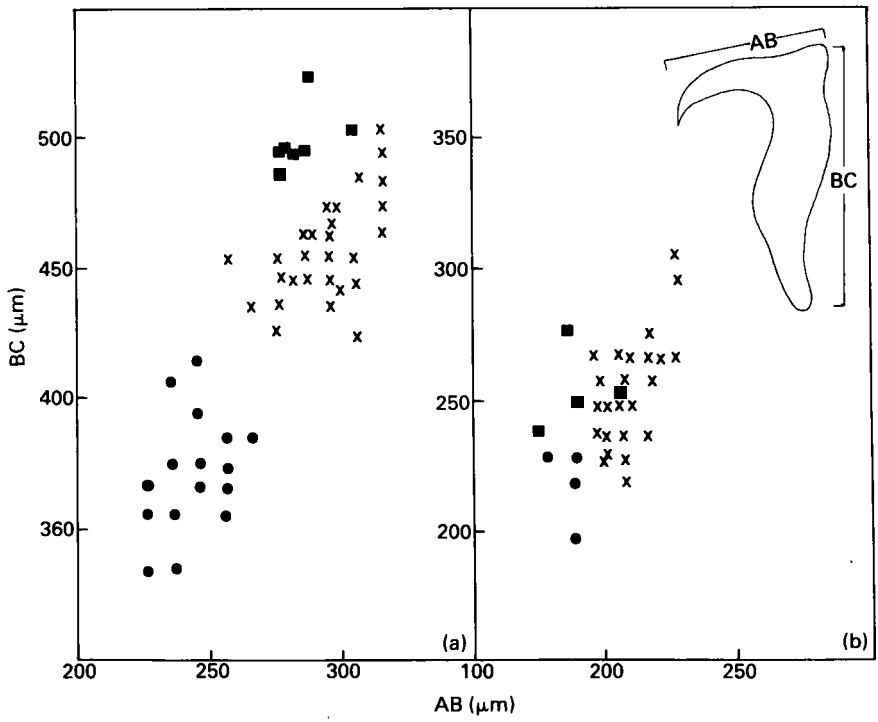

FIG 4. (a), (b) Posterior hook shank length (BC) plotted against blade length (AB, see inset) of *Raillietiella orientalis* (×), *R. boulengeri* (■), and *R. agcoi* (●) females (left) and males (right). In both sexes *R. agcoi* has smaller hooks than the two related forms. Male *R. orientalis* and *R. boulengeri* are more difficult to differentiate by hook dimensions; females are distributed into two loose cluster groups with no overlap (from Ali *et al.*, 1982b).

be inferred from single datum points and the more specimens that are measured, the more definitive and useful the character becomes.

4. *The male copulatory spicule*

Although there are undoubted intergeneric differences in the overall morphology of copulatory spicules of male porocephalids (Fain, 1961), they may be of limited use in separating species (Vargas, 1970a). In raillietiellids, however, they are of considerable taxonomic value (Gretillat and Brygoo, 1959; Ali *et al.*, 1985), showing a diversity of size and shape (Ali *et al.*, 1982a,b,c, 1984a,b; Fig. 5), and there remains considerable scope for refining and extending the use of spicule data as a discriminatory tool.

C. CEPHALOBAENIDA

It is generally accepted that the order Cephalobaenida embraces two families, the Cephalobaenidae and the Reighardiidae; the latter contains the single genus *Reighardia*, including two highly distinctive species from marine birds (Bakke, 1972; Pence, 1973; Riley, 1973a; Dyck, 1975). Two genera constitute the Cephalobaenidae, one, *Cephalobaena*, being monotypic (Heymons and Vitzthum, 1936; Cavalieri, 1970; von Haffner and Rack, 1971). The remaining genus, *Raillietiella*, by far the largest with at least 31 species, was originally divided by Heymons (1935) into five taxa, established predominantly on a variety of small differences in body shape against a background of host and geographical distribution. Fain (1961) assorted the five species described since 1935 among these same groups, but Self (1969), after reference to type material and a large collection of recently acquired specimens, concluded that there was little justification for recognizing all 19 species considered valid by Fain. Radical departures from Heymons' (1935) original scheme included a division of species from small lizards into two groups on the basis of small differences in posterior hook morphology, and the amalgamation of "slender" species from monitor lizards (family Varanidae) and worm lizards (suborder Amphisbaenia).

Nonetheless many of Self's (1969) conclusions are counterintuitive; for example, "species" from different continents and zoogeographic areas are frequently lumped together, and the many uncertainties surrounding the taxonomy of raillietiellids prompted Ali *et al.* (1981, 1982a,b,c, 1984a,b, 1985) to examine these taxa critically by evaluating more rigorous diagnostic criteria. In the majority of cases Self's conclusions were found to be unjustified, and six taxa are now recognized, based primarily on host differences—although the division of species from small lizards was retained

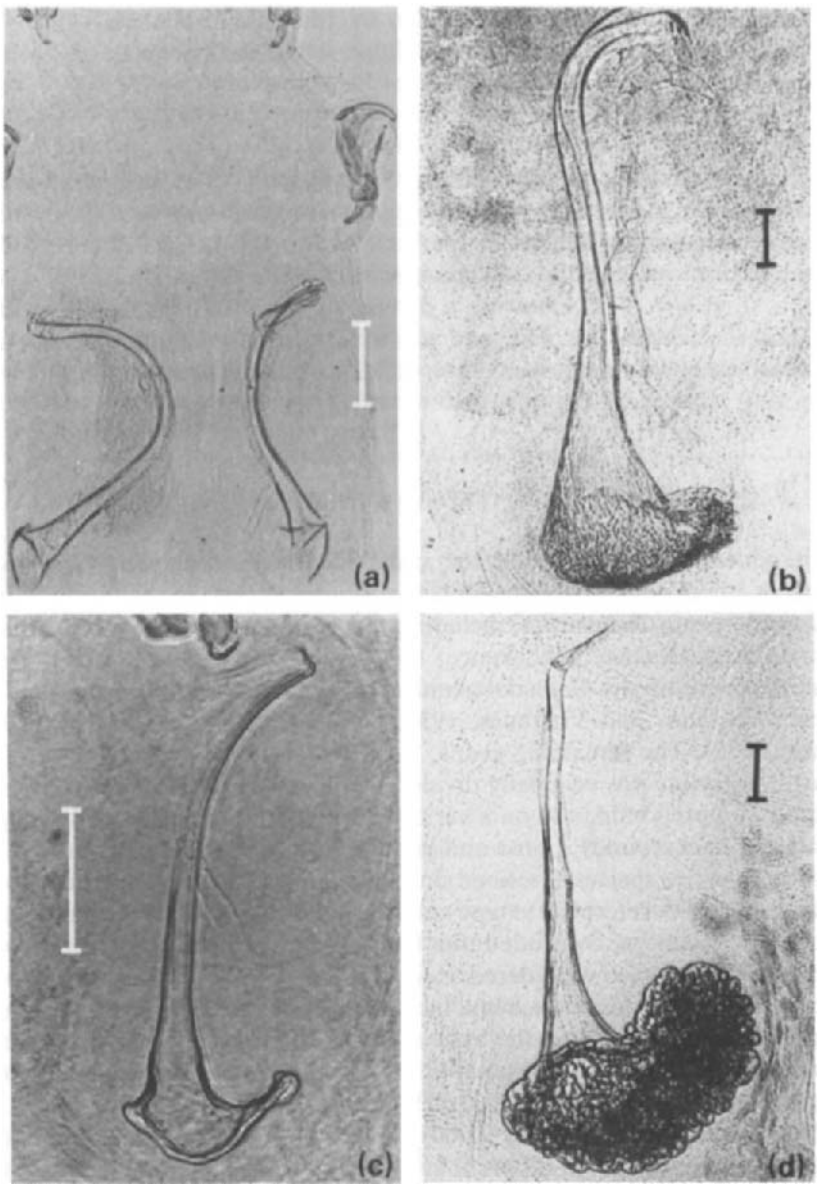

FIG. 5. Four contrasting types of copulatory spicules from raillietiellids. (a) *Raillietiella gigliolii*, the shaft is strongly curved and flares into a trumpet-shaped base. (b) *R. furcocerca*, the base is etched by a reticulum of fine grooves. (c) *R. monarchus*, showing the highly distinctive hooked base. (d) *R. orientalis*, the massive spicule base is covered by a raised tubular reticulum. (All scales = 100 μm.)

(Ali *et al.*, 1985). A number of subtle, but nonetheless distinctive, morphological characteristics, coupled with postulated differences in life cycles, indicate that the taxa represent natural rather than arbitrary groupings.

Some more recently erected cephalobaenid genera, *Megadrepanoides* (Self and Kuntz, 1957), *Mahafaliella* (Gretillat *et al.*, 1962) and *Travassostulida* (Motta and Gomes, 1968), are established on wrongly described specimens (Self and Kuntz, 1966; Self, 1969) or on premoult specimens in which so-called satellite hooks are in fact new hooks forming beneath the old (Ali *et al.*, 1985); accordingly *Megadrepanoides* is now recognized as a synonym of *Sambonia* and the others are raillietiellids. Some new additions to the genus *Cephalobaena*, proposed by Rego (1983), also belong to this latter taxon. As stated earlier, the monotypic genus *Cephalobaena* possesses several unique features (von Haffner and Rack, 1971).

D. POROCEPHALIDA

Fain's (1961) classification of the Porocephalida, a much needed revision of Heymons' (1935) outmoded scheme, was adopted (with minor changes) by Nicoli (1963) and Self (1969). It is followed here in more or less its original form but again, in the light of recent information, some further amendment is necessary (Table 1). The order is divided into two suborders, the Porocephaloidea, in which males have a single testis, and the Linguatuloidea with two testes. The latter embraces a single family, with one genus, *Linguatula*, composed of five species inhabiting the nasal sinuses and nasopharynx of carnivorous mammals (von Haffner *et al.*, 1969; Sachs *et al.*, 1973) and one (?) from the nasopharynx of caribou and reindeer (Section VI F). *L. nuttali*, a species from lions and leopards, differs from other *Linguatula* spp. in a number of minor ways, including a cleft in the caudal extremity. Von Haffner *et al.* (1969) considered this significant enough to merit the erection of a new genus, *Neolinguatula*, but in my view this discrepancy seems too trivial to warrant this discrimination.

The Porocephaloidea includes five families. The Sebekidae, recently partly revised by Self and Rego (1985), traditionally contains four genera. Three of these (*Sebekia*, *Alofia* and *Leiperia*) mature in crocodilians, although one species is also known to infect snapping turtles (Dukes *et al.*, 1971), whereas the remaining genus *Diesingia* is exclusively parasitic in freshwater chelonians (Fonseca and Ruiz, 1956). Much uncertainty still surrounds this entire group. For instance, Self and Rego (1985) consider that the monotypic species *Diesingia megastoma*, from the lungs of South American turtles, should be included within the genus *Sebekia*, despite considerable differences in hook morphology (Heymons, 1941b) and the

separation of the vagina and anus by at least seven annuli (Fonseca and Ruiz, 1956). This latter character actually places this species within the family Sambonidae, and Rego (1981b) has already suggested that the species is sufficiently different to merit its own family. This may eventually prove to be an acceptable compromise, but there is nothing whatever to justify the amalgamation of this species with *Sebekia* species. The other so-called *Diesingia* material, *D. kachugensis* (Shipley, 1910; Sambon, 1922), is known only as nymphal forms encysted on the liver of an Indian tortoise; the true identity of this species is unknown but it has few affinities with the South American species, and Fain (1961) speculated that it may be the infective stage of *Subtriquetra megacephala* (see below).

Alofia and *Sebekia* are evidently closely related although the hook morphology is quite different (Heymons, 1941c; Self and Rego, 1985). Two *Alofia* species, described by Giglioli (1922b) from unknown hosts allegedly taken in Samoa (where, incidentally, crocodilians are not found), typify the confusion which pervades this genus, and generally there is a dearth of type material. Self and Rego (1985) described a new *Sebekia* from *Caiman niger* in Brazil, Overstreet *et al.* (1985) have found another new species in *Alligator mississipiensis*, and we have a probable new form from an Australian crocodilian (Riley *et al.*, 1985). Both genera are pantropical in their distribution, particular species being associated with particular crocodilians and, because many of these host species are geographically well separated (Neill, 1971), at least some of the synonomies proposed by Self and Rego (1985) are unlikely to be correct. In many cases detailed systematic judgements must attend the arrival of better specimens.

The remaining genus *Leiperia* is composed of two species, *L. cincinnalis* from African crocodiles and *L. gracilis* from Central and South American crocodiles and caiman. However, the latter "species" is now known to be based entirely on nymphal forms (all double-hooked), allegedly of *Sebekia* and *Alofia* (Sambon, 1922; Self and Rego, 1985), and at present there is little in any of these descriptions to indicate that *Leiperia* spp. are represented at all in the Americas. I have recently received specimens of another *Leiperia* from the marine crocodile, *Crocodilus porosus*, taken off northern Australia (this awaits formal description) and thus the genus is established on at least two continents.

Another genus exclusive to crocodilians, *Subtriquetra* (Family Subtriquetridae, Fain (1961)), presently includes three species. Two, *S. subtriquetra* and *S. shipleyi*, are considered synonymous by Vargas (1975), but this seems implausible because their respective hosts are highly endemic and distinctly allopatric. After re-examining the three adult type specimens of *S. megacephala* (Baird, 1853; Sambon, 1922) I can confirm that Sambon's observation of doubled hooks is correct—in fact the hook is overlain by an

extension of the fulcrum—and this endorses the suggestions of Hett (1924) and Vargas (1975) that the species possesses a sufficient number of unique characteristics at least to justify its removal from the genus *Subtriquetra*: it may even merit its own family! Most, if not all, of the extant 21 or so species of crocodilians are likely to harbour pentastomid parasites.

The vagina and anus of females belonging to the family Sambonidae are separated by several annuli, the so-called heterogynous condition (Fain, 1961). Three *Sambonia* species from monitor lizards (family Varanidae) in the British Solomon Islands, originally wrongly described (Self and Kuntz, 1957), are morphologically diverse (Self and Kuntz, 1966). The remaining (type) species of the genus, *S. lohrmanni*, first recovered from African monitors (Noc and Giglioli, 1922), is now well characterized (Heymons, 1941a; Fain, 1961) but other, quite similar specimens from Asiatic (Heymons and Vitzthum, 1936) and Komodo monitors (Fain and Mortelmans, 1960), hitherto ascribed to the same species, should be new.

The highly distinctive genus *Elenia* (Heymons, 1932a) is also specifically adapted to monitor lizards. Unfortunately, very few specimens are available for study, and although additional material has since been described by Heymons (1939) and Riley *et al.* (1985)—which extends the known hosts and range—it is insufficient to confirm definitely that there may be more than one species. *Alofia travassosi*, from an unknown host in the Philippines, was later transferred to the genus *Elenia* because of the close correspondence in external morphology (Heymons, 1932b).

Waddycephalus spp. infect Australasian snakes and there are about ten species which are more than usually variable (Riley and Self, 1981b). More recently described small collections of adult and nymphal material, confirming some of these species, have corroborated marked differences between certain mainland and island parasite populations which infect the same snake host (Riley *et al.*, 1985; Riley and Spratt, in press). As yet, it is not clear whether these represent local races or species. This genus is the least satisfactory of those we have revised and in nearly all cases more specimens are required to establish the extent of intraspecific variation. By comparison, the taxonomy of the remaining genus *Parasambonia* (Stunkard and Gandal, 1968) is straightforward: two species are now recognized (Riley and Self, 1982; Riley and Spratt, in press).

Fain (1961) included only two genera (*Porocephalus* and *Kiricephalus*) in the family Porocephalidae and both have been the subject of recent revisions (Riley and Self, 1979, 1980; Riley and Walters, 1980). Some of the more closely related *Porocephalus* species are separated mainly on hook size (Riley and Self, 1979) and Rego (1981a) has since indirectly impugned the usefulness of this diagnostic technique, without offering any specific criticism. Rego's description of material from a fer-de-lance (*Bothrops* spp.)

does however endorse the suggestion of Riley and Self (1979) that this species may be new. The key generic character, an accessory spine overlying the outer hook (Fig. 3 in Rego (1981a), purporting to show a *Porocephalus* nymph, is in fact a *Sebekia* sp.), may have arisen independently at least twice, and if postulated differences in the life cycle are subsequently validated, the generic affinities of African and American forms should be reassessed (Riley and Walters, 1980).

Kiricephalus species constitute a morphologically distinct and homogeneous taxon but only two species (*K. pattoni* and *K. coarctatus*), which infect a large number of snake definitive and intermediate hosts, are well characterized. One is found in North and Central America and the other in South-East Asia (Riley and Self, 1980). Three other species described by Riley and Self (1980) are based on type series composed of immature specimens, and Rego's (1981b) suggestion that *K. constrictor* could be synonymous with *K. coarctatus* may be correct, particularly if it is subsequently shown that *K. coarctatus* does indeed extend well into South America; if it does, then its range encompasses several contrasting zoogeographical areas.

Fain (1961) erected the new family Armilliferidae (the alternative name Nettorhynchidae proposed by Nicoli (1963) was subsequently abandoned (Nicoli *et al.*, 1965)) to include species with circular parietal muscles arranged in thick bands so that abdominal annuli are raised and distinct. The anus opens in the same depression as the vagina (genus *Cubirea*), or a short distance in front of it but nonetheless still on the terminal segment (*Armillifer* and *Gigliolella*), or it may be separated from the vagina by up to three complete annuli (genus *Ligamifer*). This last monotypic genus is now known to be invalid; a reinterpretation of the morphology of the terminal segment firmly placed this species within the genus *Armillifer*, which now contains seven species (Riley and Self, 1981a). There are sufficiently large interspecific differences in annulus number, coupled with comparatively little intraspecific variation, to enable even nymphal forms to be reliably distinguished on this single diagnostic criterion (Fain and Salvo, 1966; Riley *et al.*, 1985).

E. CONCLUSIONS

The relatively few diagnostic criteria, with their inherent variability, may seem woefully inadequate for specific identification—in some cases this is certainly true (Ali *et al.*, 1984a)—and systematists accustomed to working with more amenable material could argue that pentastomid taxonomy has yet to become a science. The situation is not as bleak as this and, generally,

appropriate combinations of hook size, annulus number and the form of the male copulatory spicule, against a background of host and geographic distribution, life cycle differences etc., are adequate for specific diagnosis. Nearly always, though, there is a dearth of preserved material and supportive experimental work, and the subject is ripe for further evaluation, perhaps with recourse to genetical and biochemical techniques. Such studies will probably confirm the validity of most of the remaining poorly described species.

Taxonomical problems are greatly reduced when the identity of the host, and the locality, are known. A rough calculation reveals that over 50% of species have only one host species, and many hosts show a high degree of endemicism. Host specificity among most pentastomid species is thus quite marked and particular genera are exclusive to particular taxa of hosts (Table 1). By contrast, a few species, particularly some large raillietiellids (Ali *et al.*, 1982b, 1985), each infect 12–14 snake species belonging to three or four families. However, from a consideration of host dietary regimen and potential intermediate hosts, there are grounds for believing that in each of these instances there may be more than a single pentastomid species involved (Ali *et al.*, 1984b); this must remain speculative until more specimens become available.

IV. Structure and Function

A. The Body Wall and Locomotion

Pentastomids, in common with the larvae of many holometabolous insects, move by changes in the shape of the body (von Haffner, 1972). The cuticle is soft and flexible and does not, by itself, provide a suitable skeleton upon which muscles can act. Rather it is the pressure of haemolymph within the body which provides a hydrostatic skeleton for support and locomotion. Thus a fluid skeleton is the basis of muscle antagonism in (mainly) parietal longitudinal and circular muscle systems; muscle tone maintains body shape and peristaltic muscle contraction wrinkles the cuticle to achieve locomotion. In *Linguatula* spp. forward locomotion is achieved as peristaltic waves pass from tail to head (von Haffner *et al.*, 1969; von Haffner, 1972). In this genus, rows of spines project backwards from the posterior angles of each segment and, by exerting a strong frictional contact with host epithelia, presumably prevent backslip. As each wave of contraction reaches the head, the latter is elevated to allow extension; the hooks may assist in "looping" locomotion (von Haffner, 1972). Serial longitudinal muscle contractions of this complexity call for a high degree of co-ordination.

As mentioned earlier, the structure of the cuticle is distinctly arthropodan and it most closely resembles the newly synthesized procuticle or thin cuticles of arthropods which move similarly (for example, dipteran maggots —see Filshie (1970a)). This was confirmed by ultrastructural studies of the cuticle of the primitive pentastomid *Reighardia sternae* (Riley and Banaja, 1975) and the advanced species *Porocephalus crotali* (Trainer *et al.*, 1975; Fig. 6), though the latter authors conclude that the *P. crotali* cuticle is simpler in construction than that of any known arthropod. However, this is not supported by their micrographs, nor by our subsequent re-examination of the same species (Fig. 6). The outermost layer of the pentastomid cuticle, identified as a cuticulin layer (*sensu* Locke, 1966) by Riley and Banaja (1975), is overlain by lamellate secretion (Fig. 13) which originates in gland cells suspended in the haemocoel but connected to the cuticle by tenuous, chitin-lined, efferent ductules (Fig. 7). Secretion may be instrumental in attenuating the host immune response (Riley *et al.*, 1979; see Section IX), and, as such, is clearly an adaptation to endoparasitism. This differs from the situation in many insects where outer epicuticular layers are present, secreted through pore canals in the cuticle, which confer special permeability properties upon the cuticle (Filshie, 1970b).

Muscle attachments to the cuticle, involving intermediate tendinous epidermal cells containing bundles of microtubules aligned along the long axis of the stress, are again uniquely arthropodan (Banaja and Riley, 1974; Fig. 6). An innovation of pentastomid muscle attachments relates to the use of the body wall in locomotion. Parietal longitudinal and circular muscle systems form an open basketwork of muscle fibres (von Haffner, 1971; Mill and Riley, 1972; Banaja and Riley, 1974); the precise control of cuticle movement is achieved by frequent lateral attachments of individual fibres via tendinous epidermal cells which are modified to be suspensory rather than anchor-type connections associated with muscle termini (Banaja and Riley, 1974; Riley *et al.*, 1978). Alternative interpretations of tendinous cells (Trainer *et al.*, 1975) arose through a failure to identify microtubules. Growth in pentastomids proceeds through a series of moults and the events in the moult sequence follow precisely the arthropod pattern (Riley and Banaja, 1975; Riley *et al.*, 1978; Fig. 6). It is surprising that endoparasitism in pentastomids has produced remarkably little modification of the basic ground plan of the arthropod cuticle.

B. GLANDS ASSOCIATED WITH THE CUTICLE

An abundance of glandular systems, many composed of large secretory cells suspended in the haemocoel, has long been recognized as one of the

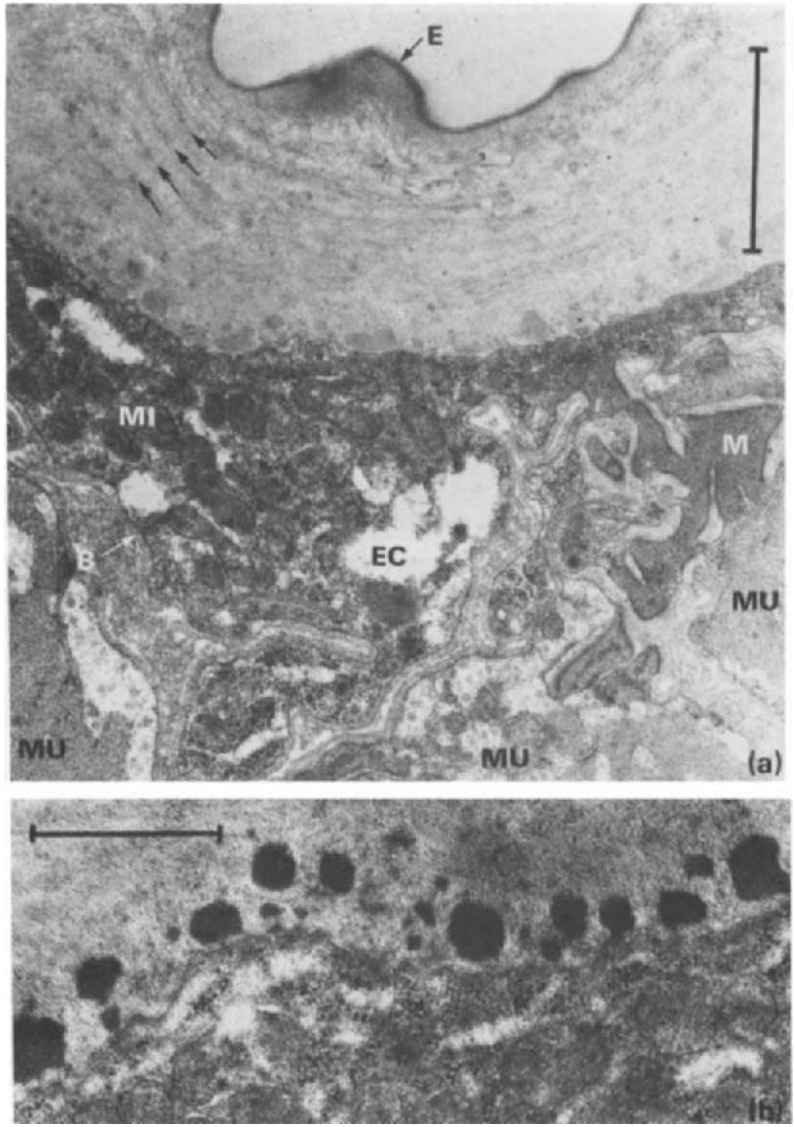

FIG. 6. (a) Part of the tegument of an infective nymph of *Porocephalus crotali* showing the dense epicuticle and fibrillar endocuticle which is organized into loose lamellae (arrows). The underlying epidermal cells contain fields of mitochondria and microtubules. Microtubules form tendinous connections between the endocuticle and the underlying muscle layers (scale bar = 2 μm). Abbreviations: B, basal lamina; E, epicuticle; EC, epidermal cell; M, microtubules; MI, mitochondria; MU, muscle. (b) Junction between the endocuticle and an epidermal cell showing a row of dense ecdysial droplets immediately before the onset of moulting. The fibrillar nature of the endocuticle is obvious (scale = 1 μm).

principal characteristics of pentastomids (von Haffner, 1924a). Doucet (1965) distinguished three broad categories of gland cells: those annexed to the viscera; sub-cuticular (parietal) glands; and cephalic (frontal and hook) glands. Histologically, glands were further subdivided into those without secretory ducts, normally an integral part of the alimentary and reproductive systems, and exocrine glands typically discharging secretion into ducts. Riley (1973a) has commented on inconsistencies associated with the nomenclature of some of the more important gland systems.

Two distinct types of exocrine gland cell are associated with the cuticle of pentastomids and, in common with insects (Noirot and Quennedey, 1974), a general feature is the presence of cuticle covering the glandular structures. Classes of gland cells can be recognized, each defined with respect to the cuticle and the manner of egress of the secretion. Epidermal glands can be compared directly with those of insects and, following the convention of Noirot and Quennedey (1974), the chloride cells (*sensu* Banaja *et al.*, 1977) would be analogous with class I cells: each cell is covered by modified cuticle (Fig. 7), secreted by the cell itself, and cell secretion must somehow penetrate this barrier, there being no duct. Class II cells are known only from termites, but the remaining class III-type cells are well represented in pentastomids: sub-parietal cells, frontal and hook glands (*sensu* Riley, 1973a; Riley *et al.*, 1979) fit into this category. Always, chitin-lined ductules, elaborated by ductule-secreting cells, are interposed between the secretory cells and the cuticle; they are continuous with the cuticle but also enter the secretory cells; their function is simply to convey secretion on to the surface of the cuticle (Fig. 7). Chloride cells and sub-parietal cells are often intimately associated in that the efferent ductules of the latter emerge onto the cuticle close to the cuticular caps which plug the necks of chloride cells (Doucet, 1965; Banaja *et al.*, 1977; Riley *et al.*, 1979; Figs 7 and 9C).

Class I cells (previously called stigmatal or cuticular glands—see Riley, 1973a) are present in all pentastomids except *Cephalobaena tetrapoda* (von Haffner and Rack, 1971). They are pyriform cells, occurring singly or in groups (depending upon the species—see Doucet, 1965), with necks converging onto caps of amorphous material contained within an annulus embedded in the cuticle proper (Figs 7 and 9C). Normally they are disposed in lines or broad bands associated with the raised part of each annulus and with the cephalothorax (Doucet, 1965). The most anterior annuli carry the greatest concentrations; gland cells tend to disappear gradually towards the caudal extremity (Doucet, 1965). Ultrastructurally these cells compare with the chloride cells of aquatic insects and many other cells known to be involved in electrolyte transport (Banaja *et al.*, 1977). Bovien (1927) commented on the presence of modified (class I) cells on the anterior cephalothorax of *Raillietiella* species and Heymons (1935) found many

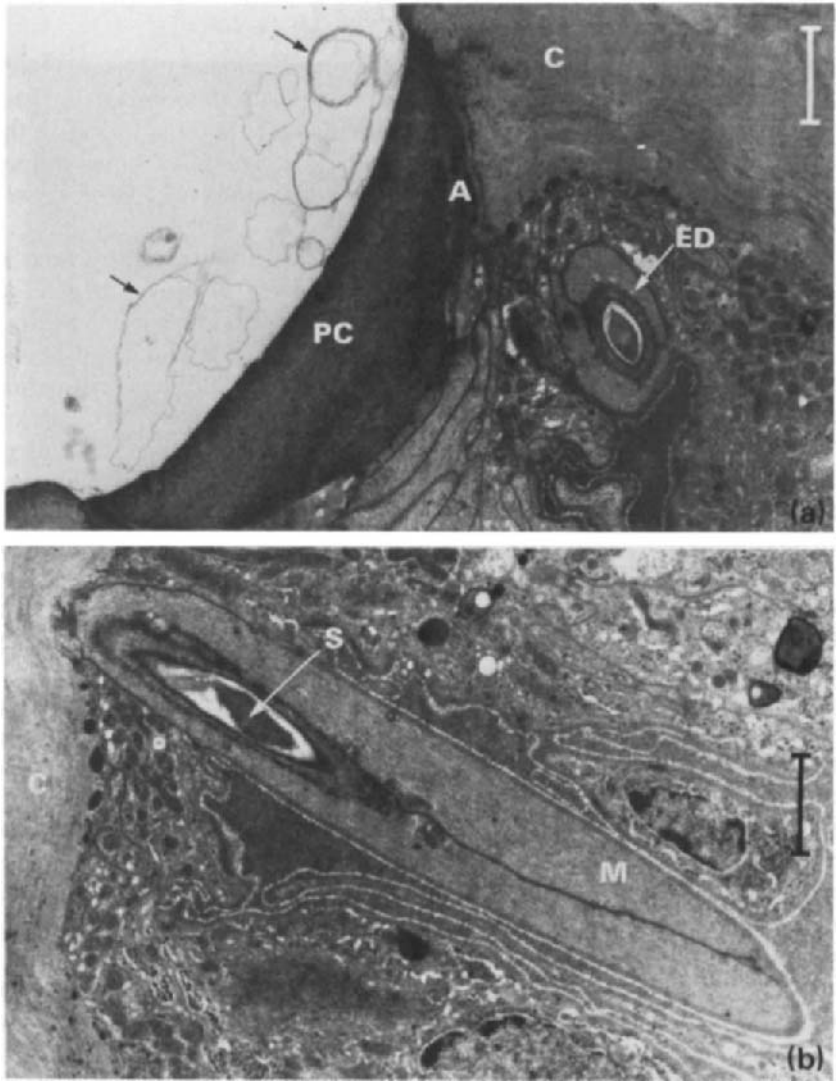

FIG. 7. (a) Cuticle of *Porocephalus crotali* sectioned through the edge of pore cap positioned above a chloride cell (not visible). An annulus supports the cap which is overlain by whorls of lamellate secretion (arrowed) emanating from sub-parietal cells. An efferent duct from such a cell, filled with amorphous secretion, is sectioned transversely (scale = 2 μm). (b) A longitudinal/oblique section through a sub-parietal cell efferent ductule of *P. crotali* at the point of penetration of the cuticle: again amorphous secretion is present. The cytoplasm of the ductule-secreting cell is full of longitudinally-orientated microtubules (scale = 2 μm). Abbreviatons: A, annulus; C, cuticle; ED, efferent duct; M, microtubules; PC, pore cap; S, amorphous secretion.

similar cells on the parapodial lobes of *R. mediterranea.* Doucet (1965) confirmed these observations in related species and demonstrated histochemically a secretory function. Other modified class I cells, found on the lateral abdominal margins of *Neolinguatula* (= *Linguatula*) *nuttali* associated with porous plates set in the cuticle, are innervated (von Haffner *et al.*, 1969; von Haffner, 1971) and may be chemosensory.

The remaining class III cells, the frontal and hook glands, which in *Reighardia* at least have a similar structure and, possibly, function to sub-parietal cells (Riley *et al.*, 1979), exhibit marked differences in disposition between the two pentastomid orders. Both types of gland in cephalobaenids are limited to the cephalothorax and their diffuse construction renders the exact limits of each difficult to define (Riley, 1973a). Each is composed of loosely aggregated secretory lobules, consisting of 2–3 large cells, discharging secretion into a minute, thin-walled efferent ductule. All the ductules emanating from the frontal gland were originally thought to converge onto the buccal capsule (Doucet, 1965; Riley, 1973a,b), and while it is true that some do, most appear to empty on to the cuticle on the anterior margin of the head (Riley *et al.*, 1979). Hook glands flank the frontal gland and their efferent ductules empty into the hook pits (Doucet, 1965; Riley *et al.*, 1979).

By contrast, the equivalent glands in porocephalids are composite and well separated, in that hook glands occupy the cephalothorax whereas paired frontal glands flank the intestine (Fig. 8); the large collecting ducts serving these structures can be readily seen by light microscopy (Spencer, 1893; von Haffner, 1924a). However, closer examination of these glands reveals that, as before, efferent ductules serve individual secretory lobules before converging onto common collecting ducts (von Haffner, 1924a); the four collecting ducts from hook glands enter hook pits and the paired ducts from frontal glands open near to the frontal papillae.

Ultrastructural aspects of frontal, hook and sub-parietal gland function in *Reighardia sternae*, investigated by Riley *et al.* (1979), are intriguing in that all seem to elaborate lamellate secretion. The entire surface of the cuticle, including the mouth and hook pits, is coated with membranes from gland cells, some of which are continuously active (Figs 7 and 13). This implied steady turnover of surface membranes may protect the parasite from the host immune response (Riley *et al.*, 1979; Section IX).

C. OTHER GLANDULAR SYSTEMS

Very little is known about glands associated with the oesophagus and rectum apart from their cytological appearance (Hett, 1924; Doucet, 1965) and this

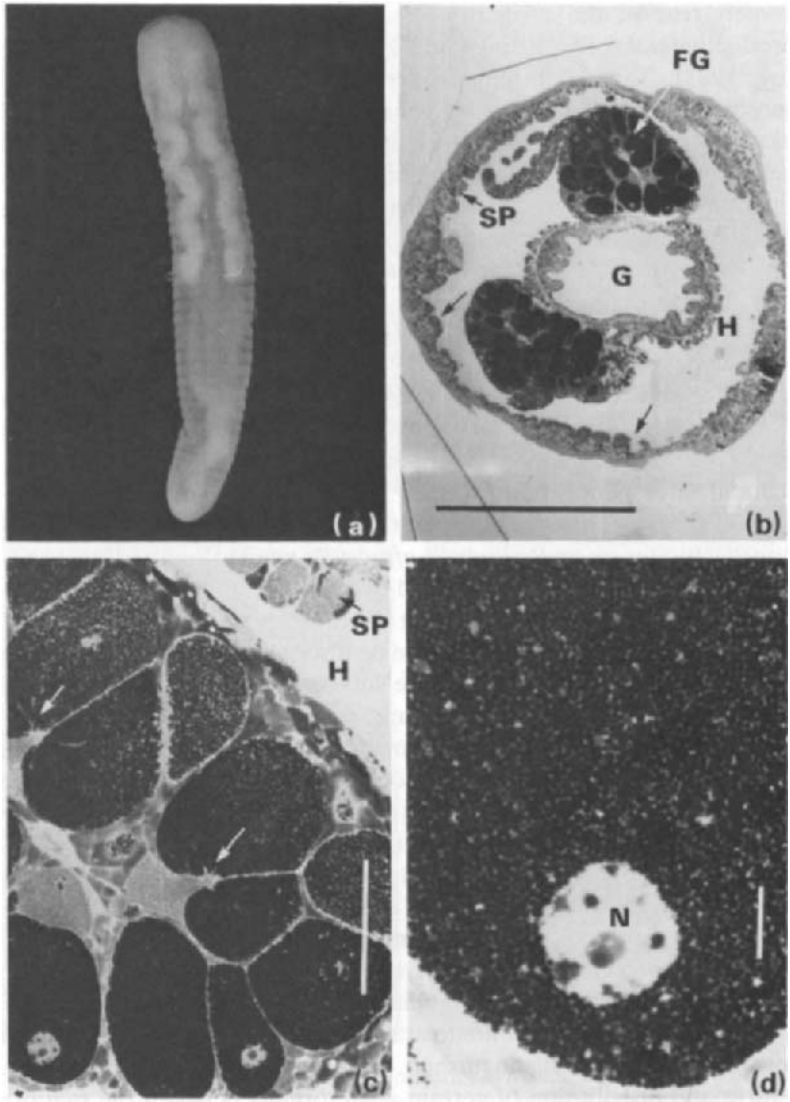

FIG. 8. (a) Living, infective nymph of *Porocephalus crotali*. The cephalothorax (uppermost) contains the hook glands whilst the white frontal glands flank the anterior half of the intestine. The nymph is 1 cm long. (b) Transverse section through an infective nymph in the region of the frontal gland. The latter is composed of large cells charged with densely staining secretory droplets. Smaller sub-parietal gland cells are arrowed (scale = 1 mm). (c) and (d) Detail of the gland cells in (b). Two collecting regions which conduct secretion to a central duct are arrowed (scales = 100 μm in (c) and 10 μm in (d)). Abbreviations: FG, frontal gland; G, gut; H, haemocoel; N, nucleus; SP, sub-parietal cell.

is mostly true of the accessory glands which are incorporated into the reproductive tract of both males and females (reviewed by Nørrevang, 1983; Riley, 1983). Thin, chitin-lined ductules serving large gland cells in the extensively developed accessory genital gland of *Reighardia* (these again rank as class III cells) conduct secretion into a transverse canal near the junction of the oviduct and spermathecae (Riley, 1973a; Böckeler, 1984c), which may be partly responsible for the nutrition of oocytes (Böckeler, 1984a). In male cephalobaenids, paired diffuse gland systems of unknown function are associated with the lower vas deferens and copulatory spicules (Riley, 1983).

D. NEUROANATOMY AND SENSE ORGANS

Doucet's (1965) meticulous account of complex arrangements of nerves and ganglia in three pentastomid species highlights constant differences in the degree of fusion of ganglia between the two orders which have since been confirmed by studies on *Raillietiella affinis*(?) (von Haffner, 1971), *Cephalobaena tetrapoda* (von Haffner and Rack, 1971), *Porocephalus crotali* (Hollis, 1979) and *Reighardia sternae* (Böckeler, 1982, 1984b).

Seven, initially separate ganglia can be discerned during the development of *Reighardia*; three fuse to form the sub-oesophageal ganglion, complete with a circumoesophageal commissure, one remains solitary, and ganglia 5–7 fuse to form another complex mass; arrangements in other cephalobaenids are interpreted similarly (Böckeler, 1984b). By contrast, a single compact sub-oesophageal ganglion is characteristic of porocephalids (Table 2) and it supplies 8–11 paired nerves (depending on the species—see Hollis (1979)). Paired nerves give rise to trunks and branches which innervate, almost exclusively, the organs of the cephalothorax (for details see Doucet (1965)). The most posterior pair, much smaller than the cephalobaenid equivalent, represent rudiments of a ventral nerve cord whose branches extend deep into the abdomen (Hollis, 1979).

Many of these nerves terminate at sense organs set in the cuticle, several types of which may be distinguished. Again there are major differences in the form and distribution of certain sense organs between the two orders (see Ali and Riley (1985), who point out some of the inconsistencies in the nomenclature of (particularly) cephalic papillae).

Chains of lateral papillae, set between the abdominal annuli, are known in at least five porocephalid genera (von Haffner, 1926; Hollis, 1979; von Haffner *et al.*, 1969; Riley and Self, 1981a; Winch and Riley, in press a; Fig. 9), and they are also present in cephalobaenids (Doucet, 1965; Ali *et al.*, 1981). Doucet (1965) and von Haffner *et al.* (1969) have demonstrated

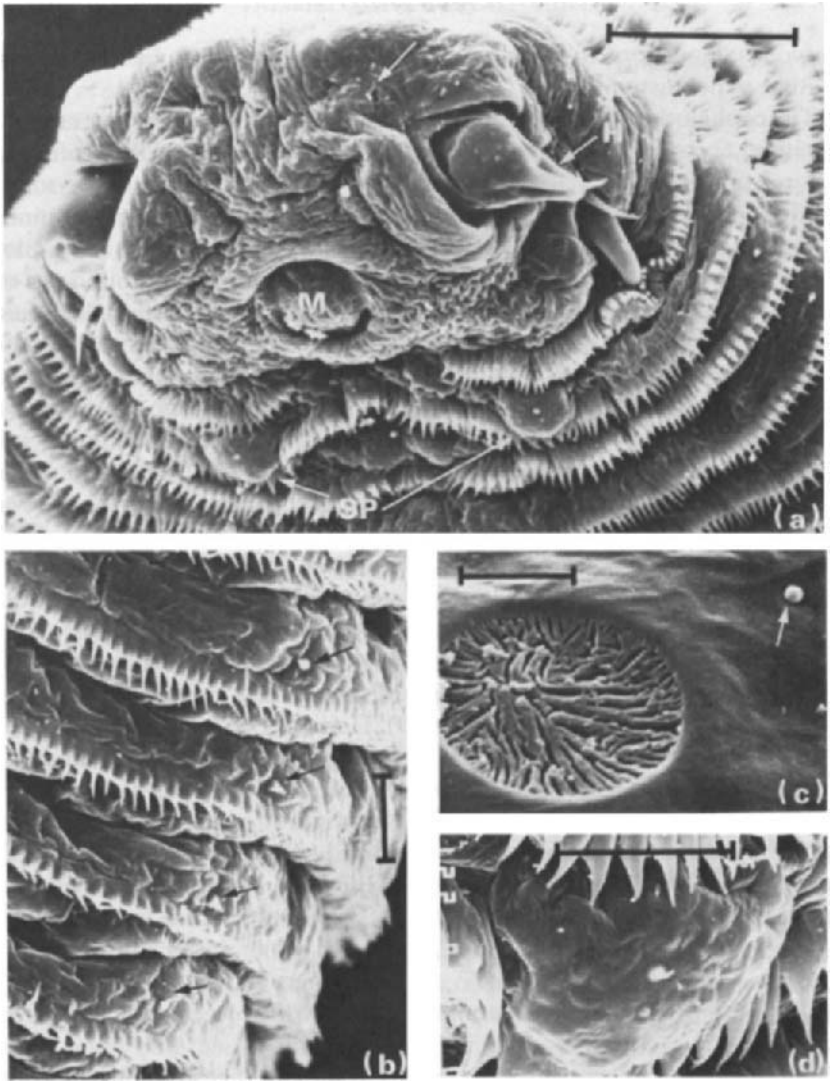

FIG. 9. Antero-ventral view of the cephalothorax of a male infective larva of *Sebekia oxycephala*. Two pairs of doubled hooks flank the mouth, the emergent ducts of frontal glands (arrowed) are above the inner hooks, and large sensory papillae, delimiting the posterior edge of the cephalothorax, sit on either side of the genital pore (scale = 100 μm). (b) Ventro-lateral margin of the mid-abdomen showing sensory papillae (arrowed) on each abdominal annulus (scale = 20 μm). (c) Grooved surface of a chloride cell pore cap set in the cuticle with the emergent ductule of a sub-parietal cell (arrowed) nearby (scale = 5 μm). (d) One of the sensory papillae seen in (a), supporting two types of sensilla (scale = 20 μm). Abbreviations: H, hook; M, mouth; SP, sensory papilla. (Micrographs courtesy of Dr. J. M. Winch.)

nervous connections to these papillae which may prove to be a ubiquitous feature of pentastomids.

The cephalothorax is richly endowed with sense organs. Prominent dorsal papillae, apparently present in all cephalobaenids except *Reighardia* spp. (Storch and Böckeler, 1979; Ali and Riley, 1985), carry, according to von Haffner (1971), a number of sensory sensillae, but these are not apparent in scanning electron microscope studies of related species (Ali and Riley, 1985). The greatest concentration of sensory structures is found on the ventral face of the cephalobaenid cephalothorax, and early light microscopical studies have shown that the frontal papilla is divided into two quite separate zones, innervated independently (Doucet, 1965). Each zone bears sensory sensillae grouped in characteristic, but conservative, patterns (Ali and Riley, 1985); additional fields of sensillae are found around the mouth. Furthermore, transmission electron microscope (TEM) studies have revealed that these sensillae show a close correspondence, in virtually every detail, to the sensillae of arthropods (Storch and Böckeler, 1979, 1982; Storch, 1979, 1984). Speculation concerning sense organ function was summarized by Ali and Riley (1985). Details of the anatomy and distribution of the complex sense organs on the cephalothorax of porocephalids, visible under the light microscope as broadly conical elevations of the cuticle (see Hett (1924) for a synopsis of papilla distribution), are provided by von Haffner (1926), von Haffner *et al.* (1969), Hollis (1979) and Winch and Riley (in press a). Frontal papillae, near to which frontal gland ducts discharge (Fig. 9), contain at least three morphologically distinct types of sensory cell (von Haffner, 1926). Hollis (1979) found additional clusters of minor cephalic papillae on either side of the head of critical-point freeze-dried specimens of *Porocephalus crotali*, and Riley and Winch (in press a) made similar observations of this and other areas of *Sebekia* larvae in fish (Fig. 9).

E. FEEDING AND DIGESTION

Relatively few studies of the alimentary tract and digestive physiology of pentastomids exist but, as far as is known, most adults feed on blood which is sucked from ruptured capillaries (Fig. 12) by an oral papilla coupled to a pharyngeal pump (Giglioli, 1922b; Doucet, 1965; Riley, 1973b). Haematin (or haemosiderin in *Porocephalus crotali*—see Self (1972)), the end-product of haemoglobin digestion, accumulates in the gut lumen and imparts a dark colouration to the intestine which is easily visible through the body wall. Streaks of haematin-contaminated mucus and discoid scars in the lungs are obvious manifestations of feeding activity in many reptiles infected with pentastomids (Deakins, 1973; Riley, 1981; Fig. 12). Encysted nymphs feed

on blood (Self, 1972; Self *et al.*, 1972), lymph and lymphoid cells (von Haffner *et al.*, 1967), or both.

A useful introduction to the histochemistry of the alimentary tract of four species is that of Doucet (1965). A short oesophagus, lined by a chitinous intima, communicates via an oesophageal–intestinal valve with an undifferentiated midgut composed of columnar or cuboidal gastrodermal cells surrounded by muscle fibres and suspended by mesenteries. Digestion in two species of cephalobaenids is predominantly extracellular because large quantities of haematin form in the gut lumen (Rao and Jennings, 1959; Riley, 1972a), but there is some intracellular digestion since iron accumulates in certain gastrodermal cells which are then shed periodically into the gut lumen. No comparable study has been attempted using porocephalids.

F. THE REPRODUCTIVE TRACT

The entire subject of reproduction in pentastomids has been reviewed by Riley (1983), and more restricted reviews have been given by Nørrevang (1983) and Self (1983); therefore only the more interesting and unique aspects need be enlarged upon here. The principal anatomical features of the reproductive tract and associated organs are illustrated in Fig. 10.

1. *Female reproductive system*

The bulk of the haemocoel of mature females is occupied by the uterus (Fig. 12), which contains eggs in various stages of development. This is true of all females even though there are fundamental differences in the form of the uterus between the two orders (Riley, 1983; Table 2; Fig. 10). Many aspects of the reproductive biology of pentastomids are highly singular and this stems, in part, from the unusual design of the female reproductive tract, particularly with respect to the relative positions of the spermathecae and genital pore, and the functional anatomy of the spermathecae themselves (Riley, 1983).

The females of many arthropods possess a seminal receptacle or spermatheca which receives sperm during insemination: often this is an entirely separate process from the actual fertilization of eggs, which may not occur until some considerable time later. When spermathecae are associated with the reproductive tract they are generally located at or near the base of the oviduct close to its junction with the vagina; in any event they are relatively accessible to the male genitalia during copulation (Chapman, 1971). By contrast, in pentastomids paired spermathecae are found at the junction of the oviduct and the *distal* uterus, and, because the uterus expands

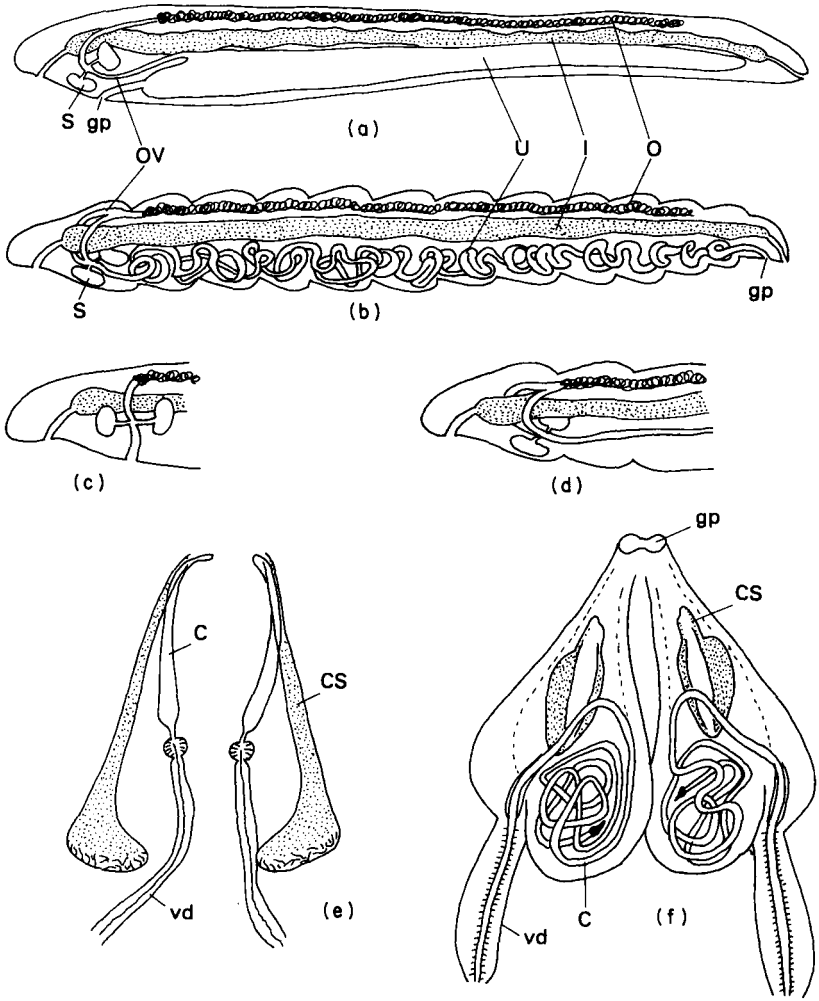

FIG. 10. Diagrammatic representations of the male and female reproductive systems of cephalobaenids and porocephalids. (a) Mature female cephalobaenid showing the relative positions of the ovary, oviduct and spermathecae and the saccate uterus leading to an anterior genital pore. (b) Female porocephalid with an elongate, coiled uterus leading to a posterior genital pore. (c) and (d) Anterior regions shown in (a) and (b) at the time of insemination. The uterus is undeveloped so that spermathecae are relatively accessible through the genital pore. (e) Paired penises and copulatory spicules of a cephalobaenid. The spicules are grooved and may serve to guide the penises to the spermathecae. (f) The same in a male porocephalid. The penises are long and coiled, reflecting the greater distance between the female gonopore and the spermathecae. The copulatory spicules may introduce the penises into the female but their extension depends upon peristaltic muscle action of the uterus/vagina. (All schematic and not to scale: modified from Riley, 1983.) Abbreviations: C, cirrus; CS, cirrus sac; gp, genital pore; I, intestine; O, ovary; OV, oviduct; S, spermatheca; U, uterus; vd, vas deferens.

enormously during development, they become progressively more remote from the vagina (Fig. 10).

The evidence suggests that during insemination sperm must be placed directly within spermathecae from the tips of the paired penises of the male. This transfer is practicable only during a critical "window" in the early development of females before the uterus has developed (Sambon, 1922; Fig. 10). Thus females display a precocious sexual development with the once-in-a-lifetime event of insemination heralding long prepatent and patent periods (Riley, 1981, 1983; Ali and Riley, 1983). Only in *Reighardia* is the patent period restricted to just a few days (Banaja *et al.*, 1975, 1976) and it now seems that this particular pattern of egg production is quite atypical (Ali and Riley, 1983; Winch and Riley, 1985).

Clearly spermathecae are vitally important organs combining two functions which at first sight seem irreconcilable: they store sperm from the time the female is inseminated to the termination of the patent period—in most species this interval is likely to be of many years duration (Riley, 1981; Ali and Riley, 1983)—and yet they are able to provide for the continuous fertilization of oocytes.

Copulation in cephalobaenids has never been observed, but Riley (1983) has shown that in *Armillifer* sp., fixed in coition and subsequently sectioned, the paired penis threads travel along the vagina/uterus to at least the level of the anterior spermathecae. The upper spermathecal duct, giving final access to the spermathecal lumen, is a tapered, heavily chitinized tube which, in certain porocephalids, narrows from an initial diameter of 20–30 μm to 3–4 μm at its point of entry into the spermathecae. Since its shape complements that of the ornamented chitinized tip of the male penis, it has been suggested that the penis must dock within the duct before the actual transfer of sperm can occur (Riley, 1983). Spermathecae probably function to isolate sperm totally from external influences, and stored sperms remain metabolically inactive and quiescent; the extreme narrowness of the access duct ensures that relatively few sperms are expressed at each contraction of the spermatheca and this provides a fine control over the number of sperms released for fertilization (Riley, 1983). Also the narrowness effectively precludes the possibility that sperms could swim unaided into the spermatheca if deposited elsewhere in more remote regions of the female reproductive tract. Hence the male genitalia are appropriately modified to conduct sperms directly into the spermathecae (Fig. 10).

Ultrastructural aspects of the functional morphology of the female reproductive tract of *Reighardia* have been dealt with by Böckeler (1984c), who drew attention to a large accessory gland, first reported by Riley (1973a), which enters the system near to the base of the oviduct. Its function is unknown but it may contribute towards the nutrition of oocytes.

With the exception of *Reighardia* (Banaja *et al.*, 1976), ovulation and egg deposition in pentastomids is a continuous process (e.g., Riley, 1981; Ali and Riley, 1983; Winch and Riley, 1985), although Nørrevang (1972, 1983) provided some evidence that in *Raillietiella hemidactyli* oocytes are not produced beyond a given time. Continuous egg release in cephalobaenids is achieved in an unusual way: Ali and Riley (1983) and Winch and Riley (1985) have shown that patency commences in raillietiellids when around 75% of eggs in the saccate uterus are small and undeveloped, and yet only large, fully embryonated eggs are released to the host lung. They postulated therefore that the vagina must be equipped with some sort of sieving device, which we have recently shown to be a relatively simple structure (Fig. 11), certainly far simpler than the equivalent sorting mechanism of acanthocephalans (Whitfield, 1970). Essentially the vagina consists of two functionally distinct regions, a chitinous cylinder next to the uterus, which is surrounded by an epidermal layer and longitudinal and circular muscle systems—this performs the sieving—and a wider anterior region lined by epidermis, which temporarily stores eggs before release (Fig. 11). The cylinder, which is substantially narrower than an egg, is filled by a thick amorphous matrix pierced by an exceedingly narrow lumen. This matrix clearly acts as a physical barrier to the exit of eggs, but equally it must be easily deformable so that, when mature eggs of the appropriate dimensions lodge in the uterus end of the system (triggering stretch receptors?), muscle contraction can force eggs through by peristalsis (Fig. 11).

2. *Male reproductive system*

The differences apparent in the form of the male reproductive tract of cephalobaenids and porocephalids (Fig. 10) reflect the proximity of the spermathecae to the genital pore of the female. Self's (1983) statement that variation in the gross morphology of the male system reflects only specific differences is patently not true.

Thus the paired penises of cephalobaenids are comparatively short and lodged within grooves in the hollow shafts of club-shaped copulatory spicules (Mahon, 1954; Fain, 1961), whereas the porocephalid penises are very elongate and coiled within sacs (Heymons, 1935) and only the base of each penis passes through the copulatory spicule, which may become partially everted during copulation (Riley, 1983; Fig. 10). Self (1983) believed that transmission of sperm to the female is accomplished by these spicule-like dilators, which may actually serve as a penis, but there is no evidence whatever to support this notion. Spicules may, in the first instance, guide the penis into the female but certainly in porocephalids their design precludes direct dilation of spermathecae (Fig. 10).

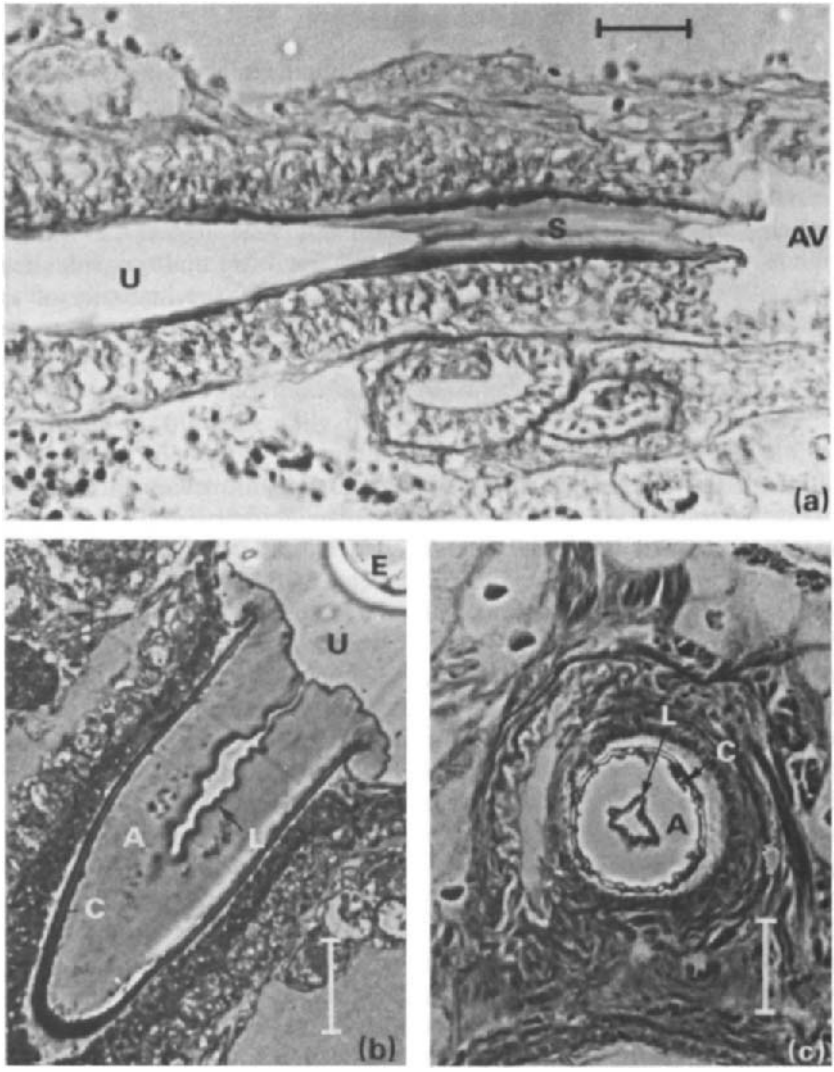

FIG. 11. (a) Wax-embedded 8 μm section through vagina of *Raillietiella gehyrae* showing uterus, sieving mechanism and anterior vagina in which eggs are stored before release. The sieve, a chitinous cylinder 25–30 μm in diameter, allows through mature eggs (approx. 100 × 80 μm) and yet retains smaller, undeveloped eggs. (b) and (c) Resin-embedded 1 μm sections of the sieve of *R. gigliolii* in oblique and transverse section showing the chitinous cylinder, almost totally occluded by amorphous material, save for a narrow lumen. Eggs must be forced through this matrix by peristalsis developed by the enveloping muscle layers. Note that an *R. gigliolii* egg measures 130 × 90 μm. (Scales = 35 μm.) Abbreviations: A, amorphous matrix; AV, anterior vagina; C, chitinous cylinder; E, egg; L, lumen; S, sieve; U, uterus. (Micrographs courtesy of Mr S. O'Malley.)

In both orders sperm is stored in paired seminal vesicles which, when full, may check spermatogenesis (Wingstrand, 1972; Overstreet *et al.*, 1985). The movement of sperm from the seminal vesicles has not been observed (Self, 1983) but Riley (1983), from a consideration of the functional anatomy of the various regions of the reproductive tract, has speculated about the probable sequence of events. Overstreet *et al.* (1985) suggest that spermatophores may be produced by male *Sebekia* but I can find no evidence in their account to support this: my observations of the anatomy of the reproductive tract of several *Sebekia* spp. reveal it to be conventional in every respect.

3. *Oogenesis*

Although the detailed anatomy of the pentastomid ovary is known in relatively few species, namely *Armillifer armillatus* (von Haffner, 1922; Nørrevang, 1972), *Raillietiella hemidactyli* (Nørrevang, 1972), *Raillietiella aegypti* (Walldorf and Riehl, 1985; Riehl and Walldorf, 1985) and *Reighardia sternae* (Böckeler, 1984a), a common structure is apparent (Nørrevang, 1983). In most species the ovary is single but in some it may be divided anteriorly or posteriorly (Heymons, 1922; Fain, 1961; Nørrevang, 1972), indicating that it was originally a paired organ (Nørrevang, 1983). Ovaries are peculiar inasmuch as mature oocytes are located on the outside of the ovary where they are bathed directly by haemolymph (Nørrevang, 1972, 1983; Böckeler, 1984a; Walldorf and Riehl, 1985). Oocytes migrate to this position via the ovarial lumen into which they are liberated from germinal epithelia; they are finally supported by a stalk cell and separated from the haemocoel by a thin basal lamina. A covering of microvilli enables oocytes to absorb some nutrients from the haemolymph but the source of yolk appears to be different at different stages of oogenesis (Nørrevang, 1983). Walldorf and Riehl (1985) have followed the previtellogenic and vitellogenic stages of *R. aegypti* eggs in considerable detail but it remains unclear whether yolk is synthesised within oocytes or produced elsewhere and then endocytosed into the oocyte cytoplasm, and, in *Reighardia*, an accessory genital gland may be partially responsible for yolk production (Böckeler, 1984a). Other aspects of oogenesis in pentastomids have been reviewed by Riley (1983) and Nørrevang (1983).

4. *Spermatogenesis*

Wingstrand's (1972) exhaustive studies of sperm structure and development in the cephalobaenid *Raillietiella hemidactyli*, reported in abridged form by Self (1983), confirmed the earlier observations of von Haffner (1924c) and

Doucet (1965) that mature spermatozoa are distinctly filiform, a necessary attribute facilitating the passage of sperms along the elongate narrow penis and through an even narrower spermathecal duct (Riley, 1983).

The testis of *Raillietiella* species, an unpaired thin-walled sac lying mostly in the posterior half of the abdomen, is connected to the dorsal body wall by a median mesentery. Its epithelium consists of two types of cell, predominantly phagocytic vegetative cells, whose role is to recycle debris suspended in the vesicular lumen, and germinal cells (primary spermatogonia); these correspond to the "nutritive" and "germinative" cells of von Haffner (1924c) (Wingstrand, 1972). The mature spermatozoon of *Raillietiella* is 100–130 μm long, and the anterior part constitutes a pseudoacrosome (which differs fundamentally from true acrosomes in its development) followed by the "body" containing a filiform nucleus, three filiform mitochondria and an axonema (plus sheaths); this structure, as already mentioned, though highly unusual, shares a number of unique details with the spermatozoa of the branchiuran crustacean *Argulus foliaceus* (Wingstrand, 1972).

V. Physiology and Biochemistry

Little is known of physiological processes in pentastomids, or their underlying biochemical pathways, but since pentastomids lack an excretory system it may be surmised that waste nitrogen (in the form of ammonium ions?) diffuses across the cuticle to be eliminated by the host. Equally, since there is no obvious circulatory system—body wall peristalsis simply agitates coelomic fluid—body fluid may be important in the transportation of respiratory gases. Indeed some species (for example *Waddycephalus*, *Elenia* and *Subtriquetra*) are bright red in life by virtue of haemoglobin in the haemocoel, and it is likely that dietary haemoglobin is broken down and resynthesized before being secreted into the haemocoel; similar processes are known in nematodes (Riley, 1973c). Haemocyanin may occur in the haemocoel of large raillietiellids (Karuppaswamy, *in lit.*).

Banaja *et al.* (1977) present evidence to suggest that pentastomids osmoregulate hypo-osmotically (that is, they maintain a blood concentration which is lower than that of their host) and, because they feed on host blood and lymph which are relatively rich in electrolytes, it was postulated that the tegumental chloride cells secrete excess ions to maintain water balance. The demonstration of intense phosphatase activity in the apices of these cells underscores their role in active transport (Riley, 1973a; Nadakal and Mohandas, 1975; Banaja *et al.*, 1977; Hollis, 1979).

Clearly pentastomids, in common with many helminth endoparasites,

must adapt to several quite dissimilar environments for the successful completion of the life cycle. Typically, eggs must withstand environmental fluctuations, the primary larva must survive the gut conditions of an intermediate host, migrate and subsequently evade host defensive strategies, and infective stages must survive excystment in the gut of the definitive host, adapt to an essentially aerobic environment in the respiratory tract of a vertebrate, and overcome yet another host immune response. Rodrick (1974, 1976) has some data to show that the various stages in the ontogeny of *Porocephalus crotali* possess the genetic capacity to respond differently to different environments: large differences in the specific activity and isoenzyme composition of lactate dehydrogenase were found in eggs, infective nymphs and adults.

Glycogen, the principal storage product of pentastomids (Doucet, 1965) is sequestered mainly within striated muscle and the gastrodermis (Nadakal and Mohandas, 1975). Details of its catabolism are unknown but moderate cytochrome oxidase activity in epidermal glands, muscles and intestinal cells, coupled with high succinic dehydrogenase activity in epidermal glands and intestinal cells of *Kiricephalus pattoni*, suggest that this species is capable of some form of oxidative metabolism (Nadakal and Mohandas, 1975).

Scattered reports of the occurrence of certain enzymes and the histochemistry of pentastomids (Doucet, 1965; Riley, 1972a; Nadakal and Mohandas, 1975; Hollis, 1979) have shown little of particular significance. Karuppaswamy (1977) has used X-ray diffraction to reveal β-chitin in the cuticle of a raillietiellid, and Cappuccinelli and Martinetto (1972) subjected homogenates of *Armillifer* nymphs to electrophoretic fractionation and recovered several major classes of protein; as Bryant (1982) concluded, "little is known of the biochemistry of parasitic arthropods"

VI. Development and Life History

A. Embryogenesis and the Egg

Early cleavage stages and subsequent development of *Reighardia* are well described (Osche, 1963; Böckeler, 1984b), though our knowledge of other species is fragmentary (Heymons, 1935; Doucet, 1965).

Formation of the egg membranes in *Reighardia* and raillietiellids begins early when the oocyte is a mere 7 – 15 μm in diameter. Patches of dense material, appearing over the oocyte surface among the bases of microvilli, accrete and fuse as it matures leaving microvilli traversing the shell like pore

canals (Nørrevang, 1972, 1983; Böckeler, 1984a; Riehl and Walldorf, 1985). Later, microvilli are withdrawn, most pore canals close, and a second, PAS-positive layer is added beneath the dense layer. A possible mechanism of eggshell formation is outlined by Riehl and Walldorf (1985).

What relationship these layers have to the final eggshells is unresolved, but it is becoming clear that most cephalobaenid primary larvae are invested by three distinct layers, whereas those of porocephalids have four (not three as implied by Riley (1983)). *Reighardia* is unusual in that the eggshell *may* have only two obvious components. The innermost, a chitinous epidermal secretion of the embryo itself termed a blastoderm cuticle (Osche, 1963; Böckeler, 1984a), is overlain by a spongy mucous layer secreted through a pore in the eggshell by a complex of cells in the mid-dorsal region of the embryo which collectively constitute the dorsal organ (Osche, 1963). *In utero* the mucous layer is delimited by a very thin layer which Riley (1983) termed a chorion. By contrast, the outermost layer of the raillietiellid egg is a definite shell (Heymons, 1926; Doucet, 1965; Esslinger, 1968; Fig. 1c) which in *R. gigliolii* is thick and brittle (Winch and Riley, 1985): the mucous layer is still present but it is now sandwiched between the two eggshells and swells only when the outer eggshell is ruptured. These three components are still present in porocephalids and in the same order (note that the dorsal organ penetrates only the inner shell membrane as in raillietiellids), but external to the whole assembly is another layer (Heymons, 1935; Esslinger, 1962b) which swells to form an obvious hyaline capsule in water (Fig. 1d). This is clearly not homologous with dorsal organ secretion, and because it lies outside the outer eggshell it must derive from female reproductive tract secretion. The PAS-rich layer seen in early oocytes (Nørrevang, 1983; Böckeler, 1984a; Riehl and Walldorf, 1985) may contribute to the "sandwiched" mucous layer.

The egg envelopes of *Subtriquetra subtriquetra* have become secondarily reduced to a single, thin, flexible membrane (Vargas, 1975), an obvious adaptation to an aquatic, free-living existence, but the presence of a dorsal organ *may* indicate the involvement of a mucous layer (Vargas, 1975).

B. EGG HATCHING AND THE PRIMARY LARVA

With the exception of *Subtriquetra*, the egg is infective to the next host in the life cycle, which necessarily acquires it as a contaminant of food or water. The factors influencing egg hatching are poorly understood: temperature alone (40°C) is sufficient to induce hatching in *Reighardia sternae* where eggs are directly infective to herring gulls (Banaja *et al.*, 1975), and the eggs of *Subtriquetra* hatch internally in the nasal passages of captive caiman,

although the significance of this is unclear (Winch and Riley, in press b). Eggs of *Porocephalus crotali* hatch in the duodenum of rodents (Penn, 1942; Esslinger, 1962b) and those of *Kiricephalus coarctatus* hatch in the upper intestine of tadpoles (Guidry and Dronen, 1980); in both species hatching occurred within 30 minutes. The hatching of *Raillietiella* eggs has been observed in the crop and intestine of insect intermediate hosts (Ali and Riley, 1983; Winch and Riley, 1985): *R. gigliolii* nymphs develop in the larva of the scarabaeid *Coelosis biloba* which has a highly specialized digestive physiology—the combination of a high gut pH and amylase activity may provide the requisite hatching stimuli (Winch and Riley, 1985).

Eggs remain viable for long periods in water (Penn, 1942; Keegan, 1943; Esslinger, 1962b), are tolerant of acids and preservatives (Salazar, 1965), but show variable responses to drying (Keegan, 1943; Esslinger, 1962b). As far as is known all eggs contain fully developed primary larvae at the time they are shed by females into the hosts' respiratory tract, and they are immediately infective to the next host.

The morphology of the primary larva is well documented (Heymons, 1935; Fain, 1961; Esslinger, 1962b, 1968; Dyck, 1975; Fig. 1a) and, although there are obvious differences in the form of the hook fulcra and penetration apparatus between the two orders (Fain, 1961), larval structures are highly conserved, and there is little variation on these respective themes (Table 2). It is assumed that, after hatching, larvae mechanically penetrate the gut wall using the penetration apparatus combined with an offset breast-stroke action of the hooked limbs (Self, 1969; Guidry and Dronen, 1980); sites of penetration are visible as haemorrhagic spots on the wall of the intestine (Esslinger, 1962a,c). Both Fain (1961) and Esslinger (1962b) described gland cells with ducts opening onto the penetration apparatus of porocephalid primary larvae, and it is possible that histolytic secretion also assists passage through tissues.

C. LARVAL DEVELOPMENT

Experimental studies of the development of *Linguatula* (Leuckart, 1860), *Porocephalus* (Stiles, 1891; Esslinger, 1962a) and *Sebekia* (Winch and Riley, in press a) in vertebrate intermediate hosts have established that the primary larva is separated from the infective stage by between six and eight moults; intermediate instars lack hooks and early instars are devoid of external segmentation. Hooks are retained by some of the instars of *Sambonia* species (Fain and Mortelmans, 1960), and by all the instars of *Reighardia* and *Subtriquetra*, which are at all times freely mobile (Banaja

et al., 1975; Winch and Riley, in press b). Larvae show active site selection: *Linguatula* prefers bronchial and mesenteric lymph nodes (Sachs *et al.*, 1973); *A. armillatus* in man invades the liver, intestine and mesenteries (Hopps *et al.*, 1971); *P. crotali* in mice and rats favours fatty tissue around the reproductive organs and intestines (unpublished observations); *Sebekia* nymphs (Fig. 9) occur free in the body cavity among the viscera (Winch and Riley, in press a); and *Subtriquetra* (Fig. 1b) invades the swim-bladder of its fish host (Vargas, 1975; Winch and Riley, in press b). *Raillietiella* species in invertebrate hosts are located on the surface of the viscera or in the fat body, and require only two moults to become infective (Ali and Riley, 1983; Winch and Riley, 1985), but at least three moults are necessary when vertebrate intermediate hosts are involved (Fain, 1961, 1964; Ali *et al.*, 1982b). Infective larvae carry single or double rows of uniform penetration spines, which immediately distinguish them from primary larvae (Fain, 1961, 1964; Ali and Riley, 1983; Winch and Riley, 1985). With few exceptions (mentioned above) larvae are encysted in intermediate hosts within the last moulted cuticle inside a capsule of host origin, although primary larvae may migrate for some time before becoming quiescent around the time of the first moult (Esslinger, 1962a; Self *et al.*, 1972).

Few examples of subsequent development in definitive hosts are known (Riley, 1981; Ali and Riley, 1983) but the death of the intermediate host appears to be the cue for excystment; Self and Kuntz (1967) observed that infective nymphs of *Kiricephalus pattoni*, in the body cavity of snake hosts killed by drowning, emerged through rents in the scales, nares etc. Riley and Self (1980) killed mice harbouring infective nymphs of *Porocephalus crotali* and found nymphs erupting through the epidermis 24–48 h later. In both cases nymphal behaviour was directed towards liberation into the stomach and thence to the body cavity of the snake definitive host; the lung was penetrated from this site within a few days (Esslinger, 1962a; Riley, 1981). Infective larval raillietiellids, pipetted into the stomachs of geckos, invaded the lungs (again via the body cavity) in as little as 4 h (Ali and Riley, 1983). Nymphal *Linguatula serrata* could be recovered from the nasopharynx of dogs in as little as 2–3 h after infection: these nymphs did not traverse tissues but migrated directly up the oesophagus from the stomach (Hobmaier and Hobmaier, 1940). In the conspicuously sexually dimorphic genus *Leiperia*, adult females are embedded in the bronchus of their crocodilian host, whereas developing stages are located in the bloodstream around the heart (Rodhain and Vuylsteke, 1932): little else is known of this unusual mode of development.

D. SEXUAL DIFFERENTIATION AND COPULATION

Porocephalids can be sexually differentiated early in development during the encysted phase in the intermediate host (see Riley (1983) for a review; Winch and Riley in press a). As far as is known, in all pentastomids females become sexually mature precociously, and copulation occurs when the uterus is undeveloped (see Section IV F; Fig. 10) and when the sexes are of similar size (Sambon, 1922; Banaja *et al.*, 1975; Riley, 1983). Sperm transfer, as evidenced by the presence of sperm in the female spermathecae, occurs around 66 days after infection in *Reighardia* (Banaja *et al.*, 1975), 80 days after in *Raillietiella* spp. (Ali and Riley, 1983), 75–86 days in *Porocephalus* (Riley, 1981), and 106 days in *Armillifer* (Noc and Curasson, 1920), but females do not become patent for another 110–155 days (depending on the species). Given that insemination is possible only during a critical period in the early development of the female, a requirement for synchronized development of males and females is manifest and it might be expected that female development would be retarded in the absence of males.

The available evidence suggests that copulation is a lengthy and complex process, that females are monogamous and that males can copulate with more than one female (Riley, 1983). In the definitive host females tend to preponderate over males (Hett, 1924), and Riley (1972b) found that, in gulls harbouring maturing infections of female *Reighardia sternae*, male worms were almost always absent, although they had obviously once been present because sperm was present in the females' spermathecae. Self (1983) stated that male and female *Raillietiella* spp. occur simultaneously over long periods of time; while this is sometimes true, our observations of large numbers of preserved raillietiellid infections (Ali *et al.*, 1981, 1982a,b,c; 1984a,b) did, in general, suggest that males die earlier than females, thereby shifting the sex ratio gradually in favour of females; this is also true in some porocephalid genera (Riley and Self, 1980, 1981b, 1982). Mature males are invariably much smaller than mature females and develop to full maturity in about half the time it takes for females to become patent (Riley, 1983).

E. PREPATENT PERIOD AND EGG PRODUCTION

Egg production in *Reighardia sternae* is most unusual, if not unique amongst cephalobaenids, in that each female produces only 2900 eggs in a lifetime over a short patent period of 1–3 days; females then die (Banaja *et al.*, 1976). During the more usual long prepatent period eggs in various states of maturity can be found in the saccate uterus, but when egg-laying begins all are fully developed (that is, they contain hooked primary larvae). By

contrast, in most (if not all) raillietiellids, patency commences when there is still a high proportion of small immature eggs in the uterus, although this proportion may gradually decline over the duration of the patent period (Ali and Riley, 1983). The vaginal sieve (Section IV F; Fig. 11) allows continuous deposition of fully developed eggs in the lungs of the host, smaller eggs being retained within the uterus until they reach an appropriate size.

Small raillietiellids contain relatively few eggs and the low fecundity of three of these species may be compensated for by strong ecological links between the egg-contaminated host faeces and insect intermediate hosts (Ali and Riley, 1983; Winch and Riley, 1985). The largest raillietiellids contain up to 200 000 eggs (Ali *et al*, 1982b), which suggests a correspondingly high fecundity offsetting massive larval mortality.

The porocephalid uterus is tubular and elongate (Fig. 10) and according to Nørrevang (1983) is up to 100 times the length of the animal (I suspect this is a wild overestimate). The number of eggs carried is roughly correlated with female size, and in larger species such as those of the genera *Linguatula*, *Porocephalus* and *Kiricephalus* gravid specimens contain 5×10^5 eggs (Pillers, 1925; Salazar, 1965; Riley, 1983). Eggs mature as they pass along the uterus towards the vagina, and in *Porocephalus crotali* are deposited at a rate of 520–2300 per female per day (Riley, 1981). It has been estimated that a female *Linguatula serrata* is capable of producing several million eggs (Baer, 1952); this sequential production line of eggs, geared to a high fecundity, is typical of many helminth endoparasites.

A long patent period is usual in both orders, at least 6 years in *P. crotali* (Riley, 1981; Riley, unpublished observations) and 1 year in *Raillietiella gehyrae* (Ali and Riley, 1983) and *R. gigliolii* (Winch and Riley, in press b); all these are underestimates, because at the time hosts were sacrificed the parasites were still producing eggs. These data become all the more impressive when it is remembered that they are achieved after a single insemination!

F. LIFE CYCLES

1. *Cephalobaenida*

Only four cephalobaenid life cycles have been investigated experimentally and one is known to be direct: von Haffner and Rack (1965) found early developing stages of *Reighardia sternae* in the stomach and intestine of a herring gull. This evidence, suggesting a direct life cycle, was partially supported by subsequent observations of the incidence of *Reighardia* in natural infections (Riley, 1972b), and experimental proof was finally obtained by Banaja *et al.* (1975, 1976). It was postulated that a migration of

female worms at the onset of patency induced vomiting in infected hosts and that egg-contaminated vomit, ingested by other gulls, was the mechanism of direct transmission. Infections were supplemented by auto-reinfection, regarded as an example of parasite opportunism providing an extra safeguard in an otherwise tenuous life cycle (Banaja *et al.*, 1976): similar reports from raillietiellid infections (Salazar, 1964; Deakins, 1973), claiming to indicate prolonged presence of males (Self, 1983), may merely represent manifestations of host pathology (Ali *et al.*, 1982b). *R. sternae* is mainly a parasite of juvenile gulls (Riley, 1972b; Banaja *et al.*, 1975), whereas the related species *R. lomviae* from guillemot and puffins is found in all age categories of host (Dyck, 1975). Dyck (1975) argued that if transmission was direct it probably occurred when parents fed their young: apparently fish are held in the beak (where eggs could be picked up) for up to 1 h before being fed to the young.

Coprophagous blattids are common intermediate hosts (Lavoipierre and Lavoipierre, 1966) of two raillietiellid parasites infecting geckos (Lim and Yong, 1977; Ali and Riley, 1983), and insects are probable intermediate hosts of other species comprising groups I, II, IV and V (Ali *et al.*, 1985). *R. gigliolii* infecting the lungs of the South American worm-lizard *Amphisbaena alba*, a facultative inquiline of the nests of leaf-cutting ants (Riley *et al.*, in press), also utilizes an insect intermediate host, the larvae of the three-horned rhinoceros beetle (*Coelosis biloba*), itself an inquiline of ant nests (Winch and Riley, 1985). Ants, thought to be a vital link in transmission, apparently deliver the parasite eggs directly to the beetle larvae.

Life cycles of the remaining *Raillietiella* spp. are unknown, although there is circumstantial evidence to suggest that one, or possibly two, vertebrates serve as intermediate hosts (Fain, 1961, 1964; Ali *et al.*, 1982b) for species of the large group III (*sensu* Ali *et al.*, 1985) from snakes. The transplant experiments of Nadakal and Nayar (1968) and Ramachandran (1977) have demonstrated the remarkable ability of raillietiellids to survive for long periods in totally unnatural hosts.

2. *Porocephalida*

(a) Introduction. Few porocephalid life cycles have been investigated experimentally but infective stages, representative of most genera, have been recovered from a variety of vertebrate intermediate hosts. Only rarely is it possible to identify nymphs specifically, but they can be tied to genera, so that general patterns of life cycles have emerged (Table 1). Certain members of the genera *Kiricephalus* and *Porocephalus* (see below) are potentially of great interest as, uniquely for an endoparasite, three vertebrates may be

involved in the life cycle. No less interesting, and in complete contrast, is the circumstantial evidence suggesting a direct life cycle in species of *Sambonia* and a *Linguatula* which infects herbivore definitive hosts. Clearly there is much scope for future research.

(b) Porocephalidae. The genus *Porocephalus*, with at least eight well characterized species (Riley and Self, 1979; Riley and Walters, 1980), includes six species from North, Central and South American snakes, all of which utilize mammals as intermediate hosts. Unfortunately there is no good character which will effectively separate the infective nymphs of these species (Vargas, 1970a,b), and the specific identity of many from naturally infected mammals (as recorded in the literature—see, for example, Rego and Vicente (1972), Rego (1980)) is at best speculative unless supported by experimental evidence; generally the latter is fragmentary.

Details of the life cycles of species infecting American pit-vipers (family Crotalidae) are summarized by Riley and Self (1979), and Riley (1981) has since followed the complete development of *P. crotali* in its rattlesnake definitive host. *P. clavatus*, a species parasitizing constricting snakes (family Boidae), utilizes larger mammals (see Self and Cosgrove (1972) for a review); wild tamarins (*Tamarinus nigricollis*) from the upper Amazon show high prevalences (42%) and nymphs persist for over two years in captive animals (Nelson *et al.*, 1966). Infective nymphs, derived from imported marmosets (*Saguinus* spp.) and fed to a laboratory reared, and therefore uninfected, boa (*Boa constrictor*), yielded adult *P. clavatus* at autopsy 10 months later (Self and Cosgrove, 1968).

P. subulifer, the best known of the two African species, infects file snakes of the genus *Mehelya* (Fain, 1961) which are primarily ophiophagous. Encysted infective nymphs and freely mobile worms have been recovered from a large number of snake intermediate hosts comprising three families (Riley and Self, 1979). This unusual snake–snake life cycle has the added complication that mammals may be implicated, and Riley and Self (1979) surmised that these were possibly paratenic hosts. But the more recent discovery of three vertebrate hosts in the life cycles of the related genus *Kiricephalus* (Riley and Self, 1980) casts doubt on this suggestion: thus mammals may be routinely involved.

Reports of *Porocephalus* nymphs apparently developing for considerable periods in quite abnormal hosts (Stiles, 1891; Nadakal and Nayar, 1968; Horvath, 1971) reaffirm that only long-term studies are likely to contribute significantly to a complete understanding of *Porocephalus* host specificity (Riley and Self, 1979).

The genus *Kiricephalus* also utilizes snakes as definitive hosts, and abundant circumstantial evidence exists to suggest that two vertebrate

intermediate hosts are essential in the life cycles of the two best known species, *K. pattoni* from South-East Asia and the Philippines and *K. coarctatus* from the Americas. Both of these infect an impressive number of snake, lizard and amphibian intermediate hosts; there is much less information concerning the other species (Riley and Self, 1980). Eggs are known to be infective to amphibians (Salazar, 1965; Keegan *et al.*, 1969; Riley and Self, 1980; Guidry and Dronen, 1980) and lizards (Yamamoto *et al.*, 1978), but snakes, including snake intermediate hosts, are refractory to infection with eggs (Keegan, 1943). Nymphs recovered from amphibians and snakes assort into distinct, non-overlapping size categories, indicating that these vertebrates are first and second intermediate hosts (Riley and Self, 1980): those in amphibians are encysted (Riley and Self, 1980) and those in snake intermediate hosts are generally free in the body cavity (Self and Kuntz, 1967), whereas adults and preadults occur exclusively in the lung of the ophiophagous definitive host.

Recently Krishnasamy and Self (1981) discovered a nymphal *K. tortus* in the orbit of a cat shark (*Hemiscillum indicum*) which, it was assumed, had eaten the snake definitive host (*Boiga irregularis*); this hypothesis was supported by the presence of other snake pentastomids (*Raillietiella* sp.) in the shark's gut.

(c) Armilliferidae. The two common African representatives of the genus *Armillifer*, *A. armillatus* and *A. grandis*, infect pythons and vipers (Fain, 1961) and, although preadults have also been recovered from two colubrids (Fain, 1960), the status of these hosts remains uncertain. Nymphs, readily diagnosed by differences in annulus number, have been recovered from a large number of mammals and, rarely, birds (Fain, 1961; Fain and Salvo, 1966). Massive nymphal infections, possibly the result of hosts ingesting entire female worms, have occasionally been found (Fain, 1961; Thurston, 1972). Such is the diversity of *A. armillatus* mammalian intermediate hosts that it might be imagined that any mammal is susceptible to infection. However, Ranque *et al.* (1974) demonstrated experimentally that rabbits were refractory although infections developed normally in five other (unusual) mammal hosts.

The predominantly South-East Asian species *A. moniliformis*, a parasite of pythons, may extend into Africa (Heymons, 1940a; Fain, 1961; Riley and Self, 1981a), and is also known to infect a wide range of mammal hosts (see Stabler and Self (1981) and Krishnasamy *et al.* (1981) for reviews). All the above three species infect man (Section VIII).

Nymphs of three other *Armillifer* species from Australia and New Guinea (Riley and Self, 1981a) have recently been described from a number of mammal hosts (Riley *et al.*, 1985; Riley and Spratt, in press)—these

tentative identifications were made purely on the basis of abdominal annulus counts. The life cycle of *A. agkistrodontis* from Taiwan (Self and Kuntz, 1966) is unknown.

Both *Cubirea* species are highly distinctive parasites of African cobras (and possibly vipers) and, though little is known of the life cycle, there are two records of so-called "nymphs" from birds (Fain, 1961). One emanates from Shipley (1898), who described obviously adult specimens of *C. annulata* from a crane (*Anthropoides virgo*)—a mistaken annulus count was subsequently corrected by Sambon (1922) during a re-examination of the only surviving specimen. The most logical explanation of this unusual finding is that the bird had recently eaten an infected snake. The remaining record is of encysted nymphs in a water hen, *Porphyrio* sp. (Heymons, 1940a). Another report of a juvenile specimen of *C. pomeroyi* from a wart snake (*Acrochordus*) in the British Solomon Islands (Self and Kuntz, 1957) is apocryphal.

G. brumpti, the sole representative of the genus *Gigliolella*, infects mainly boas (Heymons and Vitzthum, 1936; Gretillat *et al.*, 1962) and nymphs are known from three species of Madagascan mammals (Giglioli, 1922a; Heymons, 1940a; Chabaud and Choquet, 1954).

(d) Sambonidae. Only adult specimens of the three *Sambonia* species from monitor lizards in the British Solomon Islands have been described (Self and Kuntz, 1957, 1966). The remaining species, *S. lohrmanni*, found in African, Asian and Indonesian monitors, may have a direct life cycle. Fain (1961) reported the presence of numerous nymphs, 2–3 mm long, in the body cavity of *Varanus niloticus* (which also harboured adults) but, more importantly, Fain and Mortelmans (1960) autopsied a large *Varanus komodoensis* which had died in captivity and recovered a developmental series of primary larvae and the ensuing four nymphal stages from a bronchial epithelioma, the apparent cause of death. Eggs, the source of infection, had apparently been released from a gravid female in the lung. This, the only clear example of auto-reinfection known in porocephalids, was interpreted as evidence of a direct life cycle. The significance of auto-reinfection in *captive* hosts is assessed by Ali *et al.* (1982b), and we remain sceptical of the above findings for the reasons outlined by Riley and Self (1982).

Nevertheless, the undoubted fact that development can occur in a reptile, albeit in unusual circumstances, suggests that under natural conditions a reptile would be the intermediate host. Some support for this was provided by Deakins (1972), who obtained a gravid female of *S. lohrmanni* from *Varanus niloticus* and force-fed it to a snake (*Elaphe obsoleta*); more than 200 encysted or migrating nymphs and an adult (?) in the lung were discovered at autopsy after 9 months.

The other varanid parasite, *Elenia*, is known from scant museum material only (Riley *et al.*, 1985), but the life cycle may not be radically different from that described below for *Waddycephalus*; Riley and Spratt (in press) have tentatively identified a single nymph from a Solomon Island frog as a possible *Elenia* species.

Riley and Self (1981b) speculated, purely from the point of view of host dietary regimen, that members of the large and distinctive genus *Waddycephalus* utilize terrestrial vertebrates as intermediate hosts. The characteristic double-hooked nymphs have since been described from Australian mammals, skinks and frogs (Riley *et al.*, 1985; Riley and Spratt, in press). Again, nymphal diagnosis was based on annulus counts, and experimental verification is needed. The two closely related *Parasambonia* species probably also depend upon vertebrate intermediate hosts (see Riley *et al.* (1985) and Riley and Spratt (in press) for further discussion).

(e) Sebekidae. It is convenient to deal with the three genera comprising the family Sebekidae together because all, so far as is known, develop to an infective stage in fish intermediate hosts.

Sebekia oxycephala, originally thought to infect North, Central and South American crocodilians (Heymons and Vitzthum, 1936), embraces at least three species (Self and Rego, 1985; Overstreet *et al.*, 1985). The life cycle of the species infecting *Alligator mississipiensis* is well known and several freshwater fishes are involved (Holl, 1932; Venard and Bangham, 1941; Dukes *et al.*, 1971; Overstreet *et al.*, 1985). Venard and Bangham (1941) discovered that at least six species of fish are naturally infected with larval *Sebekia*, all from ponds near rivers, whereas riverine, canal or lake fish were uninfected: ecological, rather than physiological, factors were considered important in influencing distribution. Dukes *et al.* (1971) reported that the same *Sebekia* species will mature in the snapping turtle *Chelhydra serpentina*; infective nymphs were dissected from the musculature of large-mouthed bass and force-fed to four species of snake and a snapping turtle; adults were subsequently recovered from the turtle's lungs. According to Heymons (1935) snakes and lizards may also harbour nymphs, but the original source of this information (Diesing, 1835) is vague and perfunctory and these observations require confirmation.

The South American genera *Alofia* and *Sebekia* are closely related and, although hook morphology separates adults (Heymons, 1941b,c), the identification of nymphs is problematical—some of the confusion is summarized by Self and Rego (1985)—because both genera possess doubled hooks. Winch and Riley (in press a) followed the larval development of *S. oxycephala* from South American *Caiman sclerops* in fish (Fig. 9), and Heymons (1940b) gave a resumé of the development of *Leiperia cincinnalis*

in another crocodilian; unusually, the ensuing early instars exist initially in the circulatory system of these crocodiles (Rodhain and Vuylsteke, 1932; Heymons, 1940b; Fain, 1961) before females eventually become established in the bronchi.

(f) Diesingidae. Although nothing is known of the life cycle of any *Diesingia* species, it is reasonable to speculate that, since *D. megastoma* infects at least two species of piscivorous turtles (Fonseca and Ruiz, 1956), fishes serve as intermediate hosts.

(g) Subtriquetridae. One of the three species currently included in this family, *S. megacephala* from an Indian crocodile, considered by Vargas (1971) to be of uncertain taxonomic status, is known from type material only. Its putative larval stage, encysted within tortoises, was originally described as *Porocephalus kachugensis* (Shipley, 1910, Hett, 1924) and *Diesingia kachugensis* (Sambon, 1922). The life cycle of the other Indian species, *S. shipleyi* (Hett, 1924), is unknown.

The remaining species, from South American caiman (*C. sclerops* and *C. niger*), is potentially of great interest since it is the only pentastomid known to possess a free-living stage in the life cycle (Vargas 1974, 1975). Vargas (1975) flushed gravid females from the nasal passages of infected caiman and collected eggs shed into water. Unlike all other pentastomids, the eggshell of *Subtriquetra* is a thin flexible membrane which is rapidly ruptured by the larva after immersion in water. Vargas (1975) demonstrated experimentally that larvae were infective to fish, when either administered orally or allowed to penetrate directly through the skin, and were disseminated throughout the viscera after 2 days, but within the swimbladder after 3 days. Further observations of *S. subtriquetra* larvae, derived from naturally infected *Caiman sclerops* taken in Trinidad, have been made by Winch and Riley (in press b). Throat swabs from caiman revealed that larvae actually hatched internally (the significance of this is unclear) and, when transferred to water, adopted a characteristic stationary posture with the tail held aloft by the outspread limbs (Fig. 1b). Bouts of vigorous tail-bending and offset breast-stroke actions by the hook pairs occurred periodically, and the frequency and duration of these activity periods increased with larval age. Since infection depends upon chance contact with a fish intermediate host, this activity may enable larvae to disperse to more favourable sites, thereby enhancing the probability of contact. A few larvae remained active for up to 6 days but most became relatively quiescent around 4–5 days and were no longer able to penetrate fish. Active larvae were highly adept at hooking onto fish after random contact, but penetration of the swimbladder could take up to 6 days, depending upon fish size.

(h) Linguatulidae. Most adult *Linguatula* infest the nasal sinuses and nasopharynx of carnivorous mammals belonging to the families Canidae, Hyaenidae and Felidae, and eggs are infective to a range of mammal intermediate hosts, particularly large, grazing herbivores (Sachs and Sachs, 1967; Sachs *et al.*, 1973).

The best known species, *L. serrata*, is a cosmopolitan parasite of the nasal cavities of dogs, wolves and foxes (Stiles and Baker, 1935; Pullar, 1936; Griffiths and Sinclair, 1953; Sinclair, 1954; Boch and Supperer, 1977), and a subspecies (?) *L. serrata* var. *serengetiana* is known from wild dogs and jackals (Sachs *et al.*, 1973). Numerous scattered claims, originating from Stiles (1891) and endorsed by Sambon (1922), that cats (and other mammals) can serve as definitive hosts were disproved by experimental infections (Hobmaier and Hobmaier, 1940; Khalil and Schacher, 1965), which demonstrated that infective nymphs could survive in cats for a maximum of 2 weeks before being eliminated.

Eggs are expelled from infected hosts, either by sneezing or in nasal discharges (Sinclair, 1954) or via the intestine (Ehrenford and Newberne, 1981); some eggs that are swallowed hatch prematurely and auto-reinfect but are quickly destroyed (Hobmaier and Hobmaier, 1940). Commonly, cattle, sheep and goats serve as intermediate hosts (Khalil, 1972) and a natural fox–rabbit life cycle in Northern Ireland (Griffiths and Sinclair, 1953) may be responsible for the high prevalence (72%) of nymphal infections in domestic cattle in that area (Sinclair, 1954). Reports of freely migrating (excysted) *Linguatula* nymphs in intermediate hosts (Leuckart, 1860; Basson *et al.*, 1971; Sachs *et al.*, 1973) may reflect post-mortem migrations; if these do occur naturally, their significance is unclear.

The life cycles of the African representatives of the genus, *L. multiannulata*, *L. nuttali* and *L. serrata* var. *serengetiana*, are well documented (Sachs and Sachs, 1967; von Haffner *et al.*, 1967; von Haffner *et al.*, 1969; Basson *et al.*, 1971; von Haffner, 1972, 1974; Sachs *et al.*, 1973; Young, 1975).

An exciting rediscovery of a new *Linguatula* species in a ruminant has recently come to light: the parasite, previously misidentified as *L. serrata*, has been recovered from caribou (Chapin, 1926; Murie, 1935) and reindeer (Voblikova, 1961; Skjenneberg, 1965; Skjenneberg and Slagsvold, 1968; Christensson *et al.*, 1974; Rehbinder and Nordkvist, 1982) but the significance of these findings has hitherto been unappreciated (Sweatman, 1971; R. Haugerud and A. Nilssen, personal communication). High prevalences of adults and preadults have been found in the nasopharynx of yearling northern semi-domestic Norwegian reindeer: animals contain up to 27 worms, the females of which attain a maximum length of 14.5 cm. 66% of 4–6 months old reindeer ($n = 48$) were infected, but this declined to 17% in

16–20 months old deer ($n = 23$) and all those 28 or more months old ($n = 43$) were uninfected: some seasonality of egg production and transmission is suspected (A. Nilssen and R. Haugerud, personal communication). The life cycle must be highly unusual because it cannot include a carnivore. Transmission must be either direct or must involve a herbage-dwelling invertebrate intermediate host which could be accidentally ingested when reindeer feed. By any yardstick this species is more than usually interesting and it perhaps represents the zenith of pentastomid evolution. How many other ruminants carry pentastomids?

VII. Pathological and Veterinary Aspects of Pentastomid Infections

A. INTERMEDIATE HOSTS

Pentastomids, in common with all arthropods, moult frequently during development (Section VI) and thus hosts harbouring growing parasites are subject to repeated and increasing antigenic stimulation in the form of moulted exuviae. Encysted forms in intermediate hosts, immobilized within a cyst of host origin, elicit pronounced inflammatory responses; because infective nymphs can remain viable for years (Cosgrove *et al.*, 1970), and yet the infective stage is reached within weeks (Esslinger, 1962a; Ali and Riley, 1983; Winch and Riley, 1985, in press a,b), the non-moulting infective stage is most frequently encountered during routine dissections of naturally infected animals, and this can give a misleading impression of the dynamic nature of the host–parasite interaction.

Histopathological reactions in rats to developing *Porocephalus crotali* nymphs were examined by Esslinger (1962c); early nymphal instars formed the foci of granulomatous lesions which contained high concentrations of macrophages and eosinophils. As the more chronic condition associated with later instars became established, fibroblasts elaborated a fibrous capsule around the nymph which became enshrouded by plasma cells and lymphocytes. By 4 months, that is after moulting had ceased, inflammatory responses had completely subsided and the infective stage was invested by a tough, thin-walled membranous envelope: it is this stage that is most commonly encountered (Hopps *et al.*, 1971; Self, 1972; Self *et al.*, 1972; Pflugfelder, 1977). The total absence of inflammatory response against mature infective nymphs of several species in both reptile and mammal intermediate hosts was emphasized by Self and Kuntz (1967); indeed, their micrographs adequately demonstrated the extraordinary degree of

compatibility that often exists between long-established tissue-dwelling pentastomids and their hosts.

Nelson *et al.* (1966), Cosgrove *et al.* (1970) and Self and Cosgrove (1972) concluded from observations of infective nymphal porocephalid infections (mostly *Porocephalus* or *Armillifer*) in primates that there was little inflammatory response associated with live nymphs, but that dead or dying nymphs produced intense inflammatory reactions ultimately resulting in a fibrous scar; remnants of the exoskeleton may persist within the scar tissue long after the rest of the parasite has been phagocytosed, forming a cuticle granulomata. The rare pathological reactions, occasionally seen in intermediate hosts (reviewed by Self, 1972), may reflect presensitization.

Subtriquetra larvae in small fish are highly pathogenic: black mollies (fork length 30–50 mm) always die before larval development is completed, which frequently occurs when larvae attain a length of 0.8 mm (around 30–40 days after infection). Larger naturally infected fish (*Aequidens* sp., fork length 70 mm) can, however, support at least seven infective larvae, 2.5 mm long (Winch and Riley, in press b).

B. DEFINITIVE HOSTS

Some novel and interesting facets of the pathology produced by adult and preadult stages in the respiratory tracts of definitive hosts emerge from the widely disseminated literature of the subject. Statements made by some reviewers are often misleading because it is unclear whether their comments are the result of first-hand experience. For example, Self and Kuntz (1967) wrote of numerous records from reptile collectors of pentastomids being coughed up or regurgitated from stressed snakes; Telford (1971) implied that pentastomids often escaped from the lungs and wandered through the coelomic cavity, some apparently occluding the trachea and thus killing the snake by suffocation; Page (1966) stated that several forms of linguatulids seriously affected many species of snake; and Murphy and Armstrong (1978) even recommended manual removal of rattlesnake pentastomids with tweezers, but gave no confirmed safe (?) method, and furthermore suggested that the parasites could be transmitted between hosts by water!

In fact there is remarkably little evidence that pentastomids are responsible for significant pathology in naturally infected definitive hosts, although, as will become apparent, this does not necessarily apply to captive hosts. Thus Self and Kuntz (1967) recovered 100 adult *Porocephalus crotali* from an apparently healthy rattlesnake, although Riley (1981) found signs of damage in less severe chronic infections of captive animals in the form of numerous discoid scars covering the reticular and membranous regions of

the lung. Each plaque is associated with worm feeding activity (Fig. 12) and it is apparent that some loss of respiratory capacity is inevitable. Klauber (1972) surmised that *Porocephalus* species could affect host survival in certain island populations of rattlesnakes exhibiting high prevalences and intensities of infection. Fantham and Porter (1953) autopsied a timber rattlesnake (*Crotalus horridus*) heavily infected with *P. crotali* and found that the parasites had almost filled the lung cavity; lung obstruction and inflammation were apparent and there was evidence of haemorrhaging; since the animal was examined 24 h post-mortem the primary cause of death could not be determined. *Sebekia mississipiensis* can cause necrosis and haemorrhaging in the lungs and liver of alligator (Hazen *et al.*, 1978) and it can apparently kill hatchlings (Boyce *et al.*, 1984).

In contrast, Awachie (1974) reported significant mortality among geckos (*Hemidactylus brookii*) infected with *Raillietiella affinis* (?) in Nigeria; pathogenicity increased with age and intensity of infection, culminating in a gradual destruction of lung tissue with associated haemorrhaging and generalized inflammation; there was strong evidence that lizards were killed by heavy infections in natural circumstances. Related geckos (*H. mabouia*) in Dar es Salaam, Tanzania, were also heavily infected with raillietiellids (Sarda and Simonsen, 1985) and again it is likely that these parasites are important regulators of natural host populations.

Apparently carnivorous mammals can sustain high numbers of *Linguatula* within nasal passages and sinuses. Sachs *et al.* (1973) recovered between 10 and 30 parasites from hyaenas and up to 75 from lions (female parasites are fully grown at 120 and 68 mm respectively). A. Nilssen and R. Haugerud (personal communication) found up to 27 worms in reindeer. Inevitably, with burdens of this magnitude, host fitness must be impaired.

Most pentastomids inhabit the lumen of the host lung and move freely between feeding sites (Riley, 1981), but the cephalothorax of certain porocephalid females (*Leiperia*, *Elenia*, *Waddycephalus*, *Parasambonia*, *Kiricephalus* and *Cubirea*) is permanently buried in the respiratory epithelium; chronic tissue changes gradually entrap the buried anterior region within a fibrotic capsule. Deakins (1972) described a crater-like lesion in the lung of a garter snake (*Thamnophis sirtalis*) caused by the embedded head of a female *Kiricephalus coarctatus* which had penetrated into the hypaxial musculature. Clearly, with all these permanently anchored species, heavy infections will be highly debilitating.

In a detailed investigation of pentastomid pathology of reptiles dying in captivity, Deakins (1972) concluded that pentastomids were rarely the sole cause of death, but damage to the lung epithelium allowed secondary infections to become established. This latter observation may be important because, in 1977, four out of nine Dominican boas (*Constrictor c. nebulo-*

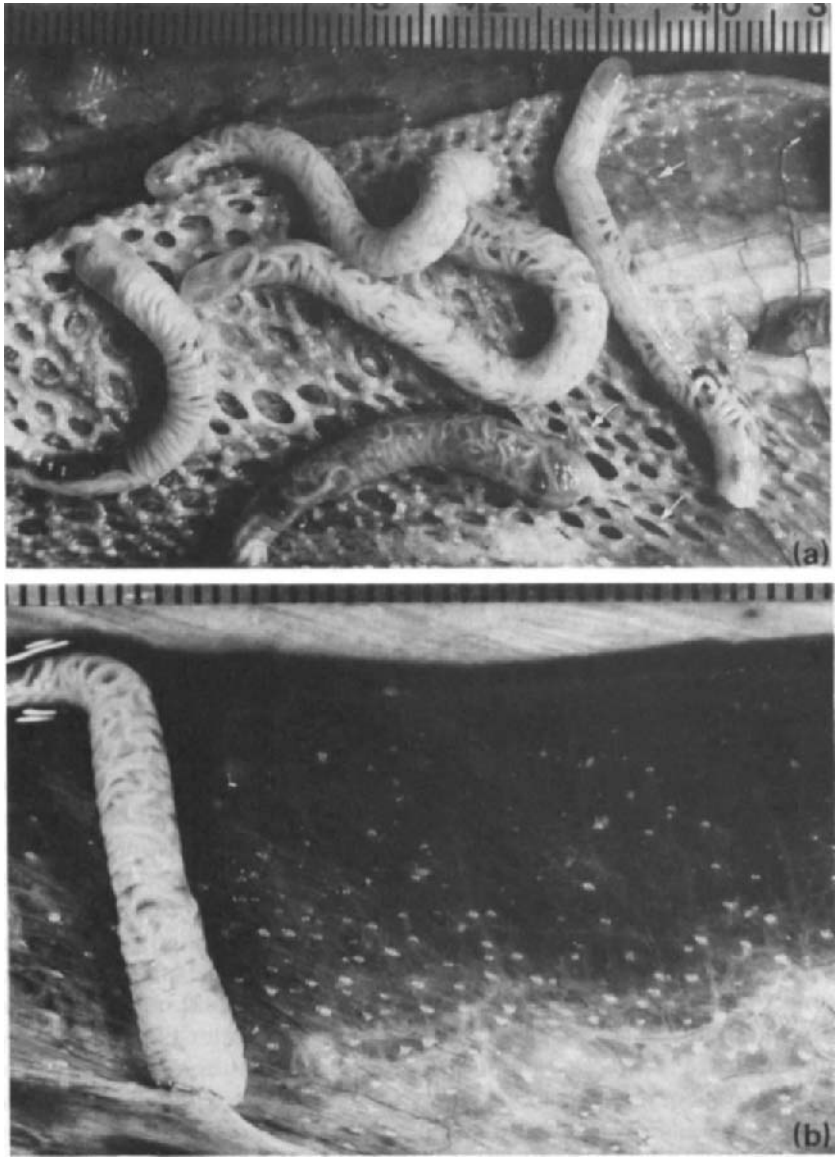

FIG. 12. (a) Five adult female *Porocephalus crotali* on the reticular lung of a freshly-killed rattlesnake. The uterine coils are plainly visible through the transparent body wall. The entire surface of the lung is pock-marked by discoid scars (some of which are arrowed) associated with past feeding activity. (b) Single female in the membranous lung where it borders the liver—the scarring is obvious (scales in mm).

sus), acquired by the Jersey Wildlife Preservation Trust, died of general bacteraemia and pericarditis, the primary cause of which was attributable to pentastomid infections (Riley and Walters, 1980; Walters, in lit.). Autopsy revealed *Porocephalus dominicana* in the lungs, which were generally eroded, inflamed and mucus-filled, and petechial haemorrhages were present in the trachea. In three cases it was postulated that the pentastomids had permitted a serious potential pathogen (*Aeromonas hydrophila*) to disseminate systemically from the lung lumen, ultimately producing bacteraemia with pericarditis (Walters, in lit.). Unusually, some of the male pentastomids were in nodules in the reticular lung, easily seen through the pleura (this is known in other snake definitive hosts—see Stiles (1891), Hett (1924) and Riley (1981)). It may be that, in the *P. dominicana* case (above), stresses associated with captivity caused the pentastomids to behave abnormally and perforate the lung wall. Obviously this must happen every time the lung is invaded from the other direction by infective larvae entering from the body cavity; haemorrhagic foci on the intestine and lung wall are testimony to this activity (Broden and Rodhain, 1910; Riley, 1981). Infections of *Sebekia* in alligator may produce or exacerbate fatal pneumonic aeromonad bacterial infections (Shotts *et al.*, 1972; Hazen *et al.*, 1978).

Equally serious are aberrant pentastomid migrations which directly or indirectly asphyxiate the host. Penn (1942) recovered two 20 mm preadults of *Porocehalus crotali* from the trachea of a water moccasin (*Agkistrodon piscivorus*) which had died of suffocation, and Deakins (1971) autopsied four alligators (*A. mississipiensis*) suffering from steatitis. In each case pentastomids were considered a contributory factor to host death, lung haemorrhage and oedema having been caused by migrating *Sebekia.* In Trinidad, all bushmasters (*Lachesis muta*) over 1 m long invariably carry heavy infections of *Porocephalus stilesi* (Hans Boos, personal communication; unpublished observations). Very commonly, when it is brought into captivity, stresses on the host animal are somehow communicated to the parasites, which are stimulated into abnormal migration, with fatal consequences. I have dissected two such animals which had died of asphyxiation and, in each, pentastomids clogged the trachea. Failure to keep bushmasters alive for any appreciable length of time in captivity has often been attributable to *Porocephalus* infections (Kauffeld, 1969).

C. CONTROL

Chemotherapy, as a means of controlling pentastomid infections, has only rarely been tried, and not surprisingly anthelminthic drugs are ineffective (Bernstein, 1972; Walters, personal communication). Kauffeld (1969) has

found Caricide (diethylcarbamazine) active against linguatulosis and recommends its use as a prophylactic, but it is unclear how the drug was administered or how its efficacy was assessed. In view of the propensity of many pentastomids for migration (outlined above) any potential anti-pentastomid drug must exert a rapid immobilizing/killing effect and it may have to be administered as a spray directly into the respiratory tract. Lastly it should be remembered that pentastomids are arthropods.

VIII. Pentastomid Infections of Man

A. *Linguatula serrata*

Linguatula serrata is of interest not only as a cosmopolitan parasite of dogs and livestock, but as a domestic zoonosis of man, who is susceptible to infection by both eggs and infective larvae (Hobmaier and Hobmaier, 1940; Schacher *et al.*, 1965; Schacher *et al.*, 1969). Eggs from dogs are clearly the origin of human infections, and in the Middle East, where *L. serrata* is endemic, prevalences of 43% have been recorded in street dogs in Beirut (Khalil and Schacher, 1965), 8% in Cairo (Khalil, 1970) and 25% in El Dakhla Oasis, Egypt (Khalil, 1973). The prevalence of nymphs in cattle in certain areas of Britain was as high as 72% (Sinclair, 1954). Although no data are available of infections in dogs, in Northern Ireland foxes are suspected reservoir hosts, possibly maintaining a purely sylvatic life cycle (Griffiths and Sinclair, 1953).

The epidemiology of *L. serrata* infections in man is complicated because both eggs and infective larvae can become established. Eggs hatch in the alimentary tract and primary larvae subsequently invade the body cavity to encyst on the viscera, producing visceral linguatulosis, whereas ingested infective larvae attempt to migrate to the nasal passages, producing nasopharyngeal linguatulosis (Khalil and Schacher, 1965; Schacher *et al.*, 1965).

In areas where *L. serrata* is endemic, particularly in the Middle East, visceral linguatulosis is probably much more common than is generally realized (Khalil, 1970). Eggs, particularly those expelled from infected dogs by sneezing, or in nasal secretions (Hobmaier and Hobmaier, 1940; Sinclair, 1954), are easily unwittingly ingested as contaminants of food, fingers etc.

The predilection sites of infective *Linguatula* larvae in "normal" herbivorous intermediate hosts are the mesenteric lymph nodes (Sweatman *et al.*, 1962; Sherkov and Rabie, 1977) and bronchial and mesenteric lymph nodes, liver and kidney (Sachs *et al.*, 1973). Less commonly they may be recovered

from other sites such as the spleen, lungs or bloodstream (Khalil, 1972; Sachs *et al.*, 1973). Serological tests for linguatulosis are yet to be developed and thus data on human infections arise only from incidental findings during autopsy; in two instances, where the sample size was large enough, nymphs were not uncommon (see Khalil (1972) for a review). Nymphs may occasionally develop in unusual sites: there are several records of monocular uveitis caused by infective nymphs in the eye chamber of patients (Hunter and Higgins, 1960; Anderson, B. and Roberts, 1962; Rendtorff *et al.*, 1962; Deweese *et al.*, 1962), and Self (1983) pointed out that such infections could develop from either ingested eggs (this is the more probable—see Fontanel *et al.* (1972)) or swallowed infective larvae (see below). Following surgical removal of the parasites, patients recovered fully, and generally there are few pathological symptoms associated with visceral linguatulosis.

In contrast, the effects of nasopharyngeal linguatulosis can be dramatic and in some cases symptoms, though acute, are severe and the condition may occasionally prove fatal. Infections are acquired when infective nymphs, encapsulated in the viscera of a number of domestic herbivores (Khalil, 1976), are ingested in raw or undercooked meat or offal. Hobmaier and Hobmaier (1940), Sinclair (1954) and Khalil and Schacher (1965) followed the early course of infection in dogs, which seems to mirror closely the situation in humans. Dogs, fed bait containing 5–50 nymphs, showed symptoms of nausea, followed by vomiting 5–40 minutes after feeding (Sinclair, 1954). Vomiting is not essential for nymphs to complete successfully the migration from the stomach via the oesophagus to the nasal sinuses because, if it is ablated by anaesthetizing the host, the time taken to become established at the final site is merely increased (Sinclair, 1954). Nymphs can reach the nasal sinuses of dogs in as little as 2 h (Sinclair, 1954; Khalil and Schacher, 1965).

In rural areas of the Lebanon raw liver of goats or sheep is routinely consumed; in the Sudan the equivalent dish consists of raw stomach, liver, lung and trachea of sheep, goats, cattle or camel. The clinical condition of parasitic pharyngitis resulting from the ingestion of infective nymphs of *L. serrata* which may be contained in these dishes is colloquially known as "halzoun" or "marrara syndrome" respectively. Although the food source of the infection has long been recognized (Watson and Abdel-Kerim, 1956), *L. serrata* was not suspected as the aetiological agent (Khalil and Schacher, 1965; Schacher *et al.*, 1965); only recently has this been confirmed by direct observation (Schacher *et al.*, 1969; Khalidi, 1972).

Symptoms, which appear soon after consumption of infected offal, begin as itching in the throat and ears followed by oedematous congestion of the buccopharyngeal mucosa, larynx, eustachian tubes and lips. Dyspnoea, dysphonia, dysphagia and headaches are common and symptoms, though

both acute and severe, are limited to the head; there is no systemic manifestation (Schacher *et al.*, 1965). Paroxysmal coughing and sneezing may dislodge nymphs, and produces immediate relief of symptoms (Schacher *et al.*, 1969; Khalidi, 1972).

Importantly, Khalil and Schacher (1965) have shown that the severity of the symptoms associated with nasopharyngeal linguatulosis in rabbits and monkeys may be exacerbated by previous infections with *L. serrata* eggs. Some of the "hypersensitive" reactions described by Schacher *et al.* (1965) probably result from this type of presensitization (Schacher *et al.*, 1969), although generally in man acute symptoms rarely extend beyond one day (Watson and Abel-Kerim, 1956).

B. *Armillifer* SPECIES

Nymphal pentastomiasis of man in Central and West Africa largely concerns *Armillifer armillatus*. Adult stages parasitize pythons (mainly *P. sebae*) and vipers (notably *Bitis gabonica*, *B. nasicornis* and *B. arietans*) and many mammals serve as intermediate hosts (Fain, 1961; Nicoli and Golvan, 1963; Amy *et al.*, 1974). Fain and Salvo (1966) confirmed that the related species *A. grandis*, which shares some of the snake hosts of *A. armillatus*, can also infect man in the Congo basin. *A. moniliformis*, the causative agent of nymphal pentastomiasis in South-East Asia (see below), and which is infrequently recorded from pythons in Africa (Fain, 1961; Riley and Self, 1981a), has yet to be implicated as a human parasite on the African continent.

Many of the data on the prevalence of *Armillifer* infections are based on incidental findings during surgery or routine autopsy, but in the case of *A. armillatus* this has been bolstered by data derived from radiological examinations (Ardran, 1948; Bretland, 1962): it all combines to suggest that, in certain areas, *Armillifer* infections are not uncommon. Details of the development of *Armillifer* infections in humans, or indeed in mammals, are lacking but, if it parallels that of other porocephalids (*Linguatula serrata* and *Porocephalus crotali*) in mammals, 6–8 moults should separate primary and infective stages (Leuckart, 1860; Esslinger, 1962a), not two as reported by Hopps *et al.* (1971) and Self *et al.* (1975). Infective nymphs, developing mainly in or on the liver, intestinal wall and mesenteries (Hopps *et al.*, 1971; Smith *et al.*, 1975) are visualized as conspicuously annulated worms, coiled within a transparent thin-walled cyst. In older infections these calcify and measure 4–6 mm in diameter (Bretland, 1962).

Recent information on the prevalence of *Armillifer* infections in man is scarce, but the following data suffice to demonstrate that in some areas the

parasite has been, and probably still is, of some significance. Thus Seiffert (1910) found a prevalence of 8% from 218 autopsies and Schaefer (1912) recorded 12.5% from 150 autopsies—both figures are from the Cameroon —and a high prevalence of 23%, from 133 autopsies, was reported from Léopoldville (Mouchet, 1913). More recent figures from radiological examinations indicate lower prevalences, although it should be remembered that this procedure reveals only calcified cysts in chronic infections, and only some cysts may calcify (Ardran, 1948). Nevertheless, Lindner's (1965) figure of 1.4% from 1000 examinations, and that of van Wymeersch and Wanson (1954) of three cases from 70 000 patients screened throughout Africa, probably indicate a significant decline in the number of human cases, a trend confirmed by the most recent figure of 60 cases from 2764 autopsies (2.2%); the highest prevalence of 7.5% was recorded in the 51–60 years age class (Smith *et al.*, 1975).

Infections are acquired in various ways. Some heavy infections have been traced to the ingestion of inadequately cleaned and undercooked snake meat (Fain, 1960; Self *et al.*, 1972; Self, 1983; Azinge *et al.*, 1978). Self *et al.* (1975) and Self (1970, 1982) point out that consumption of food or (more probably) water contaminated by snake faeces is another likely source of infection; apparently, in desert areas, with few waterholes, opportunities for transmission are increased, and records of human infections are frequently from such areas. As Coulibaly *et al.* (1972) comment, statistics of *Armillifer* infections should take into account regional variations in dietary regimen, age class of the population, etc. Among certain ethnic groups snakes are sacred but in others they are a routine source of animal protein. Eggs are known to remain viable on soil for at least 3 months (Broden and Rodhain, 1910).

Armillifer moniliformis is almost exclusively oriental in its distribution, infecting four species of python (Kugi, 1977; Riley and Self, 1981a), and among Malaysian aborigines a relatively high prevalence (45%) of nymphal pentastomiasis has been reported (Prathap *et al.*, 1969); again, highest infections occurred among tribes who consume undercooked snake meat (Self, 1970).

C. PATHOLOGY

The pathology of pentastomid infections is poorly understood and undoubtedly many factors contribute to the overall picture. Usually there is little or no host tissue reaction to mature infective nymphs and infections are largely asymptomatic. Cases of secondary infection such as pneumonia have been linked to *Armillifer* infections (Ardran, 1948) and in some of the rare,

heavy infestations of man, especially where accumulations of nymphs are concentrated in particular organs, severe compression with inflammation can occur (Cannon, 1942; Bouckaert and Fain, 1959; Fontanel *et al.*, 1972; Self *et al.*, 1975; Couliboeuf and Frézil, 1978). Hopps *et al.* (1971) calculated that nymphs increase in volume by at least 1000–fold during development to the infective stage. The relatively few cases where *Armillifer* infections have proved fatal are reviewed by Fain (1960), Self *et al.* (1975) and Smith *et al.* (1975).

It has been suggested that pentastomids cause physical damage by direct migration: Fain (1960, 1966) noted that, in certain circumstances, nymphs can excyst and migrate freely within the host, but this was qualified (Fain, 1966) by adding that "it is not known with certainty if this escaping of nymphs from their cystic membrane occurs during the life of the host or only after its death". Evidence from the literature of migrating nymphs stems largely, *if not exclusively*, from observations made during autopsy, and the underlying cause of post-mortem migration has already been identified (Section VI C). Self *et al.* (1975) offered an alternative explanation extrapolated from data on challenge infections involving *P. crotali* in mice (Self *et al.*, 1972), in which larval(?) migration was extended by a delayed hypersensitive immune response. Visceral larval migrans effects were pronounced, as was the lymphocytic response to challenge by nymphs (sic), but it is not clear from these results how old the migrating larvae were. However, an important clue was provided by Esslinger (1962a), who reported that primary larvae settled down and became lightly encapsulated after 7–8 days; significantly, the ensuing five instars totally lacked hooks. It follows that only primary and infective larvae are likely to migrate, but the micrographs of Self *et al.* (1972) implicated only the former. It is worth reiterating that, at present, there is no good evidence to suggest that encapsulated infective nymphs ever routinely spontaneously excyst in live mammal intermediate hosts.

Discamps and Albert (1974) and Self *et al.* (1975) concluded that human pentastomiasis varies enormously in its clinical and pathological manifestations—the majority of human infections are sub-clinical but with heavier infections abscesses and other lesions can produce more severe effects. Accounts of the histopathological changes associated with porocephalid infections have been given by Esslinger (1962c), Hopps *et al.* (1971) and Self *et al.* (1972, 1975).

A case of grossly disseminated pentastomiasis associated with Hodgkin's disease was reported by Smith *et al.* (1975) who, on the basis of autopsy records from the University College Hospital at Ibadan (Nigeria), calculated a 2.2% infection rate with *Armillifer*. Pentastomiasis was identified as the third commonest cause of hepatic granulomata, and malignant tumours

were associated with 33% of infections. The correlation of *Armillifer* infections with malignancy was highly significant ($p < 0.001$), although the authors concluded that while "no attempt to relate malignancy to pentastomiosis would be valid without the analysis of such important factors as age distribution of different types of malignancy, a complete analysis would be possible only on the basis of a much larger sample of autopsies".

The immunopathological processes associated with pentastomid infections of long duration are often accompanied by degenerative changes (and calcification) resulting in necrotic granulomata or cuticle granulomata (Mendeloff, 1965; Prathap *et al.*, 1969; Self *et al.*, 1972); the aetiology of such lesions is frequently unrecognized (Self *et al.*, 1975) and thus the postulated link between pentastomiasis and malignant disease (Bygbjerg and Rask, 1978) would certainly repay fresh research initiatives.

IX. Immunology

A. Introduction

Although little is known about the immunology of pentastomiasis, the available evidence suggests that there is a strong immune response, involving specific humoral and cell-mediated factors. However, with few exceptions, there is little indication that the immune response is significant in controlling pentastomid infections; rather, there are several pieces of evidence, all admittedly circumstantial, which suggest that these parasites have evolved singular and highly complex mechanisms for evading its effects (Riley *et al.*, 1979). It might be predicted that the pentastomid cuticle would constitute an effective barrier against (say) host effector cells, but the extraordinary lengths to which pentastomids have gone to ensure that, at certain times, the cuticle is entirely covered by a "protective" secretion, indicate that this is not so.

So far as is known, all pentastomids are equipped with elaborate glands which, in two species at least, have been shown to discharge a lamellate secretion on to the cuticle (Riley *et al.*, 1979; Section IV B). The postulated function of this membranous surfactant is to protect the parasite surface from the host's immune response; indeed, considering the degree of development of the glands, together with the strategic positioning of the efferent ducts, alternative explanations are effectively precluded. The mechanisms by which the membranes achieve a protective function are as yet unknown but some progress is being made in this potentially exciting area of pentastomid biology.

B. EVIDENCE OF AN IMMUNE RESPONSE

It was emphasized in Section VII that pronounced inflammatory responses are normally directed against moulting stages and moulted cuticles (Esslinger, 1962c). Encysted porocephalids dying *in situ* also elicit chronic inflammation, often resulting in the formation of granulomas (Nelson *et al.*, 1966; Prathap *et al.*, 1969), and Hopps *et al.* (1971) suggested that parasite death was influenced greatly by the degree of immunity and hypersensitivity that has developed in the host. Ogilvie and Jones (1973) considered that, in helminth-infected animals, the best evidence for cell-mediated immunity was the formation of granulomata.

Although their experimental procedures are obscure, Self *et al.* (1972) found that 8–10 *Porocephalus crotali* nymphs in mice elicited little host tissue damage and encapsulated normally. In heavy infections (45–50 nymphs), in which mice are ultimately killed, some (?) nymphs were not encapsulated but continued to migrate, mutilating tissue. This resulted in typical larva migrans responses, except that eosinophilia was not pronounced: such nymphs were finally killed and abscesses and granulomas formed. Evidence of delayed hypersensitivity was derived from observations that challenge infections were invariably killed by a marked cellular response (Self *et al.*, 1972; Self, 1972). Riley *et al.* (1979) tested this further by challenging rats which had been given 100 eggs of *P. crotali per os* with an equivalent does 1–14 weeks later; the rats were immune to reinfection. The primary infection developed normally, however, to produce about 60 nymphs. Similar experiments using mice failed to demonstrate this classical concomitant immunity (unpublished observations).

Reighardia sternae is mainly a parasite of juvenile gulls (Riley, 1972; Banaja *et al.*, 1975). A postulated age-dependent immunity may prevent its development in adult birds, despite their being equally exposed to infection (note that the life cycle is direct (Banaja *et al.*, 1975)). In an attempt to explain why adult gulls fail to become infected, Riley *et al.* (1979) administered 50 eggs *per os* to a captive adult gull which was killed 40 days later. Fifteen nymphs were recovered, all around 1.6 mm in length. Had these developed in juveniles they would have been about 5 mm long (Banaja *et al.*, 1975). Ten of these stunted worms were surgically transplanted into the air-sacs of an infection-free juvenile which was killed after 10 days and, although only five worms were recovered, these measured between 6.5 and 8.8 mm, within the expected size range of worms 50 days old. The conclusion that the immune competence of the host profoundly influences parasite growth is inescapable.

Finally Ranque *et al.* (1972) and Ranque *et al.* (1974) were able to demonstrate that, whereas *Armillifer armillatus* nymphs developed

normally in guinea pigs, rats, mice and hamsters, inducing high titres of fluorescent and precipitating antibody, in rabbits worms were rapidly destroyed and the ensuing lysis and necrosis of larvae produced granulomata and severe cirrhosis of the liver.

C. THE GLAND SYSTEMS

Of the glandular systems outlined in Section IV B, two groups are important in elaborating surface-active secretion: the frontal and hook gland complex (this is especially difficult to differentiate in cephalobaenids), together with the sub-parietal glands (Riley *et al.*, 1979; Fig. 8). Certain regions of the pentastomid surface, notably the ventral cephalothorax, hooks and the raised part of each abdominal annulus, are in particularly intimate contact with host tissue and it is highly significant that gland cell ducts erupt on to the surface in these areas. The barrier that is the cuticle is breached at intervals by the circular pore caps of chloride cells, and Doucet (1965) and Riley *et al.* (1979) have shown that, in most species, sub-parietal ducts emerge on the periphery of these pores; again a protective function for gland cell secretion is implied (Figs 7 and 9c).

Details of gland cell structure and function in *Reighardia sternae* were given by Riley *et al.* (1979). Porocephalids are in many ways more amenable to study because frontal and hook glands are disparate and compact, and, especially in infective nymphs, frontal glands, flanking the intestine, attain a spectacular relative size (Fig. 8); individual gland cells are packed with secretory droplets (Fig. 8) which may be amorphous or lamellate (Fig. 13). Intermediate stages in the transition of the former to the latter within particular cells suggest that most, if not all, of the individual gland cells are engaged in essentially the same kind of synthetic activity; secretion is eventually channelled into a central duct which opens near the frontal papillae. If hook and sub-parietal cells behave similarly—and preliminary indications support this notion—the potential for coating the entire surface with lamellate secretion can be appreciated.

Riley *et al.* (1979) resolved the thinnest lamellae in the secretory droplets of *Reighardia* as trilaminate. The equivalent droplets in *P. crotali*, which can also be resolved as trilaminate structures on the nymphal cuticle (Fig. 13), may similarly emanate from Golgi bodies. Furthermore, the individual laminae stack in precise and uniform register (although this varies with fixation: compare Figs 13a and b) and are altogether more regular. In short, these membranes are probably organized around a lipid bilayer. Thus far we have investigated gland cells in developing nymphs of *P. crotali* only. Well beyond the last moult, the proportion of frontal gland cells containing fully

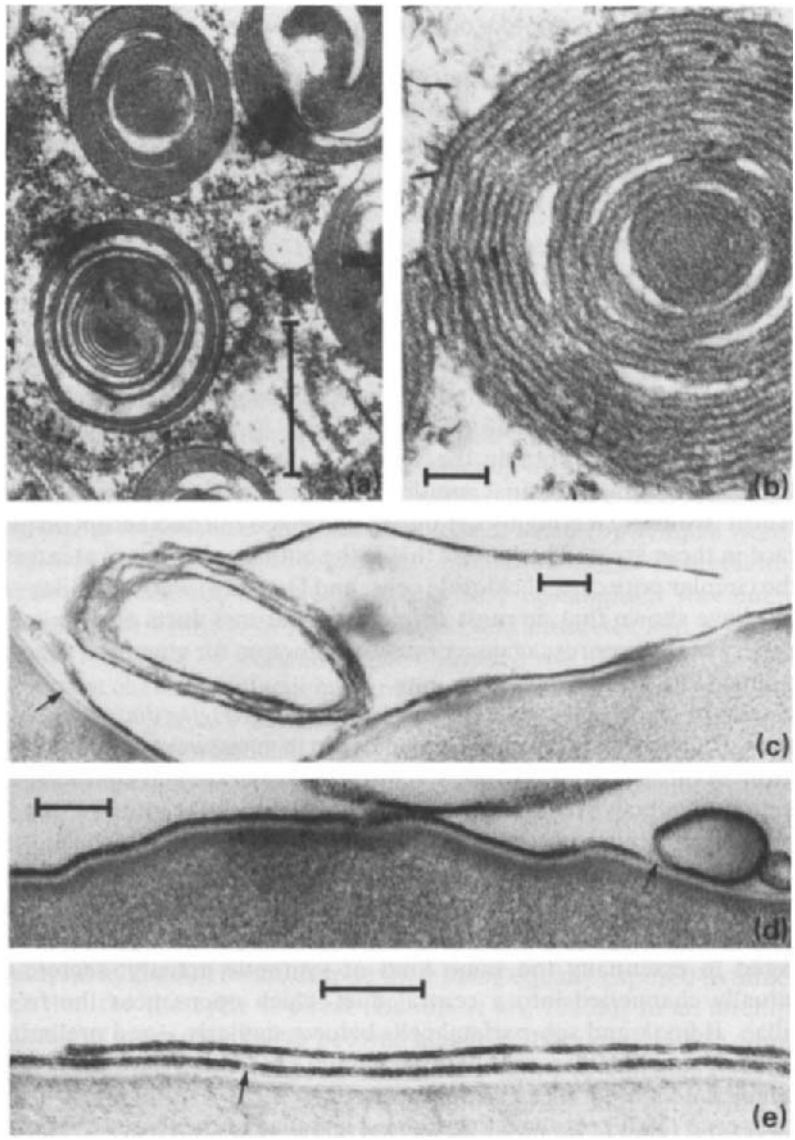

FIG. 13. Electron micrographs of lamellate secretory droplets and lamellate surfactant in nymphal *Porocephalus crotali* 85 days after infection; (b)–(e) are post-fixed in uranyl acetate. (a) Lamellate droplets in the frontal gland against a cytoplasm containing ribosomes and granular endoplasmic reticulum (scale = 1 μm). (b) Detail of a single droplet in a sub-parietal gland cell showing individual trilaminate membranes separated by amorphous material (scale = 0.1 μm). (c)–(e) Outer epicuticle showing deposition of trilamellate surfactant derived from the lamellate droplets shown in (a) and (b). Outermost layer of the epicuticle is arrowed (scales = 0.1 μm.) (Micrographs courtesy of Mr. N. C. Ambrose.)

elaborated lamellate droplets is low, and there is little evidence of active secretion; this coincides with a greatly diminished inflammatory response (Esslinger, 1962c), and it may be that the bulk of the gland system is being held in a state of readiness to overcome the defences of the final snake definitive host. Whole nymph homogenate, and frontal gland homogenate, were both effective at immunizing rats which became partially resistant to reinfection (Riley *et al.*, 1979), but these procedures failed to immunize mice (unpublished observations). There are two obvious possibilities for the function of surface surfactant: it could either bind host macromolecules, or protect the parasite by turning over continuously, perhaps leading to the production of antibody–antigen complexes which "misdirect" the immune response.

D. THE DEVELOPING NYMPHAL CUTICLE

We have recently discovered (all of these observations are, as yet, unpublished) that the morphology of the outermost layer of the *Porocephalus* cuticle changes quite dramatically during development in mice, and at least some of these changes appear to constitute a physical barrier against host inflammatory cells (Figs 14 and 15).

In the premoult nymph in Fig. 14 (56 days after infection and therefore 4th to 5th instar (?)—see Esslinger (1962b)) both the old and new epicuticles are elevated to form a feltwork of thin, "hairy", microthrix-like extensions which at higher magnifications (Fig. 14b,c) seem to be instrumental in keeping potentially marauding cells at bay. Effectively the hairs constitute a palisade of epicuticular extensions which penetrate between and even into host cells (Figs 14b,c, 15), although they are never completely endocytosed. We have not yet ascertained the importance of gland cell surfactant at this critical phase of development, but it does seem that peak inflammation coincides with peak epicuticular "hairyness". The microthrix border of the metacestodes of *Taenia crassiceps* also appears to "fend off" mouse inflammatory cells (Siebert *et al.*, 1979).

An insight into the intimacy of contact between the surface of a developing nymph of *P. crotali* (56 days after infection) and surrounding mouse tissue may be gained from Fig. 15. Clearly, the parasite cuticle is tightly juxtaposed with an enveloping mass of host granulocytes (mainly eosinophils) which are packed into a loose mesh of collagenous connective tissue laid down by fibroblasts. Although the felt of epicuticular extensions overlies the nearest eosinophils, the mass of subcellular debris which surrounds these cells (Fig. 14b,c) suggests that some have been damaged and may have disintegrated.

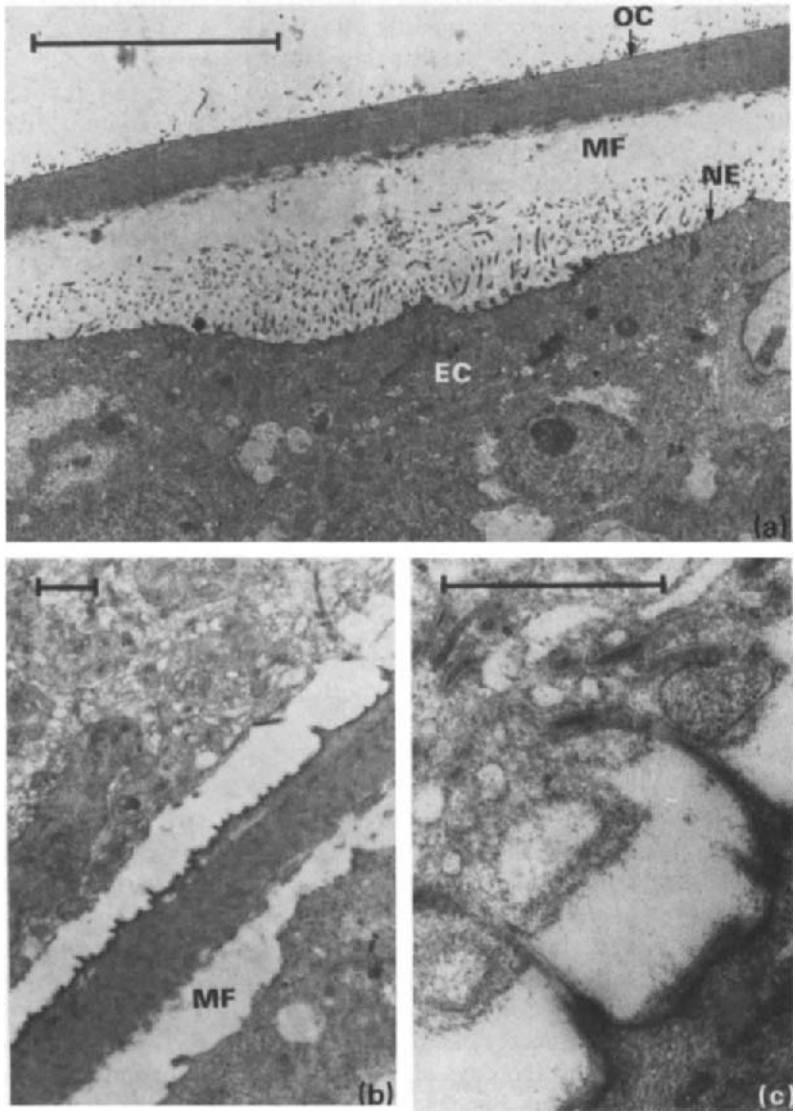

FIG. 14 (a) *Porocephalus crotali*: section through a moulting nymph (56 days after infection) showing old cuticle separating from forming epicuticle. Both epicuticles carry hairy extensions (scale = 10 μm). (b) and (c) Details of the above cuticle showing the epicuticular extensions penetrating into a mass of investing cells and subcellular debris (scale = 1 μm). Abbreviations: EC, epidermal cell; MF, moulting fluid; NE, new epicuticle; OC, old cuticle.

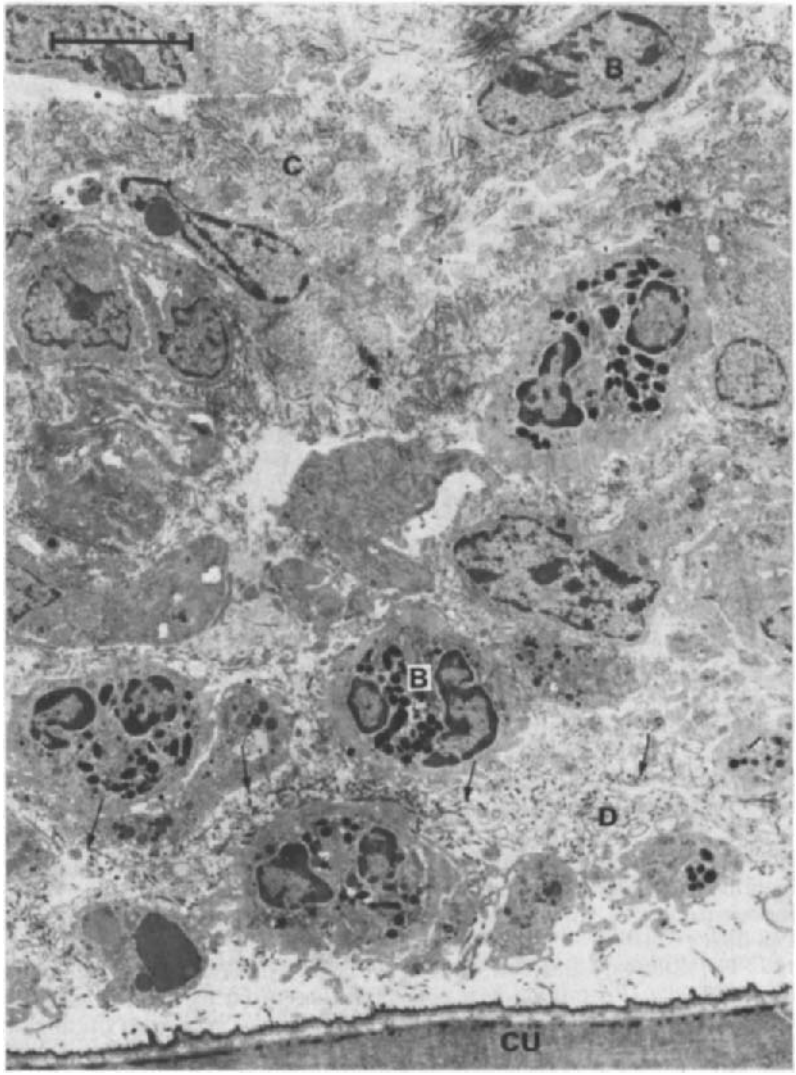

FIG. 15. Part of the inflammatory cell mass enveloping moulting nymph of *Porocephalus crotali* 56 days after infection. Investing cells are mainly eosinophils, B-cells and fibroblasts in a matrix of collagen fibres. The outer limit of the epicuticular hairs is arrowed and it is clear that some eosinophils have penetrated this layer. Subcellular debris suggests that some inflammatory cells have been damaged (scale = 5 μm). Abbreviations: B, B-cell; C, collagen; CU, cuticle; D, debris. (Micrograph courtesy of Mr. N. C. Ambrose.)

The combination of surface-active secretion, coupled with a physical surface barrier during periods of peak inflammation, enables pentastomids to survive intact the host's immune response.

Acknowledgements

I would like to thank Dr. Oliphant Jackson for advice on pentastomid pathology and Mr. Sean O'Malley for providing me with unpublished data on egg filtering. Arne Nilssen and Rolf Haugerud of the Tromsø Museum kindly acquainted me with the background to their recent studies on ruminant pentastomids and allowed me to use their data. I am particularly grateful to Judith Winch and Nicholas Ambrose for their helpful discussion and constructive criticism of earlier drafts of this manuscript and for the use of unpublished micrographs and data. Sections of this review draw heavily upon recent life cycle studies supported initially by grants from the Royal Society and Wellcome Trust and then by a Natural Environment Research Council grant (GR3/5016).

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Hypobiosis in Parasitic Nematodes—An Update

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I. Introduction

Successful transfer from an infected host to a susceptible one represents a most important achievement in the parasitic way of life. Any developmental adaptation which serves to facilitate this process is, therefore, extremely important in the epidemiology of these infections.

Hypobiosis or facultative arrested development represents such an adaptation which, by facilitating persistence of the larval forms for prolonged periods in the host, enables the parasite to capitalize on optimal opportunities for transfer. The phenomenon appears to be widespread among parasitic organisms and it has been particularly well studied in the parasitic nematodes of domestic animals. Most of the following discussion will, therefore, deal with examples from this group.

The term hypobiosis, which literally means a slowing down of life processes, was first suggested by Gordon (1973) to include such terms as arrested, retarded, inhibited, or suppressed development when these are applied to worms that have not completed their development in the host within the commonly accepted development interval for the species. Michel

ADVANCES IN PARASITOLOGY VOL. 25
ISBN 0-12-031725-7

(1974) has described it as "the temporary cessation of development of nematodes at a precise point in early parasitic development when such an interruption contains a facultative element occurring only in certain hosts, certain circumstances, or at certain times of year and often affecting only a proportion of the worms". In this definition he attempted to cover all aspects of the nature of the phenomenon as these could be inferred from the evidence available. It will be readily appreciated that it represents a complex association with the possibility of a multiplicity of interactions between the host, the parasite and their environment.

Schad (1977) emphasized that arrested development of this type involved a temporary cessation of development but that this was more than the brief halt in growth known as lethargus that is associated with moulting from one stage to another in the normal process of development. Rather, this period of arrest could be extended over considerable periods of time.

Recognition of the populations of arrested worms in an animal is often a problem when examining natural infections in a host. These populations of arrested or hypobiotic worms must be differentiated from comparable stages undergoing normal rates of development. Michel (1978) maintained that it is the uniformity of a population of arrested larvae that is the most useful criterion in differentiation. Typically, when there are both arrested and developing worms present in a population the size distribution will be bimodal. One part of the population will show wide variation in size while the other will show little variation about the characteristic size and will represent the arrested larvae. Inhibited development or stunting must also be distinguished from arrested development, and it is because this has not been done in many of the published reports on hypobiosis that some of the present confusion exists on the precise nature of the phenomenon.

In the most complete reviews of arrested development in nematodes to date, those of Michel (1974) and Schad (1977), the authors have stressed such aspects of the phenomenon as the species involved, factors associated with its induction as well as those influencing resumption of development, implications of the phenomenon for understanding the epidemiology of these conditions, and its adaptive and population regulatory significance. The present review re-examines some of the previously reviewed material and discusses additional information that has become available since these reviews, as well as attempting to integrate this information into a more comprehensive understanding of this important phenomenon.

II. Significance of Hypobiosis in the Biology of Nematodes

The significance of arrested development to the parasitic nematode has been the subject of much speculation. It has long been considered to be closely

involved with host resistance and the regulation of populations of incoming parasites. It was assumed that host resistance caused the arrested development, and that resumption of development was a result of the relaxation of immunity. That such arrested larvae could apparently resume development was demonstrated by Gordon (1948) with *Oesophagostomum columbianum*, by Michel (1952) with *Trichostrongylus retortaeformis*, and with the horse cyathostomes by Gibson (1953). Basically it was considered to be a host-regulated phenomenon which served to limit the size and pathogenicity of the population of worms that was accumulating during the season of increasing larval availability. This viewpoint was subsequently expanded to include situations where naive hosts might also regulate their burdens by immunological means when a critical threshold of parasite biomass was exceeded. Dineen (1978) proposed that control over larval development could be exerted by immunologic responses to fluctuating levels of parasite antigen about a threshold. Thus, when the number of developing worms was large, an immune response might be triggered, causing larvae that were subsequently acquired to become arrested. With loss of adult worms, host resistance would decline, allowing resumption of development of some of the arrested larvae.

It was, however, shown by other workers, primarily Anderson *et al.* (1965) working with *Ostertagia ostertagi* of cattle, that seasonal factors might also be important in the induction of arrested development in some species. Consequently, there has developed a strong argument that the function of arrested development in some nematode species is primarily a mechanism permitting survival over seasons unfavorable for external development or transmission as free-living forms (Blitz and Gibbs, 1972a and b; Michel, 1974).

In this view it is considered to be a normally occurring feature of nematode life cycles which evolved when species were confronted by periods of adverse environmental conditions that were inimical to their survival or transmission. It could thus be particularly important at the extreme ranges of a parasite's distribution where the external environment may barely permit its continued existence, as in the case of *Haemonchus contortus* in temperate areas. In other species, like *Nematodirus* spp. in sheep, where the life cycle is successfully adapted both to survival in the form of resistant eggs and the synchronization of larval hatching with rising spring temperatures and lambing, the value is less obvious.

In the case of those parasites whose infective stages survive adverse environmental conditions successfully, hypobiosis might represent an adaptation that developed in association with hosts that had extensive migratory habits. It would enable large numbers of metabolically quiescent worms to travel within hosts from one breeding season to another. Under

set-stocked conditions its importance would thus be lessened. It is interesting, in this respect, that Smeal and Donald (1982) have observed reversions in the propensity for arrest of *O. ostertagi* larvae in calves that are set-stocked compared to larvae in calves that are free-ranging.

Schad (1977) has further suggested that in those instances where seasonally related developmental arrest permitted survival during periods of adversity it could also serve as a highly adaptive mechanism for regulating populations. By allowing the large numbers of incoming larvae that are usually associated with arrested development to become metabolically quiescent at a stage in the host's existence when the environment is also degenerating for the host, it would serve to exert a sparing effect on the host. A high density of adult worms with their potential for pathogenicity represents a highly unfavorable component in a parasite's environment.

Since, in many instances of hypobiosis, maturation of the larvae coincides with the availability of susceptible host neonates, Gibbs (1982) speculated that there may be a distinct advantage to the parasite to enter the neonate as early as possible in its life and in relatively large numbers. Based on the results of Kassai and Aitken (1967), Kassai (1968) and Dineen and Kelly (1973), larvae that enter the neonate early enough could possibly capitalize on a relative immune tolerance in these neonatal hosts. This probably also holds true for a parasite like *Toxocara canis*, which is infected into pups transplacentally (Sprent, 1958) or *Ancylostoma caninum* (Stone and Girardeau, 1966) which is infected colostrally. Infections of the fetus or neonate by these means could also induce a state of immunological tolerance, allowing the worms to persist for longer as egg-laying adults in the pups.

In those cases where large numbers of infectives are simultaneously available, as with the "spring" or periparturient rise in egg counts seen with parasites like *H. contortus* (Gibbs, 1968), consequent acquisition of heavy infections by the susceptible neonates might result in a form of immune paralysis which would favor establishment of substantial populations of worms in these young animals.

III. Types of Hypobiosis

It now seems to be generally accepted that, rather than being a single phenomenon, hypobiosis in parasitic nematodes represents at least two different types of developmental behavior having different causes, although they may ultimately culminate in a similar manner. One of these types of hypobiosis is considered to have an immunological basis. It is viewed as a response of incoming larvae that enter a resistant host to constraints on their further development imposed by the immune status of that host. This type of

hypobiosis might best be referred to as inhibited development. Furthermore, it should be implicit in this assumption that there is some type of negative, possibly deleterious effect of the host on the developing larvae, resulting in the slowing down or stopping of development.

The other manifestation of the phenomenon appears to be that of an innate response within the larvae themselves to some stimulus or stimuli that may be entirely independent of the host as, for example, environmental temperature. This form of hypobiosis would represent primarily a seasonal adaptation for arrest and be analogous to diapausal development in insects as suggested by Armour and Bruce (1974) and more recently by Horak (1981). It would appear to represent a facultative pause in development which is an integral part of the developmental cycle of these nematodes directed primarily at facilitating their survival within the host over long periods of time, as well as increasing their chances for transmission at the appropriate time to a susceptible host.

Horak (1981) has suggested that there are basically two types of arrested development in parasitic nematodes which he termed nonspecific and seasonal arrested development. Non-specific arrest could occur at any time and its causes could either be host-related or parasite-related factors which affect the immediate environment of the nematode, causing inhibition in development. Seasonally arrested development, on the other hand, represents an annual event during the same season and is dependent upon nematodes being adapted to a particular environment and being responsive to external environmental stimuli acting upon the infective larvae which result in arrest at a later stage of development.

This phenomenon is not exclusive to the parasitic nematodes, however. A strictly analogous modification of development has been described with some of the free-living nematodes, in particular *Caenorhabditis elegans*. In this species a type of hypobiotic larva is produced in response to an unfavorable stimulus in the environment of the free-living nematode. That stimulus, usually overcrowding or starvation, results in formation of a facultative juvenile stage, the dauer larva, at the second molt. Dauer larvae may survive at least four to eight times the normal 2-week life span of the worms (Klass and Hirsh, 1976), and they represent a specialized dispersal form which is arrested in development, non-feeding and especially resistant to environmental stress (Cassada and Russell, 1975).

A. IMMUNE MEDIATED ARREST

As previously mentioned, the development of host resistance as a result of exposure to infective stages has long been considered to be closely

associated with the onset of certain types of arrested development, and the possibility that such arrested larvae could resume development has been demonstrated (Gordon, 1948; Michel, 1952; Gibson, 1953).

1. *Induction of immune-mediated arrest*

It is assumed that host resistance causes the inhibited development and that resumption of growth is the result of the relaxation of this immunity. Many researchers, including Ross (1963), Donald *et al.* (1964), and Dineen *et al.* (1965a; 1965b), have reported that hosts previously exposed to infections carried greater burdens of arrested larvae than unexposed, immunologically naive animals. However, as Michel (1978) has pointed out, many experimental results which purport to show that arrested development is the consequence of host resistance, on closer examination demonstrate only that adult worms are lost more rapidly from resistant than from susceptible hosts. Since arrested worms tend to persist, the ratio of arrested worms will change with passage of time. If animals are exposed to infection over a long period of time, intakes of small numbers of arrested larvae will accumulate while the numbers of adults will remain constant, thus causing the arrested larvae to represent an ever greater proportion of the population.

While arrested development has been described for an increasing number of species, the importance of host immunity as a prime inducer of arrest has still not been clearly demonstrated. For example, it was generally considered that species of the genus *Trichostrongylus*, found in the gut of sheep, showed little or no capacity for arrested development. However, Eysker (1978) and Ogunsusi and Eysker (1979) were able to demonstrate arrested development in both of the common species, *T. colubriformis* and *T. vitrinus*, at the parasitic third stage (L_3) rather than the early fourth stage (L_4) characteristic of many abomasal nematodes. These larvae are very small, 750 μm to 850 μm in length, and it was suggested by Waller *et al.* (1981), who confirmed Eysker's findings, that this is a possible reason for the lack of previous reports of arrest in these species. Eysker (1978) concluded that host immunity was mainly responsible for inducing arrest in this genus. However, Waller *et al.* (1981) disputed this conclusion as they were unable to associate inhibited development in this genus with size of worm burden, developing resistance in the host or environmental conditions experienced by the free-living stages. Furthermore, they concluded that arrested development was not a consistent feature of the life cycle of these worms.

In the case of another genus, *Nematodirus*, that shows high unpredictability with respect to propensity to arrest, Thomas (1978) and Eysker (1980) have both indicated that host resistance effects may be important in inducing arrest when it occurs, but this has not been demonstrated unequivocally.

That host immunity may play a role, albeit minor, in the induction of arrest in species which seem to be primarily seasonal arrestors has been suggested by a number of researchers. For example, Adams (1983) found that more arrested *H. contortus* were present when challenge infections were superimposed on existing infections, indicating that resident worms or a factor activated by their presence induced developmental arrest.

Michel (1978) also suggested that, in the case of some other classical examples of seasonally arresting worms, like *O. ostertagi* of cattle, there was an indication that host resistance factors could play a part in induction of arrest. Using a strain of *O. ostertagi* which had ceased to arrest in response to cold treatment of the infective larvae, Michel *et al.* (1979) found that the innate resistance seen in older animals, when combined with seasonal effects, caused over 85% of the larvae in a challenge infection to become arrested.

Recently Snider *et al.* (1981) found that previously infected calves harbored higher proportions of early L_4 of *O. ostertagi* than calves that had not been infected before challenge with multiple increasing doses of larvae. An immune response in the host was suggested by lymphoid cell infiltration of the mucosa, and this may have accounted for the greater larval inhibition exhibited by the previously infected calves.

In the case of *O. circumcincta* in sheep, which has been shown by Reid and Armour (1972) to be primarily a seasonal arrestor, Gibson and Everett (1978) have reported that lambs given daily doses of a few thousand larvae gradually became resistant to infection, and this was accompanied by the accumulation of a high proportion of arrested larvae. Smith *et al.* (1984) also observed that in previously sensitized sheep given a challenge dose of 50,000 *O. circumcincta* a high proportion of the larvae became arrested. That this was not due to overcrowding effects was shown by the fact that in non-sensitized sheep receiving the same dosage very few arrested larvae were seen. However, this might have represented a slow down in rate of development rather than true arrest, as the experiment was terminated ten days after larval challenge. Behnke and Parish (1979) have reported that, when immune mice were challenged with *Nematospiroides dubius* larvae, the larvae became arrested in the intestinal wall. Treatment of the mice with cortisone caused reactivation of development of the arrested larvae, suggesting that they were immune-suppressed.

Fox (1976) investigated the effects of host immunity as a factor contributing to arrest in the rabbit stomach worm *Obeliscoides cuniculi*, another parasite shown to be strongly responsive to environmental factors in the induction of arrest (Fernando *et al.*, 1971). Rabbits were either actively immunized with 75,000 infective larvae or passively immunized with injections of immune sera and then challenged with 5000 fresh L_3. Twenty-six

days later the population of worms arrested at the early L_4 stage was 18.3% in the actively immunized rabbits compared to 0.4% in the passively immunized and 0.1% in the controls, suggesting that host immunity could also play a part in arrest in the life cycle of this worm.

In the case of those nematodes that undergo arrest in the tissues of the host as histotrophic larvae the evidence is not clear as to the role of host immunity in inducing arrest. Many researchers in this area have implied that it is only in the sensitized or older host that larvae will arrest, but in most instances there is little direct experimental evidence in support of this assumption. Schad (1982), in his experiments on the induction of arrest in *A. caninum*, used young, 2-month old, helminth-naive pups. He found that in addition to arrest that was definitely attributable to the effects of environmental stimuli on the larvae, a proportion of larvae always arrested in his control pups. He referred to this as "background arrest". It is unlikely that this arrest was due to immune effects in these hosts. Nwaorgu and Connan(1981) found that a high proportion of the larvae of *Strongyloides papillosus* became arrested in the musculature of non-sensitized rabbits and sheep after subcutaneous injection. If the adults that developed from these injections were removed by an anthelmintic, this caused resumption of larval development and replacement of the adults. Arrested larvae seemed to function to maintain intestinal populations of adult worms and there was a possible feedback immune-mediated mechanism that operated in this instance.

In contrast to these results were those of Schad and Page (1982), who were unable to stimulate resumption of growth in hypobiotic larvae of *A. caninum* present in the musculature of pups by selective removal of adult worms with an anthelmintic. The hypobiotic larvae in the musculature of these pups survived for prolonged periods before they renewed development, and when it did occur it was spontaneous and idiosyncratic.

With *T. canis* of the dog, it appears that when acquired by a resistant host the larvae will become arrested and encapsulated in the somatic tissues rather than completing development to the adult in the gut. It is these somatic larvae that serve as reservoirs for prenatal infection in the pregnant bitch. However, if *T. canis* larvae are ingested by an "unsuitable" host, such as a rodent, the larvae will also arrest in the tissues of this host, which then serves as a paratenic host (Sprent, 1958, 1961). In this latter case it is not host immunity that is causing the arrest, but rather an "unsuitability" of the host milieu for further development.

While it thus seems that host immune effects can act to induce arrest in some of the parasitic nematodes, there does appear to be variation in the degree to which individual species respond to this stimulus. As Schad (1982) has suggested, it is unlikely that parasites that have a histotrophic arrested stage, with several alternative transmission pathways which are protective of

the larvae, would have been selected strongly for specificity of arresting stimulus. This would lead to variability in the degree to which a species responded to any given stimulus.

2. *Morphological and physiological changes in larvae associated with immune-mediated arrest*

It is of interest from the viewpoint of differentiating immune arrested larvae from seasonally arrested larvae to determine if they differ uniquely in some physiological or morphological feature from each other. However, examination of the literature that deals with immune-mediated arrest gives little information on physiological or other changes that are specifically associated with larvae undergoing this type of arrest.

Implicit in the assumption that host resistance effects can induce arrested development is the fact that such a mechanism might involve inhibitory or retardive effects on development in these larvae. Immune mediation of arrest might best be thought of as operating by way of some anti-developmental factor which would presumably have to act on incoming infective larvae in such a way that, even though molting to the next stage was accomplished, further development would be inhibited. Morphological evidence of deleterious effects on the larvae might, therefore, be expected. These might take the form of epicuticular precipitates, the accumulation of degenerative products or changes associated with the larval cells.

Changes of this sort have been described in some arrested larvae but have not been exclusively restricted to immune-induced arrested ones. For instance, Blitz (1970) observed larvae of *H. contortus* with epicuticular precipitates in one of his experimental sheep. However, these larvae appeared stunted, suggesting that they were the victims of adverse reactions to host immunity rather than undergoing hypobiosis.

Blitz and Gibbs (1971a) reported rod-shaped crystals in the intestinal cells of arrested L_4s of *H. contortus*, and subsequently these have been described by other workers. However, the larvae in other respects appeared to be normal and a link with immune induction of arrest could not be made. Stoye (1973) reported that hypobiotic larvae of *A. caninum*, from the musculature of dogs and from paratenic hosts, were morphologically identical and characterized by massive accumulations of fine granular material in their intestines, such as is found in physiologically aged larvae, but these were not attributed specifically to immune effects. However, Martin and Lee (1976) described crystals from the intestine of *N. battus* and associated the appearance of these crystals with the acquisition of host immunity.

More recently Bird *et al.* (1978) and Waller *et al.* (1979) have reported on the ultrastructure, chemical configuration, occurrence and significance

of inclusion bodies in a range of nematode species in sheep and cattle. In *H. contortus*, intracellular, rod-shaped inclusions occurred relatively constantly in seasonally arrested larvae. Formation of the inclusions was not correlated with increasing host age or with host responses to increasing exposure to infection. Furthermore, it did not appear to be related to seasonal fluctuations in the levels of larval arrest. They concluded that the crystals predominated in larvae undergoing degeneration and suggested that many larvae with crystals were rejected by the host. On the other hand, Eysker (1979) found that a high percentage of arrested early L_4s of *H. contortus* contained crystalline inclusions, whereas much lower percentages of late L_4s and early L_5s had crystals. He also observed that before the fourth molt the crystals underwent disintegration, indicating that worms with crystals could develop further. He associated the occurrence of these crystals with normal arrested development. With *O. ostertagi*, Waller *et al.* (1979) saw crystals only in adults and L_4s recovered by cross-infection in sheep. These crystals were considered to cause blockage of the gut which could have adverse effects on the parasite. Recently Pelinski (1980) has reported needle-shaped crystals in the bodies of *H. contortus* and *H. placei* L_4s showing seasonally arrested development.

Evidence that there are morphological features characteristic of immunologically arrested larvae is therefore equivocal. The crystals and other inclusions that have been described are apparently found in larvae arrested either immunologically or seasonally. Certainly in some instances they appear to be associated with degenerative processes in the larvae that ultimately lead to their expulsion from the host.

A physiologic difference in susceptibility to anthelmintics, as described for seasonally arrested larvae (Anderson, 1977; Watkins, 1982), has also been reported for immune arrested larvae. Behnke and Parish (1979) found that immune arrested larvae of *N. dubius* in mice were insusceptible to the activity of pyrantel pamoate, an anthelmintic which is 99% effective against adults.

It thus appears from the limited evidence that morphologically and physiologically no unique feature has been described which can be used to differentiate immune arrested larvae from seasonally arrested larvae.

B. SEASONALLY INDUCED ARREST

This type of hypobiosis appears to be a facultative, innate developmental response primarily associated with survivability and facilitation of transmission for those species that possess the capability. It appears to be the nematode equivalent of diapause in insects, and many of the features

characterizing this type of behavior are similar to those seen in insect diapause. Like diapause it appears to occur in response to a stimulus that is usually environmental but could also be multifactorial. It tends to be restricted to one stage in the life cycle of the worm, usually the fourth larval or L_4 stage, but an earlier stage, the L_3 or infective stage, appears to be the recipient of the inductive stimulus.

There is now good evidence that it represents an innate characteristic of certain nematode species. It has been demonstrated that it can have a genetic basis, and may be selected both artificially and under natural conditions. It has been most frequently associated with, and consequently studied in, the Strongyloidea, where there is a free-living phase in development.

1. *Factors involved in the induction of seasonal arrest*

A number of potential inductive stimuli for this type of hypobiotic development have been investigated, but in only a few instances have definite stimuli been implicated as causing arrest in a particular species. Part of the reason for this is that, in most instances when we are studying a "conventional" or taxonomic species, we are in fact dealing with a species complex encompassing numerous strains or geographical races. Such strains have evolved to fit particular environments and may represent a wide range of graded responses to any given environmental or other stimulus that may induce hypobiosis. Thus the same "species" in the hands of different investigators from differing geographical locations may give very different results.

(a) Environmental stimuli. In insects, diapause is usually initiated as a response to environmental signals which herald the coming of adverse conditions (Chapman, 1982); similarly, in certain nematode species environmental stimuli appear to be important.

(i) Temperature. In temperate climates seasonally induced arrest seems to be linked to the advent of cool temperatures. Armour *et al.* (1969) first suggested that chilling of the L_3 of *O. ostertagi*, such as occurs during autumn and winter, might be the primary stimulus for induction of arrest in this species. This was later confirmed by Armour and Bruce (1974), who further suggested that this type of arrest represented a diapause-like phenomenon. However, cold as a stimulus for arrest in *O. ostertagi* in north temperate populations has not been unequivocally implicated. Thus Michel *et al.* (1974) reported that constant low-temperature storage was less effective than the experience of natural conditions on pasture in inducing arrest, and Armour (1978) found that exposure to gradually falling fluctuations in

temperature and decreasing day length were most effective in inducing arrest in *O. ostertagi*.

Fernando *et al.* (1971) and Hutchinson *et al.* (1972) showed that arrested development in the rabbit stomach worm *O. cuniculi* could be induced by maintenance of the third larval stages at low temperature (4°C) for periods of 6 weeks, and subsequently Watkins (1982) used this very effectively in his selection experiments for propensity to arrest in *O. cuniculi*. Additional evidence suggestive of a temperature induced arrest was provided by Blitz and Gibbs (1972a) and McKenna (1973), who showed that exposure of *H. contortus* L_3s to autumn conditions would induce a high degree of arrested development in these worms.

Subsequent work by a number of researchers has provided confirmatory as well as indirect evidence that exposure of larvae to low environmental temperatures could induce arrest in a number of different species. Thus Waller and Thomas (1983) reported that high levels of inhibition in *Nematodirus* spp. seen in autumn appeared to be the result of seasonal factors acting on the larvae, and were independent of the size of infection or duration of exposure of the lambs. Herd *et al.* (1984) suggested that hypobiotic development observed in their experiments on sheep was induced by seasonal stimuli acting on the infective larvae rather than by host immune responses, since hypobiosis was observed in November but was absent or negligible in August. Hypobiotic larvae were derived from L_3s ingested during October and November, a period when temperatures fell as low as 5°C and conditions were appropriate for cold-conditioning. Eysker (1981) reported that exposure of infective larvae of *H. contortus* for 5 weeks to 15°C or 16°C very effectively induced arrested development, whereas exposure to lower temperatures was considerably less effective. *O. circumcincta*, however, required lower temperatures, and exposure for 5 weeks to 6°C was effective in this instance.

In some instances fluctuating temperatures appeared to be more effective in induction than constant temperatures; thus Schad (1982) found that if *A. caninum* L_3s were exposed to regimes in which temperature was varied between 5°C and 15°C (simulating autumn fluctuations in temperature) significant levels of arrest could be induced, ranging from 64.3% to 87.7%. Adjustment for background arrest gave levels of 65.1% which were comparable to levels of arrest observed under field conditions. He commented that, even though the potential to arrest might reside in only a portion of the free-living population if potential adults either failed to develop or were expelled, the result was a parasite population constituted largely of arrested larvae. In additional experiments, sudden chilling at 7 days also produced comparable levels of arrest, which suggested that precipitous changes in temperature rather than prolonged

low-temperature storage was a specific signal for induction of arrest with *A. caninum*.

In tropical and semi-tropical areas seasonally induced hypobiosis is more usually linked to the onset of hot, dry conditions. Thus Shimshony (1974) has reported that some goat nematode parasites showed arrest during the dry season in Israel. In the southern USA Williams and Bilkovich (1971), Williams *et al.* (1983) and Baker *et al.* (1981, 1984) have observed peak arrest of *O. ostertagi* in spring, with accumulation of larvae over the hot, dry summer months.

Likewise in Australia, seasonal occurrence of inhibited development in *O. ostertagi* reaches a maximum in spring in permanently grazing cattle (Anderson, 1968; Smeal *et al.*, 1977). Unlike *O. circumcincta* in temperate zones, which arrests in autumn and winter (Reid and Armour, 1972), in warmer climatic regions this species behaves similarly to its bovine counterpart. Thus in Australia arrested development of *O. circumcincta* tends to reach its maximum in larvae ingested in spring (James and Johnstone, 1967; Anderson, 1972; Southcott *et al.*, 1976). These larvae remain arrested until midsummer (January) but mature by autumn (April). Also in Iraq, Altaif and Issa (1983) have reported high levels of arrest in *O. circumcincta* during the hot months of summer.

Further evidence as to the relative independence of arrest in *O. circumcincta* from host immune effects is the work of Donald *et al.* (1982). They found large populations of arrested L_4s in lactating ewes 7 weeks after the start of lambing, when the postparturient rise in egg counts was at its peak, these larvae being acquired from pasture. The ewes had more arrested L_4s at this time than their total L_4 burden before lambing. Lactating ewes have been shown to be immunologically less competent than non-lactating ewes and therefore more susceptible to infection by incoming larvae that develop normally and produce eggs which contribute to the periparturient rise (O'Sullivan and Donald, 1970; Chen and Soulsby, 1976). From the results of their experiment it appeared that lactation and the concurrent depressed immune state did not prevent larvae from becoming arrested, and that the stimulus for arrest must be other than immunological.

Presumably in these instances of arrested development in warmer climates, the stimulus for arrest is not cold and might be increasing temperatures, increasing moisture during the rainy season, or even the onset of dry conditions associated with summer.

(ii) *Humidity*. Since factors other than environmental temperature might be operative at the time of year when seasonal arrest occurs, there has been some speculation as to the possible role of moisture or humidity as one such alternative. Ogunsusi and Eysker (1979) have shown that inhibited development of *H. contortus* in Nigeria occurs towards the end of the rainy season

and, as has been mentioned, this pattern of arrest is generally seen in other tropical and sub-tropical areas. It is tempting, therefore, to speculate that increasing environmental moisture such as would occur during the rainy season could act as a seasonally associated inducer of arrest in certain geographical areas.

Connan (1978) attempted to show that fecal culture water content could act as a stimulus to induce arrested development in larvae of *H. contortus*. To this end two types of cultures were used, one "dry" and one "wet". "Dry" cultures contained approximately 58 g percent of water whereas "wet" cultures contained approximately 77 g percent of water. Significantly higher levels of arrest were obtained in the larvae from the "wet" cultures than from the "dry" cultures. However, there also appeared to be reduced infectivity associated with the larvae from the "wet" cultures, and Connan (1978) suggested that the difference in behavior of the larvae might be explained on this basis. In one trial, infectivity of "wet" cultures was zero and in the remainder of the experiments it was generally lower than with larvae from the "dry" cultures. If loss of infectivity affected primarily non-conditioned larvae it might partly explain the difference in results between the two methods of culturing. Connan (1978) therefore suggested that arrest might require a stimulus or could be innate in a few of the infective larvae used, with differential loss increasing the proportion in lambs infected from the "wet" cultures.

(iii) *Photoperiod.* Since one of the very reliable and consistent indicators of seasonal change is day length or photoperiod, and this stimulus appears to be most important in initiating diapause in insects (Chapman, 1982), it was considered to be a likely signal to initiate seasonal onset in nematodes as well. An attempt was, therefore, made by Gibbs (1973) to show that photoperiod might be an important inducer of arrest in some instances. Larval cultures of *H. contortus* were subjected to two light regimes. One represented a short (10 hour) the other a long (16 hour) photoperiod and L_3s were exposed to these two light regimes for a period of 6 weeks at 20°C. Arrested development in the larvae which had been exposed to the short photoperiod was significantly ($p < 0.01$) increased (70%) over that in the larvae held at the long photoperiod or to that in freshly cultured larvae.

On the other hand, Connan (1975) was unable to relate storage conditions of L_3s of *H. contortus* to a propensity for arrested development. He cultured infective larvae in darkness at 25°C then subjected them to various regimes, including exposure to pasture conditions for increasing 10-day intervals up to 40 days, storage inside in darkness at 4°C, or storage outside in darkness for 40 days. He observed little difference in the degree of arrest between the different groups, but found that larvae held in culture for 12 days showed high levels of arrest when given to sheep. This led him to

suggest that unidentified environmental factors acting during the 12 days of culture were capable of inducing a potential for arrest. In this work he did not eliminate the possibility that short photoperiod might have been a factor; however, Cremers and Eysker (1975) were also unable to demonstrate any significant effect on the extent of arrested development in larvae of *H. contortus* when L_3s were exposed to variations in day length.

The evidence that photoperiod can play a role is thus still equivocal.

(b) Host-related factors. Since changing environmental effects influence the host as well as the free-living stages of the worms, it is entirely possible that alterations in the host in response to these environmental changes could act as signals for larvae entering these hosts, influencing their development and thus inducing seasonal developmental arrest.

On the premise that, since photoperiod can influence host physiology (Ortavant *et al.*, 1964) and this might in turn influence nematode parasite behavior, Blitz (1970) conducted an experiment using two groups of ewes infected with *H. contortus*. The ewes were subjected to two photoperiod regimes, one using a fixed long day of 18 hours of light and 6 hours of darkness, the other the prevailing environmental period. Larvae from these ewes which were dosed to groups of parasite-free lambs held under similar conditions of photoperiod showed no difference in rate of arrest between the two groups.

Subsequently, Armour (1977) gave, in autumn, freshly cultured *O. ostertagi* L_3s and larvae conditioned for several weeks at 4°C to stabled calves or to calves held in a climatic chamber programmed to simulate spring conditions. Only those calves given the conditioned larvae had significant burdens of arrested larvae at necropsy, indicating that there was no effect of the host on the induction of hypobiosis.

Coadwell and Ward (1977) studied the relationship of annual variation in the growth of *H. contortus* in experimental infections of sheep to arrested development. Fifty-six worm-free sheep were each infected with single infections of 50 000 *H. contortus* L_3s at varying times over a 2-year period. The sheep were killed from 13 to 89 days after infection and the lengths of both male and female worms measured. Using these data, growth lengths were computed for each sex. The results showed that there was a close relationship between relative worm lengths and time of year. The relationship was cyclical and closely followed a sine curve with a maximum at day 90 and a minimum at day 275 of the year.

Arrested development was first recorded at day 217 of the year and arrested larvae were present in all subsequent infections until day 26 of the following year. These results showed that arrested development appeared to be a part of a seasonal cycle in the growth pattern of the worms and was a

component of a gradual change rather than an isolated sudden change. It occurred in autumn with a clear demarcation between the beginning and end of the period. Because of the nature of the infecting process, Coadwell and Ward (1977) were able to rule out implication of a host immune response, but the results suggested that the host had a strong influence on development of the worms. The authors concluded that the most reasonable explanation of their results appeared to be that either one or a group of substances in the sheep were available for ingestion by the worms in varying amounts according to the time of the year. When the levels of this particular substance or substances rose or fell, arrested development at the L_4 stage started and continued until the appropriate part of the cyclic pattern recurred. Coadwell and Ward (1977) claimed that they were able to rule out any effects of changes in environment on the preparasitic stages because of the facts that the strain of worms used was shown by Connan (1975) to be unresponsive to such stimuli and that their larvae were stored at 5°C for not more than 60 days. I question this, as there is evidence of low temperatures having some effects on arrest in *H. contortus*, and 60 days would appear to provide an adequate length of time for such factors to operate (McKenna, 1973; Eysker, 1980).

An alternative explanation of these results might also be that of Waller and Thomas (1975), who suggested that the strain of *H. contortus* in Britain had an innate capacity for showing developmental arrest during the autumn months of the year.

(c) Parasite-related factors. Since seasonally related, arrested development appears to be an innate capability present in some nematode strains but not in others, it is reasonable to expect that some of the factors relating to the induction of this type of development would be exclusively parasite-related. Such factors would primarily include worm interactions and genetically regulated propensities for arrest that might exist in certain strains.

(i) *Worm interactions.* In many instances larval arrest seems to be initiated at times of year when larval intakes tend to be maximal, coming as it usually does at the ends of periods of optimal environmental conditions for larval development and accumulation. This has led to the suggestion that the sheer magnitude of such larval intake may result in crowding or overcrowding of larvae which might act as signals or stimuli for the initiation of arrest of development. Much of the experimental evidence for these types of density-dependent effects on developmental arrest has been reviewed by Schad (1977). As he has pointed out, the belief that, when a large fraction of a large dose of infective larvae arrests it is an immunologically mediated phenomenon, is based on the fact that the greater the dose the greater the antigenic stimulation and the greater the host's response. In the case of species that do

not arrest until the late fourth stage or fifth stage this is not difficult to imagine. However, for species that arrest as L_3 or early L_4 such dose-dependent effects would have to be triggered immediately on larval entry into the host. For such species, control of arrest by way of the host's immune system would, therefore, seem unlikely.

However, there exists the real possibility that there could be a direct effect from the parasite recognizing its own population density through some pheromone-like substance. There is no direct evidence that such a phenomenon is operative with the parasitic nematodes, but neurosecretory cells have been demonstrated in parasitic nematodes (Davey, 1982). While there is no observation which directly links neurosecretion to growth and development in nematodes, neurosecretory cells are associated with the production and release of hormones governing growth in a variety of taxa.

There is, however, some evidence in support of the hypothesis that pheromones may play a role in controlling arrest, obtained from observations of retarded development in the free-living nematode *C. elegans*. Under conditions of abundant food and low population density *C. elegans* develops through four larval stages (L_1 to L_4) reaching the hermaphroditic adult stage within 3 days of hatching. However, in response to starvation or overcrowding, development can be arrested at the second molt. This arrested stage is called a dauer larva and is a nonfeeding form specialized for survival and dispersal (Cassada and Russell, 1975). In this form the larvae can survive for months until, encountering food, they molt and resume development (Klass and Hirsh, 1976).

Golden and Riddle (1982) have reported the existence of an environmental cue, a pheromone, which triggers formation of dauer larvae in wild type cultures and prevents further development of these larvae. Dauer larvae may be induced in starved cultures or in populations of high density before exhaustion of food. When placed in fresh medium the larvae quickly and synchronously recover and resume development. Golden and Riddle (1982) were able to show that worms responded to both a food signal which was heat-stable, dialysable, neutral and hydrophilic and was found in bacterial cultures and yeast extract, and to the pheromone. It appeared that the relative proportion of the two signals, and not their absolute concentrations, was important in obtaining dauer larvae. The pheromone was isolated and shown to be fatty acid-like but not a prostaglandin. There is also evidence that other species of rhabditid nematodes produced pheromones that were active on their species. The concentration of the pheromone and the availability of food are apparently monitored by the chemosensory organs of the L_2 and the dauer larvae and the integration of these opposing signals determines the course of larval development.

The existence of such a triggering substance originating within the worms

themselves, whose induction is initiated by any one of a number of potentially adverse stimuli, presents an attractive alternative explanation to that of an environmental cue directly causing changes within the larvae.

It has been suggested that the prior presence of adult worms may act to direct incoming larvae into an arrested state. Michel (1963) infected two groups of calves daily with 1500 cold-conditioned larvae of *O. ostertagi*. One group was treated weekly with an anthelmintic effective against adult worms and the other was left untreated. Arrested larvae accumulated in this latter group but not in the treated group. After anthelmintic treatment was stopped, the previously treated calves acquired large numbers of arrested worms, suggesting it was the presence of adult worms rather than host immunity that had caused the accumulation of the arrested worms. Somewhat contradictory evidence was given by Fernando *et al.* (1971). Three groups of rabbits were injected with *O. cuniculi*—one with fresh larvae, one with cold-conditioned larvae and the remaining group with a mixture of the two types of larvae. Rabbits receiving mixed infections of larvae did not have higher levels of arrested larvae than those receiving only cold-conditioned larvae. The rapidly developing fresh worms did not exert an arresting effect on the slower growing conditioned ones.

(ii) *Genetic bases for arrest.* Differences in propensity to arrest that appear to be genetically related have been reported for different geographic strains of the same species of nematode. Basically two types have been described. One represents an obligatory (inherited) propensity to arrest that is seasonally related but does not require an obvious external stimulus for its induction. The other type is genetically controlled, extends through a spectrum of arrest propensities in the various geographic strains of a species, may be selected for and appears to be dependent on some external stimulus for its induction.

(a) Obligatory innate arrested development. Only one report of this type of arrest has been made to date and this was merely a suggestion. Waller and Thomas (1975) observed substantial arrest in the L_4 stages of *H. contortus* in July in northern England. The occurrence of arrest at this time of year seemed to rule out the possibility of an environmental temperature effect on the free-living stages, since declining autumn temperatures such as have been described for *O. ostertagi* by Armour (1970) were not involved. It was also difficult to visualize a temperature-linked stimulus during the summer when temperatures tended to level off. They therefore suggested that the strain of *H. contortus* in their area exhibited an innate capacity to show arrested development as a normal part of its life cycle.

(b) Genetically controlled, externally induced arrest. It has been observed that differences in propensity for arrest exist between different strains of the same species of worms occurring in different geographical locations or

subjected to different cultural procedures. For example, Armour *et al.* (1967) showed that strains of *O. ostertagi* could lose their propensity to arrest by repeated culture under laboratory conditions which precluded exposure to the environmental conditions necessary for the induction of arrest. Gordon (1974) presented preliminary results on Australian strains of *H. contortus* which suggested that clines existed in the capacity of this species for arrest associated with the severity of the winter. Thus a strain from South Australia, which is one of the colder parts of that country, had a marked seasonal propensity for arrest, whereas a strain from a sub-tropical area near Brisbane lacked this ability.

Also in Australia, Smeal *et al.* (1980) showed that two geographically isolated populations of *O. ostertagi*, one in beef cattle on the tablelands of New South Wales and the other in dairy cattle on the coastal lowlands, differed significantly in their propensity for arrest in the spring. On the northern tablelands, in beef cattle, the degree of arrest was low (0–5%) in winter and high (63–72%) in spring. In the coastal region, where dairy farming predominated, low levels of arrest were observed at all seasons, with a maximum of 12.85% in the spring. They attributed these differences in levels of arrest to genetic diversity between the two populations, probably arising as a result of dissimilarities in climate. Subsequently, Smeal and Donald (1981, 1982) confirmed these findings but also showed that management practices could act in maintaining the genetic differentiation between the two strains of worms. In one experiment (Smeal and Donald, 1981) pasture plots in a warm coastal region were separately contaminated in the autumn, winter and spring with eggs of a coastal strain of *O. ostertagi* from dairy calves. Simultaneously, an equivalent set of plots was contaminated with a strain obtained from a beef cattle management system from the tablelands region. Reciprocal transfer of the coastal strain of eggs was also made to the tablelands, where pasture plots were contaminated during the winter. Successive groups of parasite-free calves were grazed on the plots at four-week intervals and then killed and examined for worms. In both environments, irrespective of the season of pasture contamination, arrested development reached its maximum in spring, and the two strains differed only in the proportion of the population arrested, which was significantly higher for the tablelands strain. There was no difference between strains for either numbers of larvae on pasture or total worms in calves. This further suggested that the difference in extent of arrest between the two strains was genetically determined. Smeal and Donald (1982) subsequently reported that, on a coastal farm in New South Wales where both beef and dairy cattle production were carried on simultaneously, they had observed marked differences in arrest between populations from the dairy cattle and from the beef cattle. The proportions of arrested, early fourth-stage larvae in the

worm burdens of tracer calves grazed on pastures contaminated by the beef calves was over 60% in the late spring, whereas a maximum of only 12% was reached on those plots contaminated by the dairy calves. These differences were considered to be due to different management practices for the two types of cattle. Because calving periods for beef cattle are usually restricted to one season of the year, usually the spring, arrested development in these cattle in spring could have a selective advantage to the parasite, since it would coincide with a less favorable (hot, dry) environment for development of infective larvae on pasture. Arrested development in the beef cattle strain was thus a genetic strategy for survival. In the dairy animals, calves were born throughout the year and kept in pens until weaned at 8 weeks of age. After weaning, they were transferred to small fields which were continuously grazed by other young calves until they were 8–10 months of age. The continuous introduction of susceptible calves to the same pasture thereby ensured high rates of pasture contamination and a rapid succession of new generations of pasture larval populations. There would be little selection for arrested development under these conditions. Dairy management, therefore, ensured continuous high levels of pasture contamination. With this system infection rates were always high and arrest of larvae would have less survival value to the worms. It therefore appeared from the experiments that population differences in the degree of arrest could be accounted for by the different management systems.

These results and those of the previously mentioned workers suggest that the factors governing the propensity for arrest are plastic and that the facility to alter development to adapt to various environmental constraints is fairly readily acquired. It should thus be possible to select deliberately for this trait in populations of worms showing a capacity for this type of development. In this connection, Michel *et al.* (1973), using the "Weybridge" strain of *O. ostertagi*, in which the propensity for arrest had decreased from 24% to 5%, published the first report of apparent selection for an increased ability to arrest. They showed that after one generation of cold treatment the progeny of previously arrested worms exhibited 27% arrest, whereas the progeny of previously non-arrested worms, under the same conditions, exhibited 11% arrest.

Subsequently, Watkins (1982) and Watkins and Fernando (1984), through a process of selection, were able to increase substantially the propensity for arrest in response to cold treatment in a population of *O. cuniculi*, the rabbit stomach worm. Selection was done by subjecting infective larvae to cold treatment at 3°C–6°C for 28 days before they were given to rabbits. After 25 days the rabbits were killed and the worms recovered from their stomachs. All the early fourth-stage larvae were collected and reintubated into a second recipient rabbit. The infections in

these rabbits became patent in 16 to 23 days and from the resulting eggs infective larvae were cultured and again cold-treated as previously described. These larvae were then given to rabbits and the procedure for collection of the early fourth stages repeated. These procedures were continued until five generations of worms subjected to cold treatment had been produced. Over five generations of selection the progeny of previously arrested worms increased their propensity for arrest after cold treatment from a maximum of 15% to a maximum of 90%. When selection pressure was maintained, high levels of arrest continued to be observed. However, when the high-arrest strain was passaged without cold treatment of larvae, the propensity for arrest decreased. In the populations highly selected for arrest a significant proportion of the infecting dose was observed to arrest without cold treatment. Furthermore, the percentage of arrest after cold treatment was found to be correlated with the percentage of arrest before treatment. This indicated that the degree of arrest was very much dependent on the worms and that the influence of the host in determining the extent of arrest was of secondary importance. Watkins (1982) speculated that, had arrest in these worms been controlled by only one gene pair, the progeny produced by the worms in the first recipient rabbits would have exhibited close to 100% arrest. Since this did not occur he concluded that the basis for arrested development in this instance involved multiple gene pairs (polygenes). Each polygene might actually have controlled only one aspect of development but the additive effects of many such genes would result in worms that either developed normally or showed arrest. He hypothesized that a worm would exhibit the characteristics of arrested development if its polygenically determined genotype exceeded a particular threshold value.

Selection for high arrest would, therefore, increase the frequency of alleles which contributed to arrest in the population as a whole. Some individuals might have higher numbers of slow development genes than others and would be deeply arrested. Others might only exceed the threshold by small amounts and thus be less deeply arrested.

Watkins (1982) observed in generations six and seven of his selected worms that up to 36% arrest could occur spontaneously in non-cold-treated worms. This result resembled those obtained by Blitz and Gibbs (1972a) working with *H. contortus*. They observed levels of arrest from 20% to 45% in August and September in worms freshly cultured and given to lambs so that environmental stimuli were not considered to have acted. They termed this type of arrest "nonspecific" and attributed it to an interaction between the host and the viability of the parasite. On the other hand, arrest which was attributable to an environmental stimulus was found to fall between 92% and 100% and was called "specific" arrest. Nonspecifically arrested worms

may therefore be individuals whose genotypes exceed the threshold for arrest. Specifically arrested worms would be the result of gene–environment interactions. In selecting for arrest it would be expected that eggs passed early in the infection of a recipient rabbit would be produced by worms which emerge shortly after transfer. Eggs produced later in infections would probably be produced by late-arresting parents. Selection for deep arrest would thus be best accomplished by selecting these later eggs and Watkins (1982) was able to do this. He further suggested that a multiple gene hypothesis could account for the observed decrease in propensity to arrest that he observed under conditions of passage using non-cold-treated larvae. In a population with a high propensity to arrest, non-random mating must take place. Larvae that underwent "nonspecific" arrest (deep arrestors), having the highest concentration of genes influencing the propensity to arrest, would be excluded from the mating populations of worms which exist in the rabbit early in infection. Passage of worms under conditions of non-cold-treatment would cause a shift toward a lower frequency of alleles contributing to arrested development.

Watkins (1982) presented an intriguing proposal, based on his data, as to how ability to arrest could be propagated in a natural population of this parasite which arrests in autumn and winter in northern temperate regions (Gibbs *et al.*, 1977). If it was assumed that eggs that were deposited on 18 August would mature to the infective stage over 25 days and that the larvae then experienced chilling for 30 days, they would be available to infect rabbits by 12 October. Early maturing worms would produce eggs by 14 December but environmental conditions would be inimical to larval development (too cold). Eggs from later batches of maturing worms (late arrestors) would be deposited on 29 March, 19 April and 10 May and could give rise to infective larvae that would not only propagate the species but also the ability to arrest in response to cold. Genes associated with arrest would be carried over to be disseminated among the summer generations of worms which undergo rapid life cycles. Early maturing larvae would tend to leave no progeny, whereas worms which produce offspring late in infection would do so. Selection for a period of arrest to suit the particular environmental conditions would thus be possible by these means. Such a scheme might also apply to other seasonal arrestors that utilize a cold stimulus as a signal for the initiation of arrest.

Other researchers have proposed a balanced polymorphism to explain the differences in propensity to arrest that have been observed within some species. Thus Sollod (1967) suggested that populations of *O. ostertagi* might consist of arresting and non-arresting morphs. Seasonal differences in the relative abundance of arrested larvae would thus be due to changes in the proportions of infective larvae of each morph present on pasture. Armour

(1970) basically supported this view that there might be two larval morphs of which one was susceptible to the factors causing arrest and which, by some selective process, became dominant in autumn. He further argued that the larvae that were susceptible to arrest seemed more capable of survival under conditions simulating autumn. However, in field experiments, Michel *et al.* (1974) reported high levels of arrest in calves grazing pastures in winter but none in calves grazing the same pastures in spring, while the number of infective larvae on the herbage remained approximately constant over this period. Michel *et al.* (1975) supported these findings with similar results when larvae were stored for up to 25 weeks at either 6°C or 10°C. Arrest in the larvae rose to a maximum, then fell over the period while there was negligible mortality or change in infectivity of the stored larvae. In an attempt to explain these variations in propensity to arrest between strains of the same species, Michel *et al.* (1974) suggested that there was a continuous distribution of susceptibility to arrest among populations of a species with individual subpopulations (strains) differing in mean susceptibilities to the quantity of stimulus required for arrest.

Michel (1978) further argued, on the basis of the existence of natural stimuli for arrest and the changing responses of larvae with time, against theories involving balanced polymorphism. However, as Smeal and Donald (1981) have pointed out, this argument strictly is directed against polymorphism as the only explanation for seasonal arrest and is not a case against a polymorphism consisting of worms which do or do not respond to an environmental stimulus in which the relative frequencies of the two forms vary in response to selection.

2. *Physiological and morphological changes associated with seasonally induced arrest*

Examination of the literature relative to the nature and mechanisms of the changes induced in seasonally arrested nematode larvae shows that this is scanty, and most of the available information is still speculative.

(a) Effector mechanisms. Since seasonally induced arrest appears to be genetically controlled, it is interesting to speculate how such control might be accomplished.

As has already been mentioned, seasonal arrest is invariably associated with a particular stage in the normal development of these parasites and accompanying this is usually a process of molting whereby the parasite sheds the cuticle of one stage for that of another. Rogers and Petronijevic (1982) have pointed out that molting is critical to the development of parasitism in nematodes because it affords the worm the opportunity to alter its cuticle in

order to present an appropriate interface to a series of different environments. Experiments on molting in larval nematodes have indicated that this is under genetic control, and transcription and translation of mRNA in the intermolt periods are essential for completing development and preparation for molting to the next larval stage (Pasternak and Leuschner 1975; Bonner *et al.*, 1976).

In addition to those genes which control the continuous basic processes of parasitic nematodes, Rogers and Petronijevic (1982) have suggested that there are also sets of genes that are characteristic of the different stages which are active or inactive at appropriate times in the development of the worm. They therefore proposed that, at any one stage in worm development, the gene set controlling the succeeding stage is suppressed. Supression was considered to be the result of an accumulation of an inhibitor of gene activity; most probably juvenile hormone because of its negative effects in regulating gene activity in worms (Rogers, 1979).

However, Davey (1982) has stated that there appears to be a clear distinction between the processes of molting and of growth or increase in mass in nematodes. Even though associated, these processes may be separately controlled.

Observations by Bruce and Armour (1974) of the L_4 of *O. ostertagi* using the electron microscope have revealed that neurosecretory granules which were prominent in the nerves of the posterior end of the L_3 remained so in diapausing L_4, but became depleted in developing L_4. While there is no observation which directly links neurosecretion to growth and development in nematodes, neurosecretory cells are associated with the production and release of hormones governing growth in a variety of taxa. Davey (1982) has therefore speculated that the differences between the two types of larvae constitute evidence for a posterior neuroendocrine center controlling development in nematodes.

Sommerville (1982), however, cautioned against complete acceptance of the proposed role of juvenile hormone as the supressor of the gene set for growth in arrested L_4s. As he pointed out, L_4s in arrest have completed the third molt, increased in length and undergone some sexual differentiation before growth ceases. Clearly at this point the gene set for the adult stage has been turned off. Juvenile hormone causes suppression to occur at the stage of transcription. It is thus limited to periods during development when the cell cycle is at an appropriate stage. In the case of arrested development, whether associated with the immune response of the host or with other stimulation of the free-living stages, the larvae have passed this critical stage because the gene set for the adult stage has already been activated. It is unlikely, therefore, that growth has ceased because of the activity of juvenile

hormone, but its cessation may possibly be due to an alternative substance or substances.

Watkins (1982) suggested that there must be some delay in the initiation of the actual mechanism responsible for the suppression of gene activity. The stimulus for arrested development is received at the L_3 stage but its effects are not manifested until shortly after molting to the L_4. He proposed a hormonal mechanism whereby the hormone acted as a transcription inhibitor by binding to chromatin in the L_3. The L_3 could then exsheath, grow and molt to the L_4 before the hormone exercised its effects. As an alternative mechanism he suggested that cold treatment of the L_3 might result in a failure to synthesize a generalized growth factor such as a thyroxin-like molecule. Thyroxin has been shown to influence respiration and affects cell nuclei, stimulating systems of DNA-dependent, RNA polymerase necessary for mRNA production (Griswold & Cohen, 1972). If this were so, the molt from L_3 to L_4 could be completed in the absence of the thyroxin-like material. The nematode would thus lack the transcriptional and translational apparatus necessary for growth in the L_4 stage and would thus be inhibited in further development.

It has been observed in some of the species that arrest in response to cold stimuli that, as exposure of the L_3 to cold treatment proceeds, ability of the worms to arrest reaches a maximum and then declines (Hutchinson *et al.*, 1972; Watkins, 1982). In an attempt to explain this feature Hutchinson *et al.* (1972) postulated that kinetically variant isoenzymes might be induced in the process of acclimation. This would imply that at the time of optimum cold treatment arrest resulted from a lack of enzymes capable of activity at that temperature. However, with prolonged cold treatment, new isoenzymes might be produced which were capable of operating at the lower temperature. Such a concept is not incompatible with the speculation concerning failure to synthesize a thyroxin-like molecule proposed by Watkins (1982).

The dauer larvae of *C. elegans* differ from other developmental stages in physical properties, physiology and behavior. As has been mentioned, these larvae represent a specific stage resistant to adverse conditions, usually overcrowding or starvation. The changes characteristic of such larvae have been well studied and the larvae show some distinct physical, physiological and behavioral differences from normally developing larvae. Thus they are relatively thin and dense because of radial shrinkage of their bodies (Cassada and Russell, 1975). The body wall cuticle is relatively thick and the larvae are resistant to detergent treatment (Swanson and Riddle, 1981). The excretory gland is inactive, as shown by an absence of secretory granules (Nelson *et al.*, 1983). The dauer larvae also show behavior that is not observed in other stages. They are lethargic, pharyngeal pumping is sup-

pressed and they tend to crawl up objects projecting from the substrate (Cassada and Russell, 1975). However, there are also marked ultrastructural changes in a variety of tissues and organs which have been described by Albert and Riddle (1982). In particular a number of dauer-specific alterations in neural structures have been observed. These changes, which involved the inner labial neurons, amphidial neurons and the deirids, demonstrate a plasticity in nematode morphology not previously described. Other than the work of Bruce and Armour (1974), ultrastructural studies of this sort have not been carried out on parasitic hypobiotic larvae, and one is led to speculate that similar changes might also be associated with these larvae had they been looked for. Were changes of this sort to be present in the parasitic larvae, it would greatly facilitate unravelling of that important question, are immune arrested and seasonally arrested larvae morphologically different? If not, are there really two types of arrest?

(b) Changes in biochemical or metabolic activity. Studies have suggested that seasonally arrested larvae may in some way be altered so as to be metabolically less active than normally developing larvae (Armour and Bruce, 1974). This has important implications for the parasites in terms of their susceptibility to the anthelmintics used in their control. Thus arrested larvae of *O. ostertagi* have been reported as having reduced susceptibility to many anthelmintics (Anderson, 1977) and this was recently supported by Watkins (1982) from the results of his studies on the effects of thiabendazole on arrested L_4s of *O. cuniculi*. Another implication is that such relatively inactive larvae would be less pathogenic to their hosts. This seems to be so, as some animals may harbor large populations of these larvae which produce no obvious pathological effects compared to those seen when they mature (Gibbs, 1964).

(c) Host immunity evasion strategies. It is now well established that parasites elicit vigorous immune responses in their hosts and that the resistance to reinfection thus acquired is mediated by way of humoral and cellular effector mechanisms (Ogilvie and De Savigny, 1982). The capability of arrested larvae to remain for relatively long periods in substantial numbers in what must be resistant hosts in the face of a hostile environment must therefore mean that they possess some sort of successful evasive strategy. Mitchell (1982) has proposed three broad types of mechanisms to account for the prolonged survival of parasites in their immune hosts. These are: (1) reduced or altered antigenicity/immunogenicity of the parasite; (2) modification of induction or expression of the host immune responses; and (3) modification of the intramacrophage environment. Most of the suggestions are still speculations for which few experimental data bearing on their

existence or importance exist, and this is particularly true with the parasitic nematodes. However, Thomas *et al.* (1975) reported that viable *H. contortus* larvae, which were used as source of antigen in complement fixation tests, showed a decrease in antigenic potency following storage for 2 months at 4°C in contrast to fresh larvae killed by freezing and held at −15°C, whose antigenicity was unaffected. They postulated, based on the work of Hutchinson *et al.* (1972), that these changes in antigenic potency could be due to physiological changes in the larvae. Furthermore, they suggested that this reduced antigenicity might permit a higher rate of larval establishment in a sensitized host without provoking an immune response.

Recent work by Smeal and colleagues, reported by Smeal (1982), on arresting strains of *O. ostertagi* in Australia, however, suggests that, while antigenic disparity does indeed exist between arresting and non-arresting strains of this parasite, contrary to what one might expect, the arresting strains appear to be more antigenic than the non-arresting strains. Using two strains of *O. ostertagi*, one an arresting strain derived from beef cattle, the other a non-arresting strain from dairy cattle, they examined local cellular abomasal mucosal responses and the kinetics of lymphocyte populations generated by experimental infections of each strain. It was observed that infections with the arresting strain caused a much more intense mucosal cellular response than did the non-arresting strain. The arresting strain also generated significantly higher numbers of cells containing IgG, IgG_2, and IgM, but not IgA, than the non-arresting larvae. However, it was not determined whether this response was involved in protective immunity to subsequent challenge.

IV. Release or Resumption of Development

Factors responsible for the initiation of resumption of development of the arrested larvae are still not well understood. As Michel (1978) has intimated, since there could be a variety of causes that can lead to arrest, so could there be a number that initiate resumption of development.

A. Immune-Mediated Arrest

In the case of larvae that are arrested by virtue of host resistance effects acting to suppress further development, one would expect that removal of or release from the suppressor stimulus would result in resumption of development by the larvae. This suppressor stimulus might be the overall host immune response to infection by the worms.

Attempts at stimulation of resumption of development in arrested larvae by immunosuppression of the host have not, however, given generally satisfactory results. Thus Gibbs (1968), using chlorambucil, an immunosuppressive alkylating agent, was unable to stimulate resumption of development of arrested *H. contortus* in ewes in spite of a marked lymphocyte depression in these ewes, suggesting that they were severely immunosuppressed. However, resumption of development was later observed in these ewes after treatment was discontinued at the same time as a periparturient egg count rise was observed in other ewes. Similar results were obtained using adrenocorticotrophic hormone, another immunosuppressive agent. Likewise, Prichard *et al.* (1974) were unable to initiate redevelopment of arrested larvae of *O. ostertagi* in calves by treating the calves with dexamethasone trimethylacetate (a corticosteroid). The corticosteroid treatment appeared to be immunosuppressive as shown by a significant lymphopenia in the treated calves. They concluded that it appeared unlikely that a depression of immunity was responsible for the resumption of development of arrested larvae. However, it should be remarked that, in both these experiments, the arrested larvae were seasonally arrested and perhaps as a consequence could not respond to an immunosuppressive stimulus.

In the case of *N. dubius* in mice, Behnke and Parish (1979) have reported that treatment with cortisone of mice in which larvae were arrested in the intestinal wall caused reactivation of development. In this case the larvae were apparently inhibited as a result of reinfection of immune mice. On the other hand, prednisolone treatment of dogs carrying histotrophic arrested stages of *A. caninum*, although producing some degree of immunosuppression as judged by lymphocyte transformation essays, did not release larvae from dormancy (Schad and Page, 1982). In fact, the dogs treated with the steroid harbored significantly greater populations of arrested larvae than did the untreated controls. Arrested larvae appeared to resume development spontaneously over the duration of the experiment.

The evidence is not strong that immunosuppression of the host will result in resumption of larval development.

B. SEASONALLY INDUCED ARREST

It has been suggested that seasonally arrested development is very similar to diapause in insects (Armour and Bruce, 1974; Horak, 1981). In this event the duration of the arrest should be of a fixed, predetermined length equivalent to the period of adverse conditions over which the larvae need to be arrested. Maturation of these larvae would, therefore, most likely be triggered by some internal mechanism within the arrested larvae them-

selves, although it is conceivable that some external stimulus, either host or environmental, might also be operational. This would culminate in a fairly synchronous maturation of most of the arrested larvae at an appropriate time of the year. This appears to be the case with some of the trichostrongyles like *H. contortus* (Gibbs, 1968; Connan, 1978) and *O. ostertagi* (Armour and Bruce, 1974). However, there is also some controversial evidence. Thus in two major studies on emergence from arrest with *O. ostertagi*, Michel *et al.* (1976a,b) demonstrated patterns of continuous rather than synchronous maturation. In their first report yearling calves were infected naturally in each of 3 years by grazing pastures in the autumn. The calves were housed in December and resumption of development was studied by worm counts done on animals killed at intervals. Emergence from arrest occurred at a steady rate of almost 500 larvae per day but from March onwards nearly all remaining larvae developed over a short period of about 1 month. In the second report, three groups of calves were infected over a period of 6 weeks with daily doses of cold-treated L_3s. Group 1 got larvae from 1 November, Group 2 from 6 January and Group 3 from 14 April. Five days after the last larval dose, thiabendazole was administered to clear adult worms. Approximately 85 000 larvae were arrested in each calf. Calves were killed at intervals over the next 139 days and worm counts were conducted. A linear decrease in numbers of arrested larvae occurred amounting to emergence at about 635 larvae per day. All three groups of cattle showed resumed development at about the same rate, irrespective of time of year, with no sudden resumption of development.

These results were different from those of Armour and Bruce (1974) who used two groups of calves inoculated with cold-treated L_3s of *O. ostertagi*. One group was infected in January and one group in April (13 weeks later than the first group). Necropsy counts on calves killed at periodic intervals following infection indicated that little emergence occurred over the first 16 weeks but that synchronous maturation occurred between weeks 16 and 18 after infection. The 16–18 week period of arrested development was approximately the period when winter conditions in Britain are unsuitable for development of the free-living stages. The authors concluded that the induction of inhibition by the environmental stimulus of chilling, the fixed period for the duration of arrest within the host, and the spontaneous maturation of the arrested larvae were characteristic of a diapause phenomenon. The major difference between the results of the two groups of researchers is that Armour and Bruce (1974) reported a persistence in the arrested state for the first 16 weeks followed by sudden maturation, whereas Michel *et al.* (1976a) had a similar result but there was some emergence throughout their experiment. In the 1973–74 results of the latter workers, the holding pattern of arrest was not obvious as there was continuous

emergence; in 1974–75, if all animals were considered, the holding pattern could be seen, but numbers of developing and adult worms were also seen throughout. Michel *et al.* (1976a) concluded that a small constant number resumed development daily, the rate being greatly increased in March, or even earlier if there was a breakdown in resistance. Michel *et al.* (1976b) also concluded that simultaneous development of large numbers in spring would be demonstrable only if large numbers of larvae, greater than 85 000, were present initially.

It has been demonstrated by Armour and Bruce (1974) and Michel *et al.* (1975) that, with passage of time, larvae that have been conditioned to arrest will lose this ability if they are not ingested by a suitable host. Beyond a certain period of time such conditioned larvae, even if they are acquired by a susceptible host, will have lost the ability to arrest. This seems to indicate that, once larvae are conditioned, the physiological processes that have to be completed before they can resume development proceed irrespective of whether or not they are ingested.

In a clear demonstration of the fixed period of arrested development in *H. contortus*, arrested larvae were surgically transferred by Blitz and Gibbs (1971b) from naturally infected ewes to parasite-free ewes maintained under worm-free conditions. Ten to 12 weeks after the transfer the worms matured, causing a marked rise in fecal worm egg counts which coincided with a marked mid-April rise observed in the egg counts of sheep in other experiments.

If the period of seasonally arrested development is constant for a particular species, it would be expected that larvae acquired early in the season would mature sooner than those acquired later. To test this assumption, Connan (1978) carried out a study on the resumption of development of *H. contortus* in sheep in Britain. Two groups of sheep were naturally infected by grazing on contaminated pasture from 10 to 27 September and from 22 November to 9 December, respectively. The sheep were then grazed on uninfected pasture and moved at regular intervals until housed in January. Tracer lambs grazed with the ewes carried burdens that were 93% to 100% arrested. Of 14 sheep in the early group, all were pregnant except two, which were barren, and one ram. Of the 15 sheep in the latter group, all were pregnant except one which was barren and one ram. Ewes lambed during the first 2 weeks in February. However, the periparturient rise in egg production did not occur until April for both groups. Two ewes from the early group and one ewe and a ram from the later group were killed on 20 March. The ewes contained 100% arrested larvae while the ram's burden was 92% arrested. It therefore appeared that parturition did not cause redevelopment of the arrested larvae. Connan (1978) had hoped to show that the periparturient rise was due to the resumption of development of arrested larvae, but

expected the rise to occur in the later exposed group some 10 weeks after that in the early group. The rise in both groups occurred simultaneously, which was interpreted to mean that resumption in development in the spring occurred in response to some unknown stimulus associated with spring. However, if the larvae on pasture entered diapause in response to an autumn stimulus the duration of the diapause would be related to the time of receipt of the stimulus. Termination of diapause would therefore occur spontaneously after an equal time in the larvae in both groups, irrespective of how long the worms had been present in the host.

V. Relationship Between Resumption of Development, Pregnancy and Lactation

There has been a great deal of work demonstrating that resumption of development or activation of quiescent larvae can be influenced directly or indirectly by changes occurring in the host that are related to pregnancy or lactation. This is perfectly reasonable if it is accepted that one of the prime purposes of hypobiosis is to facilitate transmission of the parasite from one host to another. What more appropriate time for such transfer to be effected than when the susceptible neonate is available?

In the case of those nematodes that are transmitted prenatally or transmammarily the trigger for activation or resumption of development appears to be endocrinal and associated with one or more of the hormonal changes of pregnancy, parturition or lactation. The timing of, for example, the transcolostral infection by *Strongyloides ransomi* in pigs has been shown by Batte and Moncol (1968) and Stewart *et al.* (1976) to be precise, larvae appearing in the mammary gland for only a few days. Warren (1971) and Mia *et al.* (1975) have shown that infection of calves with *Toxocara vitulorum* takes place by way of the colostrum and milk shortly after birth, and parasite eggs regularly appear in the feces of calves at 3 weeks of age. In this connection Oshima (1961) has shown that treatment with prolactin of mice infected with *T. canis* stimulated the larvae to resume their migration. Also in point is the work of Stoye (1973), who was able to stimulate passage of larval *Ancylostoma caninum* into the milk of lactating infected bitches by administering oestradiol alone or in combination with progesterone. Stoye and Krause (1976) also demonstrated the effects of oestradiol and progesterone on resumption of development; they infected female pups a few days after birth with 20 000 L_3s of *A. caninum*; when the pups matured they were bred and the excretion of larvae in milk was recorded as a measure of larval reactivation. The females were ovariectomized at 28 days after parturition, and lactation maintained by twice daily dosage with prolactin. Oestradiol at 2.5

mg plus progesterone at 50 mg per animal was injected as a microcrystalline suspension and caused excretion of larvae in the milk. When oestrogen was increased, larval excretion increased proportionately.

The reports of Zamirdin and Wilson (1974), Wilson (1977) and Wilson *et al.* (1978a,b) have shown that the migration of *S. ratti* is linked to neuroendocrine-related changes accompanying the sucking stimulus in rats. Wilson (1977) suggested that there are probably hormone operators which influence the concentration of larvae in the lactating mammary gland. However, Wilson *et al.* (1978a) were unable to demonstrate that histotrophic (arrested) larvae played a very important role in contributing to transmammary infections in their experiment, but these experiments were not carried out on multiply infected rats. Multiple infections would sensitize rats, and larvae entering such hosts would be subjected to immune constraints. It is thus possible that in a sensitized host larvae would be directed to the tissues of the host, which is the assumption of other workers studying systems of maternal transmission.

The results from these studies all suggest that there is a direct effect of one or more host hormones on the larvae which activates them to migrate into the milk.

In some cases the arrested larvae can remain in the tissues for relatively long periods of time. Lyons and Biggs (1983) reported that arrested larvae of *Uncinaria lucasi* could remain alive for as much as 4 years in the blubber of northern fur seals (*Callorhinus ursinus*).

In the case of trichostrongyles of ruminants, as exemplified by *H. contortus*, synchronous maturation of the arrested larvae in the lactating female results in the well known "spring rise" in fecal parasite egg counts that has been described by a number of workers (Brunsdon, 1964; Gibbs, 1968; Connan, 1968). Maximum expression of this rise in egg counts in sheep has been associated with parturition and lactation, especially the latter. Salisbury and Arundel (1970) suggested the term "periparturient rise" to describe this phenomenon because of its association with parturition rather than exclusively with season of the year.

Most workers believe that the periparturient rise reflects a fall in the immune status of the host associated with the endocrinological changes of parturition and lactation—a periparturient relaxation of immunity (Connan, 1968; O'Sullivan and Donald, 1970). In this connection Chen and Soulsby (1976) have demonstrated reduced blastogenic responses to specific antigens or non-specific mitogens in pregnant and lactating ewes. Dineen and Kelley (1972) have also shown that one immunologic defect in lactating rats, leading to retention of worms, was failure of induced cells to differentiate into effector cells.

O'Sullivan and Donald (1970, 1973) have postulated that the temporary

suspension of the immunologic capacity of the ewe could result in resumption of development of arrested larvae in the case of those undergoing immune-induced arrest, resulting in increased rates of establishment of newly ingested larvae or in increased fecundity of female worms, which would lead to the increased egg counts that were seen. Michel (1974, 1976) has argued that the periparturient rise can be explained more simply by postulating that the normal mortality of adult worms was suspended in lactating animals. However, this is too simple an explanation, as shown by Donald *et al.* (1982) who found that there was a greater rejection of incoming larvae of *T. colubriformis* and *Ostertagia* at or before the L_4 stage in non-lactating than in lactating ewes. In a recent experiment Gibbs and Barger (1986) observed a periparturient rise in fecal egg counts in pregnant ewes following maturation in early spring of arrested L_4s of *H. contortus* and failure of the ewes to expel the resulting adult worms. Peak egg counts were seen in September just before lambing. Similar to the findings of O'Sullivan and Donald (1973), lactating and non-lactating ewes were equally refractory to new infections with *H. contortus* but lactating ewes acquired greater burdens of *O. circumcincta* and *T. colubriformis* than did dry ewes. When immunosuppression of lactation coincides with the presence of larvae developing in the gut of sheep it enables the larvae to reach adulthood and to remain for extended periods of time.

The effects of lactation may best be interpreted in view of its known ability to suppress the immune expulsion of worms through its hormonal interferences, in particular that due to prolactin. The principal lactogenic hormone is prolactin and its fluctuations coincide with the way in which lactation interferes with the host response to parasites. In the ewe the rise in fecal egg counts may begin before lactation (O'Sullivan and Donald, 1970; Brunsdon, 1964; Gibbs and Barger, 1985). It is also known that injection with estrogen will cause a rise in plasma prolactin (Chen and Meites, 1970); thus barren ewes injected with 2 mg of diethyl stilboestrol per day in corn oil showed a rise in fecal egg counts some 14 days after commencing the injections (Gibbs, 1968). The mode of action of worm expulsion seems to be a diphasic process, the first part mediated by antibody (Jones and Ogilvie, 1971) and the second by specific lymphoid cells (Dineen and Kelly, 1972) acting by way of specific lymphokines which may affect metabolism in the worm (Dineen *et al.*, 1973). Connan (1973) has suggested that the specific effect of lactation may be to block the release of lymphokines.

In the case of tissue dwelling histotrophic forms of arrested larvae, immunosuppression can also play a part in facilitating the development and retention of adult worms. Lloyd *et al.* (1983) have shown that pregnancy and lactation induced a marked suppression of immunological responses (lymphoid transformations induced *in vitro* by phytohemagglutinin mitogen and

T. canis antigen) in bitches infected with *T. canis*. Eosinophilia was also suppressed and these changes were coincident with the establishment of heavy infections of *T. canis* in the intestine. Eosinophils have been shown by Wassom and Gleich (1979) to play an important role as effector cells against parasites in the gut mucosa.

There thus appears to be a complex interrelationship between changes in the host particularly associated with pregnancy and lactation, and the activity associated with arrested larvae. These may involve the effects of various host endocrine factors acting directly on the larvae, or they may be the result of the immunomodulatory effects of these hormones indirectly influencing the expression of the phenomena associated with resumption of development.

VI. Reflections and Conclusions

From the foregoing review of the relevant literature, it appears that most workers in this field have tended to favor one or other of two viewpoints as to the role of hypobiosis in the biology of nematodes. Some of the earlier workers saw the phenomenon as primarily a result of host resistance which served to regulate populations of worms based on the extent of infective larval intake by the host. More recently the process has come to be regarded as a means of synchronization of parasitic development with changes taking place in the environment, either externally or in the host. This allows worms acquired over an extended period to persist in the host when conditions are unsuitable for further development or when conditions might be unfavorable for development and transmission of free-living stages. Further, the ability of arrested forms to resume development over relatively short periods of time or at strategically important points in the life cycle of the host ultimately results in the accumulation of large numbers of adult worms and consequently of infective stages at epidemiologically important points for the parasite. From this viewpoint, hypobiosis represents a physiological adaptation of parasitic nematodes aimed primarily at facilitating survival in the face of a changing environment.

Currently, hypobiotic development tends to be viewed as occurring in two forms. One form is associated with responses of the incoming larvae to immune-related stimuli in the host and perhaps might more properly be referred to as inhibited development. The other form is considered to represent a type of diapause development which is dependent on some external or extra-host inductive signal. However, perusal of the available evidence shows that such distinctions are by no means as clearly defined as has been suggested.

In attempting to visualize a mechanism that might be involved in inducing immune-mediated arrest, it is reasonable to assume, based on the general effects of immune processes on invading organisms, that such effects might be deleterious to the larvae. They should cause depression of growth or stunting and such a check on development would perhaps best be referred to as inhibited development. One would therefore expect in such populations of inhibited larvae to see variations in size distribution and other evidence of deleterious effects such as epicuticular precipitates or the accumulation of degenerative products in larval cells. However, examination of the literature on immune-induced arrest shows that this is not the case. Rather, such immune-arrested larvae are in most respects essentially normal and such populations are generally characterized by a great uniformity in size distribution. To all intents and purposes such larvae are indistinguishable from so-called "diapausing" larvae. While immune-mediated, inhibited development undoubtedly can occur, such changes as are produced in the larvae appear to be detrimental to further development, and the most probable fate of such larvae is to be rejected by the host. It thus seems highly likely that in cases of immune-mediated arrest, the immune state is acting merely as a signal to the incoming larvae, rather than as a protective mechanism of the host aimed at neutralizing or destroying invading organisms. As was pointed out earlier in the discussion, there appears to be a great variation between species as to the extent to which some seem to respond to host immunity as an inducer of arrest as compared to others.

Likewise, the assumption that the non-immune-mediated type of arrest is strictly analogous to diapause in insects is probably also too sweeping a conclusion to draw. Perhaps in strains of certain species like *O. ostertagi*, where arrested development has been linked to a definite environmental stimulus associated with a particular season of the year, and when the period of larval conditioning for arrest extends over a limited period of time, some 16–18 weeks, such a conclusion may be justified. However, even with these species there may be arrest in response to host resistance stimuli under certain circumstances. Furthermore, of particular importance in demonstrating the variability that exists in this phenomenon which contributes to the difficulty of presenting a unified concept of hypobiotic development, is the occurrence of intraspecific variation—the differences that exist between different geographical strains of the same species as to the timing of their period of arrest and the utilization of appropriate stimuli. Thus, as has been mentioned in the case of *O. ostertagi*, a positive inductive stimulus in the form of low environmental temperatures has been demonstrated in strains in north temperate regions. However, it has been shown that this is very much a matter of geographical idiosyncracy. Under other environmental conditions, such as those in Australia or the southern USA, the stimulus for

induction is different and may be increasing temperature or change in photoperiod, or may even be host-mediated, but it is certainly not decreasing temperature. The basic mechanism that results in arrested development in this species must therefore be capable of activation by a variety of stimuli, depending on the location.

However, even in those situations where the period of arrest seems to be relatively fixed and resumption of development is somewhat synchronous, Michel (1978) has pointed out that it may be an oversimplification to think of this as dependent on a biological clock mechanism as described for diapause in insects. The time interval from conditioning to both deconditioning and renewed development is variable. It has been shown that deconditioning can be precipitated by an abrupt increase in the environmental temperature of the larvae, and it has also been shown that in debilitated animals arrested larvae can develop in large numbers some months earlier than in healthier animals.

Characterization of the type of arrest in some of the other parasitic nematodes, particularly those with a histotrophic phase in the somatic tissues, is even more difficult since features characteristic in some cases and uncharacteristic in others of both immune-mediated and diapause-like arrest may be observed. Thus it has been shown that *A. caninum* larvae will arrest in response to some external stimulus, but the resulting arrested larvae are not limited in their period of conditioning and will remain for indefinite periods in the tissues, resuming development in response to a host-induced hormonal stimulus, or idiosyncratically.

T. canis is stimulated to arrest by entrance into a resistant host. This may be an immune canine or it may be a paratenic rodent host. In either case the host is "unsuitable" for further development to the adult stage. These larvae enter the somatic tissues where they become encapsulated, histotrophic larvae. Like those of *A. caninum*, the larvae are not conditioned to arrest for a limited period and can persist for long periods in the tissues. They will resume development either after a hormonal stimulus in the pregnant bitch or by entrance into a susceptible pup when the paratenic host is consumed.

Michel (1978) has indicated that nematodes of nearly all species have an innate capacity to interrupt their development at an early parasitic stage and, by depressing their metabolic activity, to extend their survival. This suggests that the propensity to arrest is already well established in parasitic nematodes and that, depending on the selection process, a variety of stimuli might serve to induce this type of developmental behavior. That such a propensity to arrest has a genetic basis has been discussed; furthermore, selection for arrest seems to be fairly readily accomplished within relatively few generations. Such an innate capacity would go far to explain the fact that within a particular species a variety of stimuli have been shown to induce

arrest, depending on the particular situation in which the organism finds itself.

Inductive stimuli might thus include not only environmental cues like temperature, humidity or photoperiod but also host resistance factors in the form of acquired immunity or age or species resistance. Increasing immunity of the host might thus act simply as another stimulus for signalling the seasonal arrest of larvae. After all, even in the case of the strictly seasonal arrestors, host immune levels probably reach their maxima at the end of the grazing season when stimulation by intakes of infective larvae is greatest. At this time the environment is deteriorating for the parasite as well as for the host, and it would thus represent a most appropriate time for developmental arrest of larvae to occur. Furthermore, this stimulus would be active on the infective larvae immediately they enter the host. Thus the same larval stage would be in receipt of the inductive stimulus irrespective of whether this was extra- or intra-host. This could in turn serve to stimulate the appropriate gene set for arrest. As to how this cue is mediated one can only speculate, but it is conceivable that the actual stimulus for induction of the arrested state may take the form of a pheromone produced by some of the larvae as they encounter the appropriate stimulus, as has been shown for *C. elegans*.

It thus appears that there is most probably not two kinds of arrest in parasitic nematodes but only one. It might be considered to represent the parasitic nematode equivalent of diapause in insects but has some unique features of its own. Where diapause in insects is characterized by great uniformity, hypobiosis in nematodes is much more variable and less predictable.

It represents a modification of development that is primarily aimed at facilitation of survival or persistence of these parasitic nematodes over periods of adversity. Selection pressures to this end, therefore, appear to be of prime consideration. Adaptability to these selection pressures appears to be under genetic control and to be readily selected for. The basic mechanism that results in arrested development must have the capability of activation by a variety of stimuli, depending on the situation. Any of a variety of cues or stimuli associated with entry of the parasite into a stressful situation could therefore serve to induce this state.

Acknowledgements

The support of the Maine Agricultural Experiment Station for some of the work referred to in this review is gratefully acknowledged.

Thanks are due to Mrs. Rita Yerxa for her diligence in typing this manuscript.

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The Eggs of Monogeneans

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I. Introduction

The life cycles of monogeneans are relatively simple compared with those of other parasitic platyhelminths (cestodes and digeneans). Most mono-

ADVANCES IN PARASITOLOGY VOL. 25
ISBN 0-12-031725-7

geneans inhabit the skin and gills of fishes and these sites are accessible to free-swimming larvae (oncomiracidia) hatching from freely deposited aquatic eggs[1] (Fig. 1). This simple life cycle has sufficient evolutionary plasticity to enable monogeneans to adapt to fishes living in a range of marine and freshwater habitats and with strikingly different ways of life, such as bottom-living flat-fishes and fast-moving pelagic species. But greater evolutionary potential is revealed by the remarkable survival of monogeneans on those fish-like ancestors of the tetrapods that colonized the terrestrial environment, leading to monogenean life cycles of sometimes surprising complexity (Fig. 2).

No previous attempt has been made to undertake a comprehensive review of information on monogenean eggs, and yet our understanding of the role of the eggs in the biology of monogenean parasites has expanded during the last 30 years. Of general interest to all students of parasitology are the recent discoveries of hatching rhythms and hatching factors, which in many monogeneans are of fundamental importance in infection of new hosts, and the intriguing changes in reproductive biology that have accompanied the progressive attenuation of the ties between tetrapod hosts and the aquatic environment. Moreover, monogeneans deserve special attention because

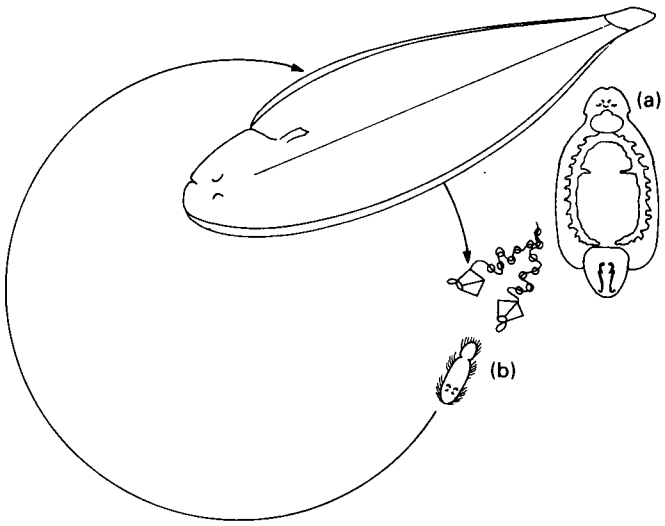


FIG. 1. The life cycle of *Entobdella soleae*. The adult parasite (a) inhabits the lower surface of the common sole (*Solea solea*). Ciliated larvae (b), hatching from tetrahedral eggs on the sea bed, invade the upper surface of the sole and migrate to the host's lower surface. Based on work by Kearn (1963a).

[1] A shelled zygote or embryo, accompanied by vitelline cells, will be referred to in this review by the traditional, although incorrect, term "egg".

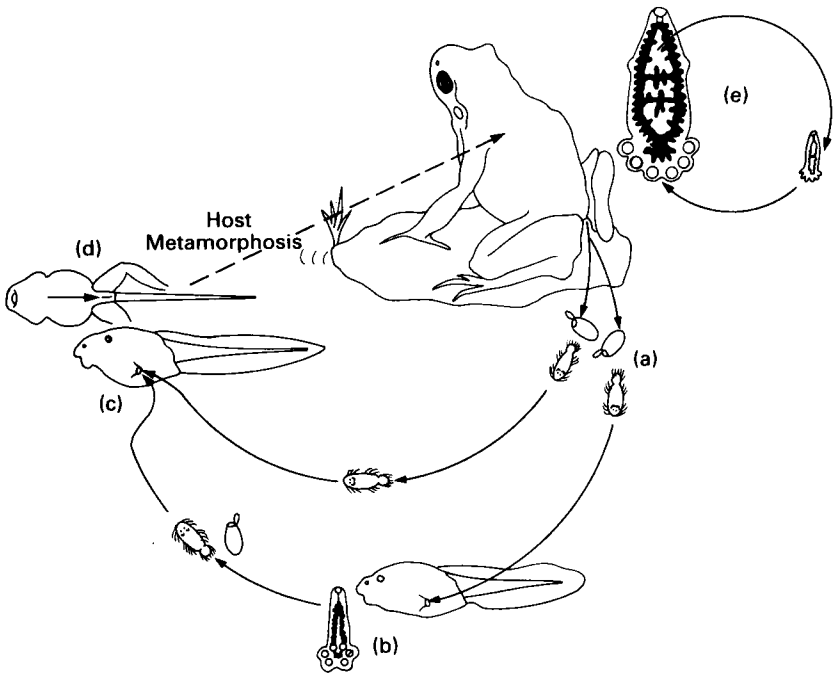


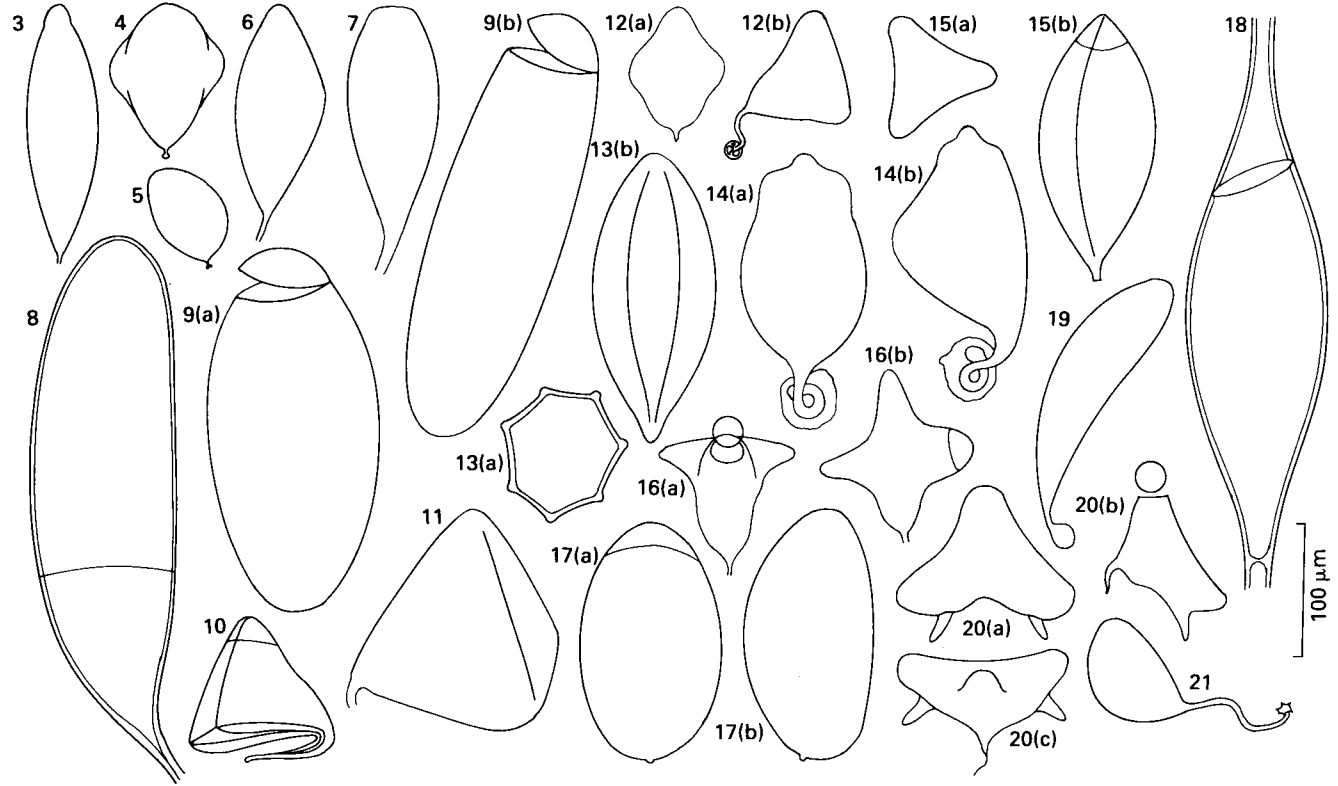
FIG. 2. The life cycle of *Polystoma integerrimum*. Ciliated larvae (a) emerging from eggs laid by adults in the frog's bladder invade tadpole gills. Larvae encountering the gills of young tadpoles undergo neoteny (b). Larvae from bladder parasites or from eggs laid by neotenous parasites encountering the gills of older tadpoles (c) grow slowly and migrate at night over the ventral skin of metamorphosing tadpoles (d) to the bladder. Ovoviviparity in bladder parasites leads to infection of the same frog (e). Based mainly on the work of Combes (1968).

they provide opportunities for direct observation of the egg assembly process and display a surprising variety of egg shapes and egg appendages which have attracted little functional interpretation.

Few observations have been made on egg assembly in cestodes and in digeneans and their eggs are much more conservative in shape, relatively small in size and mostly lack appendages. It therefore seems a desirable exercise and an opportune time to review our knowledge of monogenean eggs.

II. THE DIVERSITY OF MONOGENEAN EGGS

Monogeneans show a remarkable diversity in the shape and size of their eggs (Figs. 3–21) and in their egg appendages (Figs. 22–33), although this


3
4
6
7
9(b)
12(a)
12(b)
15(a)
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18
5
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8
9(a)
19
16(b)
13(a)
16(a)
20(b)
11
17(a)
100 µm
10
20(a)
21
17(b)
20(c)

FIGS. 3–21. The range of size and shape of monogenean eggs. All eggs drawn to the same scale. Scale bar = 100 μm. FIG. 3. *Plectanocotyle gurnardi.* FIG. 4. *Dactylogyrus chranilovi*, redrawn from Iziumova (1969). FIG. 5. *D. vastator*, redrawn from Nybelin (1924). FIG. 6. *Leptocotyle minor.* FIG. 7. *Hexabothrium appendiculatum.* FIG. 8. *Diplozoon paradoxum*, redrawn from Bovet (1959; reproduced with permission). FIG. 9. (a) (b) Two eggs of *Discocotyle sagittata*, reproduced with permission from Owen (1970), *Parasitology* **61,** p. 280. FIG. 10. *Calicotyle kröyeri*, redrawn from Kearn (1970a). FIG. 11. *Entobdella soleae.* FIG. 12 (a) (b) Two views of the egg of *Horricauda rhinobatidis*, reproduced with permission from Kearn (1978a), *International Journal for Parasitology* **8,** p. 309. FIG. 13. Egg of *Rajonchocotyle batis* seen in cross-section (a) and in side view (b), based on drawings by Cerfontaine (1899). FIG. 14. (a) (b) Two views of the egg of *Entobdella australis*, reproduced with permission from Kearn (1978b), *Australian Journal of Zoology* **26,** p. 210. FIG. 15. Egg of *Dictyocotyle coeliaca* seen in cross-section (a) and in side view (b) redrawn from Kearn (1970a). FIG. 16. (a) (b) Two views of the egg of *Dendromonocotyle kuhlii.* FIG. 17. (a) (b) Two views of the egg of *Diplorchis ranae*, redrawn from Ozaki (1935). FIG. 18. *Diclidophora merlangi.* FIG. 19. *Acanthocotyle lobianchi.* FIG. 20. (a) (b) (c) Three views of the egg of *Squalotrema llewellyni*, redrawn from Kearn and Green (1983). FIG. 21. *Diplectanum aculeatum*, redrawn from Oliver (1969). Figs. 4, 5, 9, 10, 12(b), 13(b), 14, 17, 19, 21 represent the whole of the egg and the appendage, if present; in the remaining figures only the root of the appendage is shown.

diversity has not always been fully appreciated. Baer and Euzet (1961) stated that monogenean eggs are of two main types: fusiform eggs produced by polyopisthocotyleans and tetrahedral eggs produced by the remaining monogeneans (so-called monopisthocotyleans). Kingston *et al.* (1969) made a similar distinction but, from a broader survey made by Bychowsky (1957, p. 89), a picture of greater variability emerges. Monogeneans produce eggs that may be almost spherical (Fig. 5), ovoid (prolate spheroids) (Figs. 8, 9), fusiform (spindle-shaped) (Figs. 3, 18) or with flattened surfaces. The most common shape with flattened facets is the tetrahedron (triangular pyramid) (Fig. 11) but spindle-shaped eggs may also have flattened sides (Figs. 13, 15). Tetrahedral eggs appear to be unknown in other parasitic platyhelminths and I have failed to find references to eggs of similar shapes in other invertebrates.

The size range of monogenean eggs is illustrated in Figs. 3–21 by a selection of eggs drawn to the same scale. The eggs of *Dactylogyrus vastator* (Fig. 5) and *Diplozoon paradoxum* (Fig. 8) are drawn adjacent to each other in the diagram and illustrate one of the smallest and one of the largest eggs, the volume of the egg of *D. paradoxum* being about 22 times that of the egg of *D. vastator*. The egg of the polystome *Pseudodiplorchis americanus* is exceptional in terms of size as well as in terms of features of the eggshell (see below); when the body of the fully developed oncomiracidium lies straight within the egg, the egg may slightly exceed 600 μm in length (Tinsley, personal communication).

Related monogeneans may produce eggs of different shapes. Some microbothriid[2] monogeneans produce spheroidal or fusiform eggs (e.g. *Leptocotyle minor*, Fig. 6) but *Pseudoleptobothrium aptychotremi* makes tetrahedral eggs (Kearn, unpublished observation). Most capsalids lay tetrahedral eggs (e.g. *Entobdella soleae*, Figs. 11, 29) but the eggs of *E. corona* and *E. guberleti* are spheroidal (see Hargis (1955) and Caballero y Caballero and Bravo-Hollis (1962), respectively). Kearn (1978b) observed that the egg of *E. australis* is symmetrical and urn-shaped when viewed from one direction, but when rotated about its longitudinal axis through 90° has a triangular profile (Fig. 14a,b). Oliver (1969) has described the egg shapes of several diplectanid parasites. The eggs of *Diplectanum aequans*, *Furnestinia echeneis*, *Protolamellodiscus serranelli* and several species of the genus *Lamellodiscus* are tetrahedral whereas *Diplectanum aculeatum* (Fig. 21), *Cycloplectanum americanum* and *C. echinophallus* have spheroidal eggs. The eggs of *Dactylogyrus vastator* are spheroidal in shape (Fig. 5) but *D.*

[2] The names used for the major groups of monogeneans are those employed by Llewellyn (1970, 1982).

chranilovi lays eggs that are usually tetrahedral (Fig. 4), although Iziumova (1969) reported that oval eggs are occasionally produced.

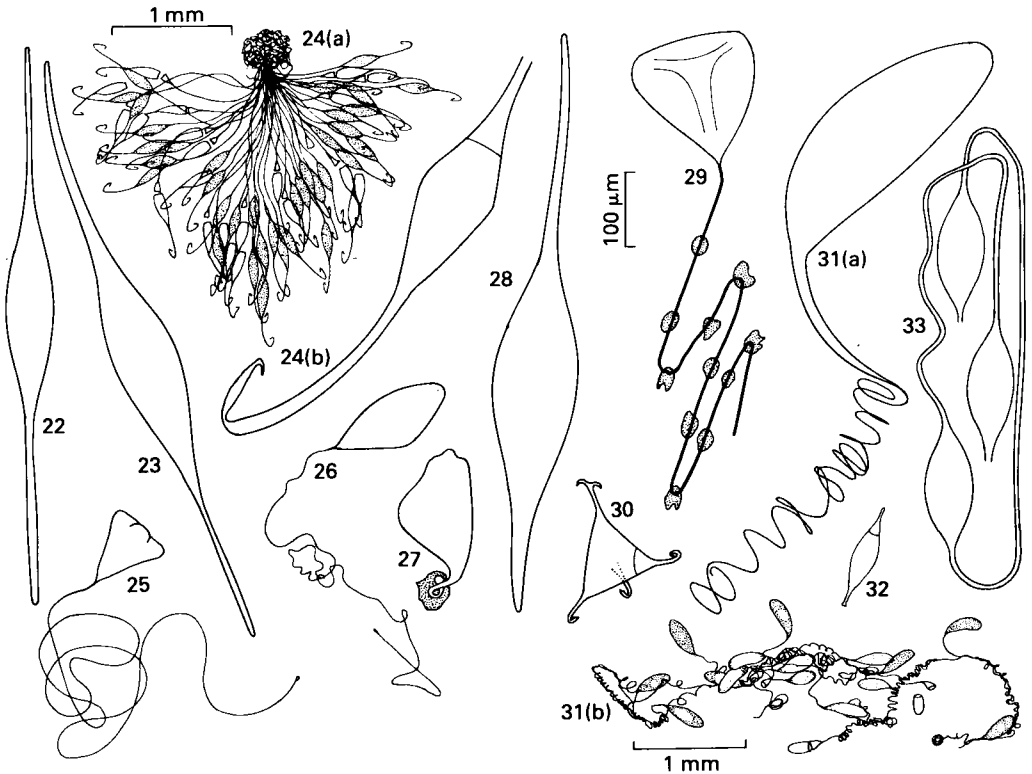
Almost all the monocotylid monogeneans that have been studied lay eggs with shapes based on the tetrahedron. Monocotylids have colonized a broad range of habitats, but *Merizocotyle* sp. from the nasal fossae (Kearn, 1968), *Calicotyle kröyeri* inhabiting the cloaca (Fig. 10), *Horricauda rhinobatidis* from the gills (Fig. 12) and *Dendromonocotyle kuhlii* from the skin (Fig. 16) all have eggs which resemble, some more closely than others, a regular tetrahedron. The egg of *C. kröyeri* has two unusual features. First, two of the edges of the tetrahedron bear thin flanges of eggshell material and secondly the surface of the egg is coated with adhesive material (Kearn, 1970a).

There are two monocotylids with eggs of an unusual shape, each of which appears, so far, to be unique among monogeneans. The egg of *Dictyocotyle coeliaca* from the body cavity of rays has a fusiform profile in side view (Fig. 15b) but when viewed from its proximal or distal end the egg is seen to be triangular in cross-section (Fig. 15a). The egg of *Squalotrema llewellyni* is thought to be derived from a tetrahedral shape by flattening of one of the four corners (Fig. 20; Kearn and Green, 1983); a hollow spur, with its cavity communicating freely with a central cavity within the egg, projects from the shell close to each of two of the corners.

There are no records of tetrahedral eggs being laid by acanthocotylid or by dionchid monogeneans. The eggs of *Acanthocotyle lobianchi* have an unusual shape which might be described as a modified spheroid; the egg is elongated, rounded at the distal extremity but tapering proximally (Figs. 19, 47). The eggs of dionchids are spheroidal (Fig. 42a).

Tetrahedral eggs are unknown in polyopisthocotyleans. Their eggs are rather conservative in shape; many such as *Discocotyle sagittata* (Fig. 9), produce spheroidal eggs, and others, such as *Diclidophora merlangi*, produce fusiform eggs (Figs. 18, 28). The eggs of *Rajonchocotyle* spp. are of special interest because the fusiform egg has prominent longitudinal ridges (Figs. 13b, 38b). Cerfontaine (1899) published a drawing (reproduced in Fig. 13a) of a transverse section through an egg of *R. batis*, which showed that there are seven ridges dividing the egg surface into seven facets; the eggshell of *R. emarginata* is similarly constructed (Fig. 38b).

The eggs of the polystomes *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis* deserve special mention. The parasites inhabit the bladders of desert toads, and the eggs develop fully while retained inside the uterus of the parasite (Tinsley and Earle, 1983). According to Tinsley (personal communication), when the larvae packed inside the uterus are examined superficially they appear to lack a cover, but closer examination reveals the presence of a thin, crinkled, completely flexible sac enclosing each oncomiracidium. The shell stretches, perhaps by unfolding of the


1 mm
24(a)
24(b)
22
23
25
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100 µm
29
30
31(a)
31(b)
32
33
1 mm

FIGS. 22–33. Egg appendages of monogeneans. FIG. 22. *Pseudaxine trachuri.* FIG. 23. *Gastrocotyle trachuri.* FIG. 24. *Microcotyle gotoi* (a) eggs attached in a group by the entangled opercular appendages; (b) single egg with incomplete opercular appendage. From Bychowsky (1957), p. 90 reproduced with permission from the Zoological Institute, Academy of Sciences of the USSR, Leningrad. FIG. 25. *Haliotrema balisticus.* FIG. 26. *Leptocotyle minor.* FIG. 27. *Entobdella australis*, reproduced with permission from Kearn (1978b) *Australian Journal of Zoology* **26,** p. 210. Note adhesive material on the appendage. FIG. 28. *Diclidophora merlangi.* FIG. 29. *Entobdella soleae.* Drawn from a scanning electron micrograph of an egg attached to glass prepared by Mr. J. Rees. FIG. 30. *Calceostomella inerme*, reproduced with permission from Euzet and Ktari (1973) *Bulletin de l'Institut national scientifique et Technique d'Océanographie et de Pêche de Salammbô* **2,** p. 610. FIG. 31. *Diplozoon paradoxum.* (a) Single egg (b) eggs attached in a group by their entangled appendages. FIG. 31(b) reproduced with permission from Bovet (1959) *Bulletin de la Société neuchâteloise des sciences naturelles* **82,** p. 235. FIG. 32. *Quadriacanthus* sp. From an unpublished sketch by Dr. M. El-Naggar. FIG. 33. Eggs of *Squalonchocotyle catenulata* joined in a chain by their appendages. Redrawn from Guberlet (1933). All of the eggs are drawn to the same scale, represented by a 100 μm scale bar, with the exception of Figures 24(a) and 31(b) to which separate 1 mm scale bars are appended.

crinkles, when the oncomiracidium elongates its body, and it also stretches to accommodate the increasing size of the growing embryo. Hence the shells of these polystomes are quite unlike the relatively rigid shells of most monogeneans, and their properties will receive special attention later (Section VI).

The possible significance of the shapes and sizes of monogenean eggs will be discussed in Section VIII.

In contrast with most digenean and cestode eggs many monogenean eggs have filamentous apical extensions of the eggshell material. Bychowsky (1957, p. 90) proposed the terms "filament" and "little foot" to distinguish between extensions of the distal (= leading) and proximal (= trailing) apices of a fusiform egg during its passage from the ootype into the uterus. However, because of the difficulty of applying these terms to tetrahedral eggs, some of which may have extensions of the eggshell at all four apices (see Fig. 30), I later proposed that these extensions should be called "appendages" (Kearn, 1963b).

There are many monogenean eggs, especially those laid by parasites of freshwater vertebrates, in which appendages are either absent, as in *Discocotyle sagittata* (Fig. 9), or very short, as in *Dactylogyrus vastator* (Fig. 5). The eggs of some marine monogeneans, such as *Diclidophora palmata*, also lack appendages. On the other hand, appendages may be remarkably long, tapering distally, flexible and coiled. The lengths of these entangled appendages are difficult to measure but Whittington (personal communication) has estimated that the egg appendages of the microbothriid *Leptocotyle minor* (Fig. 26) may lie between 2.5 and 3 mm in length, and those of the polyopisthocotylean *Hexabothrium appendiculatum* measure up to about 3.2 mm in length. The appendages of the freshwater polyopisthocotylean *Diplozoon paradoxum* differ from those of *Leptocotyle* and *Hexabothrium* in arising from the opercular pole rather than from the abopercular pole of the egg and in their exceptional length, between 20 and 25 mm according to Bovet (1967) (Fig. 31a). Tetrahedral eggs may also bear long, slender, coiled appendages, for example the eggs of the ancyrocephaline monogenean *Haliotrema balisticus* (Fig. 25).

It is common for the fusiform eggs of polyopisthocotylean monogeneans to possess needle-like appendages at both opercular and abopercular poles, for example in *Pseudaxine trachuri* (Fig. 22) and in *Diclidophora merlangi* (Fig. 28). The tiny fusiform freshwater egg of a species of the ancyrocephaline genus *Quadriacanthus* has similar, but shorter, opercular and abopercular appendages (Fig. 32).

Egg appendages may bear adhesive material. In the capsalid *E. soleae* an appendage measuring about 880 μm in length carries between eight and 11 colourless droplets of adhesive material spaced at intervals of about 60 μm

along the appendage (Fig. 29; Kearn, 1963b) but in *E. australis* the appendage is much shorter and bears a single terminal droplet of adhesive material (Fig. 27).

Some monogeneans possess hook-shaped appendages, for example in the fusiform eggs of the polyopisthocotyleans *Diclidophora luscae* and *D. denticulata* (Fig. 34b,c) and in the tetrahedral egg of the calceostomatid *Calceostomella inerme* (Fig. 30).

Eggs may be joined together in various ways by their appendages. Eggs with sticky material on the appendages may be joined to each other by this adhesive in small groups as in *E. soleae* (Kearn, 1963a) and in *A. lobianchi* (Fig. 47; Kearn, 1967). Eggs may be attached together by the fusion of the opercular and abopercular appendages of adjacent eggs as in the hexabothriid *Squalonchocotyle catenulata* (Fig. 33). A third way in which eggs may be attached together to form a bundle is by the entanglement of long, slender, coiled appendages as in *Diclidophora luscae* and in *D. denticulata* (Fig. 34).

The way in which egg appendages are assembled will be dealt with in Section IV A and the functional significance of egg appendages will be considered in Section VII.

III. The Egg Assembly Apparatus

A. General

Egg production in monogeneans involves a co-ordinated sequence of events that serve to bring together a single oocyte (or zygote) and numerous vitelline (or "nurse") cells (Fig. 35) in the egg mould or ootype, where the vitelline droplets, located peripherally in the cytoplasm of the vitelline cells, are released and coalesce around the package to form the eggshell. The proteinaceous shell then undergoes an enzymatic "tanning" process (sclerotization) involving phenolic substances, with the consequence that the shell becomes progressively darker, gradually changing colour from water-white to yellow or brown. This basic packaging process may be elaborated in some monogeneans by the construction of egg appendages or by the provision of adhesive material.

The general disposition of the germarium and the various female reproductive ducts has been illustrated for a range of monogeneans by Baer and Euzet (1961, fig. 175). That part of the ducting system that is concerned with the assembly of eggs is illustrated for a capsalid monogenean (*Entobdella soleae*) in Fig. 36 and for a polyopisthocotylean monogenean (*Diclidophora*

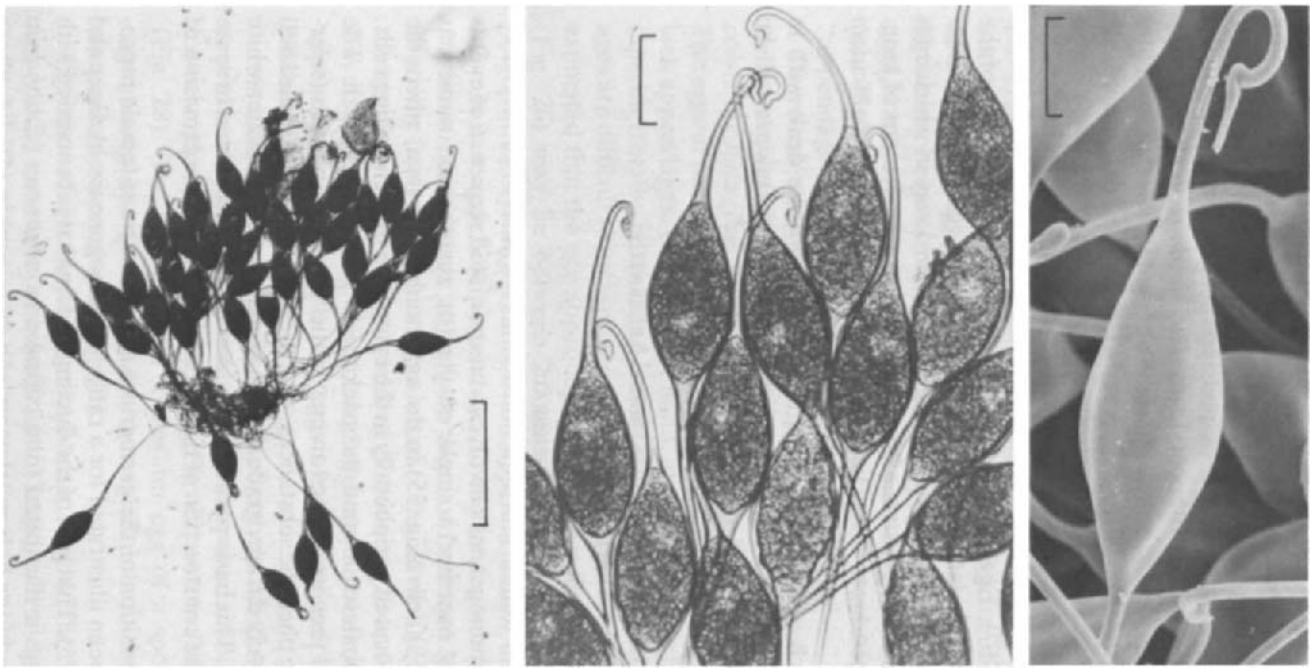


FIG. 34. (a) An egg bundle of *Diclidophora luscae*. Scale bar = 500 μm. (b) A few of the eggs from the bundle viewed at a higher magnification showing the hook-like opercular appendages. Scale bar = 100 μm. (c) An egg of *D. denticulata*. Scanning electron micrograph by Prof. D. W. Halton. Scale bar = 50 μm.

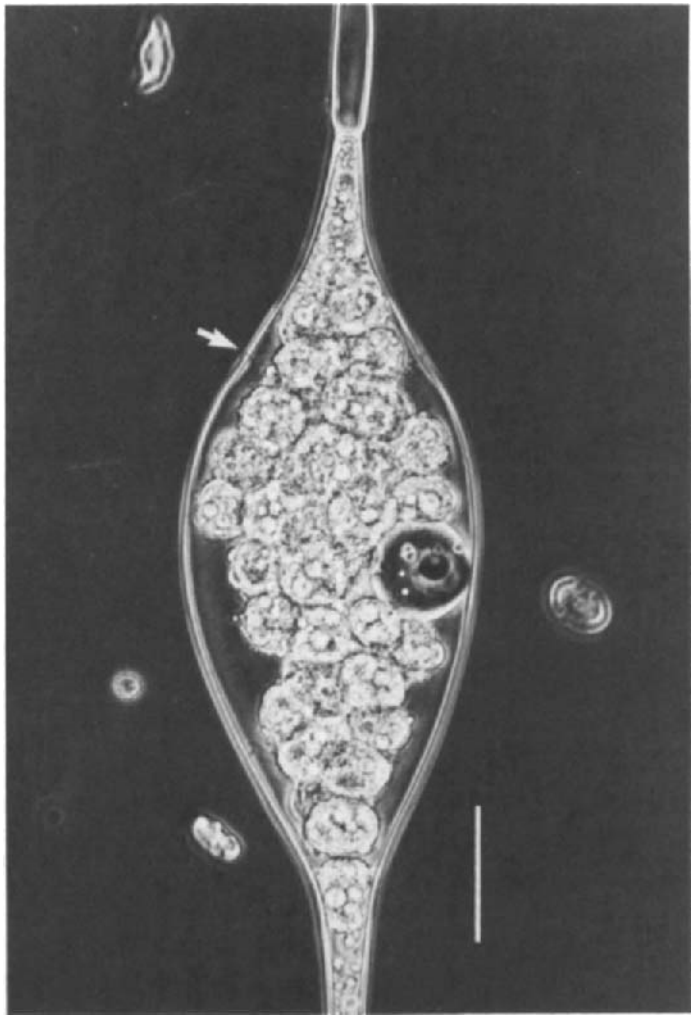


FIG. 35. Freshly laid egg of the hexabothriid polyopisthocotylean *Neonchocotyle pastinacae* from the gills of *Dasyatis pastinaca*, seen with the phase contrast microscope. The dark cell is the zygote and the light cells vitelline or "nurse" cells. The fusiform shell is extended to form a needle-like appendage at each end of the egg. The position of the opercular discontinuity in the shell is shown by the arrow. Scale bar = 50 μm.

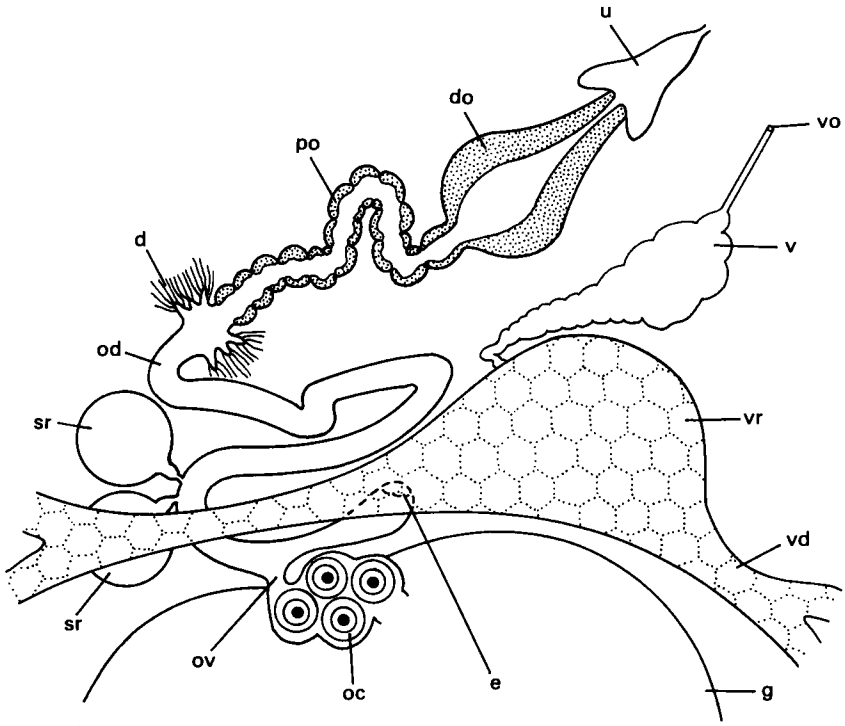


FIG. 36. The egg assembly apparatus in *Entobdella soleae* in ventral view. d, Ducts of ootype glands; do, distal tetrahedral chamber of ootype; e, exit of common vitelline duct from the vitelline reservoir; g, germarium; oc, oocyte; od, ovo-vitelline duct; ov, oviduct; po, proximal tubular region of ootype; sr, seminal receptacle; u, proximal region of uterus (distal region not shown); v, vagina; vd, transverse vitelline collecting duct; vo, vaginal opening; vr, vitelline reservoir. It is assumed that the vagina opens into the vitelline reservoir, although the opening has not been detected (Kearn, 1970b). Reproduced with permission from Kearn (1985) *International Journal for Parasitology*, **15**, 189.

denticulata) in Fig. 37. A major difference is the presence in polyopisthocotyleans of an additional tubular connection, the genito-intestinal canal, between the female reproductive tract and the intestine. There is an isolated report by Yamaguti (1958) of a genito-intestinal canal in the dactylogyridean *Geneticoenteron lateolabracis*. Bychowsky (1957, p. 360) expressed doubts about reports by Johnston and Tiegs (1922) of a vitello-intestinal communication in the monogeneans *Protogyrodactylus quadratus* and *Trivitellina subrotunda*.

In *E. soleae* the vagina containing inactive sperms is presumed to open into the vitelline reservoir but sperms are also stored in the seminal

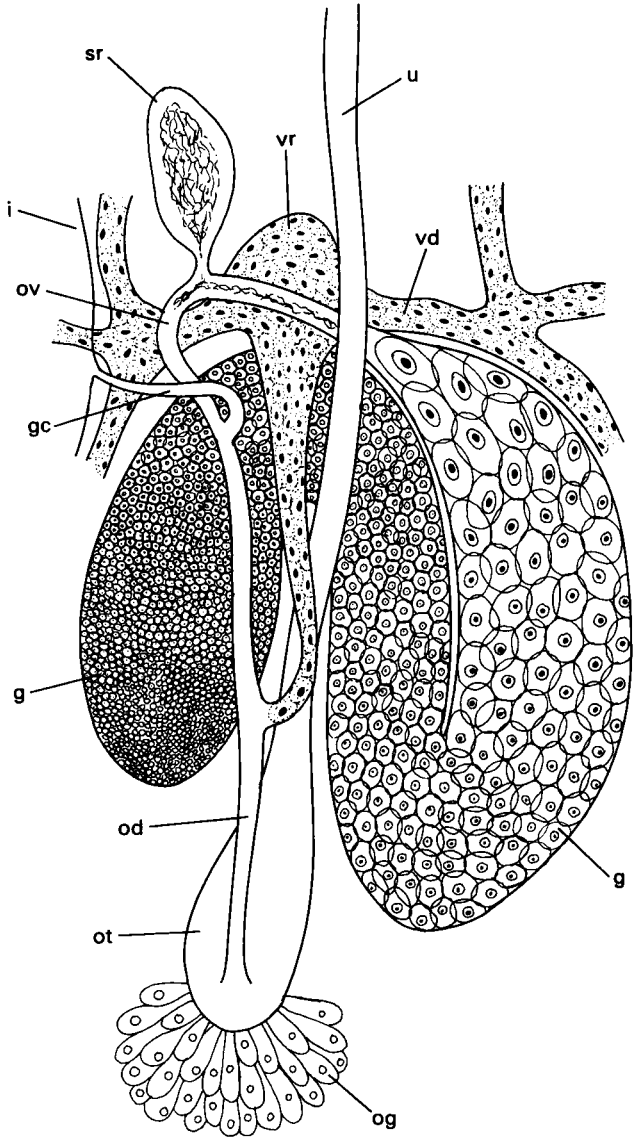


FIG. 37. The egg assembly apparatus of the polyopisthocotylean *Diclidophora denticulata* (= *Dactylocotyle denticulatum*) in ventral view. Redrawn from Cerfontaine (1896). gc, Genito-intestinal canal; i, intestine; og, ootype glands; ot, ootype. Other lettering as in Figure 36.

receptacles (Kearn, 1970b). It is not known when or how sperms are transferred from the vagina to the seminal receptacles but it is assumed that the latter provide a source of sperms for fertilization since the sperms in these receptacles are continually active.

Although Cerfontaine (1896) claimed to have found a short vagina (not shown in Fig. 37) leading to the seminal receptacle in some specimens of *Dactylocotyle denticulatum* (= *Diclidophora denticulata*), this was not confirmed by Frankland (1955) and there is evidence that, in the related *D. merlangi*, the spined penis attaches to the body surface of another adult parasite and that sperms pass through the tegument and find their way to the seminal receptacle (Macdonald and Caley, 1975). However, vaginae are present in other polyopisthocotyleans and the way in which vaginae may have evolved from parasites lacking vaginae and indulging in hypodermic impregnation has been considered by Llewellyn (1983).

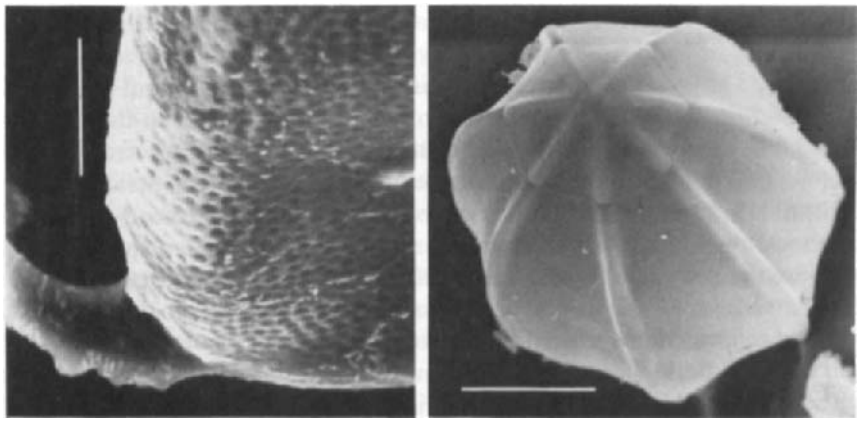
B. THE OOTYPE

While studying living specimens of monogeneans and especially *Epibdella* (= *Benedenia*) *sciaenae* and *Calceostoma elegans* (= *C. calceostoma*) van Beneden (1858) referred to "un organe spécial . . . dans lequel les germes, entourés de leur masse vitelline, séjournent quelques secondes, y subissent la pression des parois, reçoivent leur véritable forme . . .". Van Beneden coined the term "ootype" for this egg mould.

Bychowsky (1957) was aware of the variety of shapes of monogenean eggs and the general correspondence between the egg shape and that of the ootype. Lesser features are also impressed on the egg by the egg mould. For example, studs on the inner surface of the ootype in *E. soleae* appear to produce corresponding pits in the outer surface of the eggshell (Fig. 38a; Kearn, 1963b). It is remarkable that an egg mould made of soft tissue and capable of vigorous muscular deformation (see below) is able to reproduce repeatedly and faithfully an egg of relatively uniform shape and size. However, Kearn (1985) noted that the egg of *E. soleae* spends a few minutes, sometimes much longer, in the ootype after the cessation of ootype movements and before expulsion into the uterus, and it was suggested that this resting period might be important to permit some degree of hardening of the shell so that the egg takes up the shape of the egg mould.

In *E. soleae*, Kearn (1985) found evidence for a small but significant increase in the size of the egg as the parasite increased in size.

In those cestodes that assemble tanned eggs there are reports of two kinds of Mehlis' gland cell, so-called "mucous" and "serous" cells (Löser, 1965a). In the digenean *Fasciola hepatica*, Gönnert (1962) and Threadgold and



(a) (b)

FIG. 38. Scanning electron micrographs of monogenean eggs. (a) *Entobdella soleae*, showing the pitted outer surface and the root of the appendage. Scale bar = 20 μm. (b) *Rajonchocotyle emarginata*, viewed from the opercular end and showing the opercular discontinuity. Scale bar = 30 μm.

Irwin (1970) described similar "mucous" and "serous" cells with ducts intermingling and penetrating the ootype wall throughout its length. Ebrahimzadeh (1966) made an extensive comparative study of digeneans which indicated that in some species the duct openings of the mucous and serous cells are segregated such that the former open proximally in the ootype and the latter distally. In other digeneans the serous cells were reported to be absent but were said to be functionally replaced by a secretory ootype epithelium. Serous glands are said to be absent in those cestodes that lack a tanned shell (Löser, 1965b).

Since the process of assembly of tanned eggs seems to be basically the same in all parasitic platyhelminths, monogeneans might be expected to possess similar cells associated with the ootype. However, the overall picture in monogeneans is confused. The careful histological work of Goto (1894) revealed evidence of two distinct groupings of cells, one group of cells with ducts penetrating the ootype wall throughout its length and a second group of cells with ducts which converge on the ootype entrance. Each of these groups of cells has been independently described as "Mehlis' gland" (Rennison, 1953; Thurston, 1964). Goto's work revealed an apparent distinction between so-called monopisthocotylean monogeneans, possessing only glands opening at the entrance to the ootype, and polyopisthocotyleans, with glands opening through the wall of the ootype in addition to the

ootype entrance glands. Light microscope observations on *Entobdella soleae* (Fig. 36) and on *Polystomoides malayi* (Rohde and Ebrahimzadeh, 1969, fig. 1) appear to support this distinction, but Rohde and Ebrahimzadeh did not find glands opening through the ootype wall in three other species of the genus *Polystomoides*. The only transmission electron microscope (TEM) work on the monogenean ootype is that of Stranock and Halton (1975) but their findings are inconsistent with those of Goto. Stranock and Halton (1975) made no reference to ootype entrance glands either in the "monopisthocotylean" *Calicotyle kröyeri* or in the polyopisthocotylean *Diplozoon paradoxum*. Moreover, they reported two distinct kinds of gland cell with ducts penetrating the ootype wall in both of these parasites, and they equated these cells with the mucous and serous glands of cestodes and digeneans. Hathaway and Herlevich (1976) also used these terms for two kinds of gland cell with ducts penetrating the ootype wall of the polyopisthocotylean *Octomacrum lanceolatum*. In the polyopisthocotylean *Polystoma integerrimum*, Williams (1960a) gave histochemical evidence that the gland cells opening through the ootype wall secrete mucus, but Kohlmann (1961) in the same animal equated these cells with the serous cells of digeneans and claimed that the ootype entrance glands are mucous cells.

An additional complication in some monogeneans is the fact that the egg or its appendage may be provided with adhesive material. In *C. kröyeri*, there is an adhesive coat on the egg surfaces but the origin of this material was not considered by Stranock and Halton (1975).

Another inconsistency between digeneans and monogeneans is the report by Stranock and Halton (1975) of a secretory ootype epithelium in addition to mucous and serous ootype glands. Similarly, *P. malayi* was said to have a secretory ootype epithelium as well as ootype entrance glands and glands opening through the ootype wall (Rohde and Ebrahimzadeh, 1969). The work of Goto (1894) indicated that the ootype lining may be syncytial in some monogeneans and cellular in others. My own observations (unpublished) on histological sections of the ootype of *E. soleae* indicate that it may be syncytial but this requires confirmation with TEM. Stranock and Halton (1975) claimed that the ootype linings of *C. kröyeri* and *D. paradoxum* are cellular in nature.

Against this confused background, speculation about the possible homologies of ootype glands in monogeneans has little value and must await a thorough comparative study of the ootype regions of the different kinds of monogeneans, using TEM and semi-thin sectioning.

The ootype wall of monogeneans is well endowed with muscles that, in a few monogeneans that have been studied, are arranged in two layers, inner circular fibres and outer longitudinal muscles (Williams, 1960a; Rohde and Ebrahimzadeh, 1969). Sphincter muscles have also been described at the

proximal end of the ootype (Williams, 1960a; Kohlmann, 1961; Rohde and Ebrahimzadeh, 1969; Ktari, 1971) and at the distal end (Kohlmann, 1961). Goto (1894) described in some capsalid monogeneans a cervix-like extension of the distal end of the ootype into the uterus; he regarded this extension as a valve serving to prevent back-flow into the ootype. It is conceivable that increased fluid pressure in the uterus, generated by contraction of the uterine wall, might compress the lips of the "cervix" so preventing back-flow into the ootype. A similar cervix-like structure occurs in *E. soleae* (Fig. 36).

Curiously, Stranock and Halton (1975) reported that the cellular lining of the ootype of *D. paradoxum* is ciliated, but Bovet (1967) stated that the ootype of *D. paradoxum* is devoid of cilia. Cilia have not been reported in the ootypes of other monogeneans.

IV. Egg Assembly

A. General Account

Dawes (1940) considered that observation of the mode of egg formation in living monogeneans is a matter of considerable difficulty. However, some capsalid monogeneans (*Entobdella*, *Benedenia*) have several features which recommend them for such studies. After removal from the host the parasites readily attach themselves to the bottom of a glass vessel and continue to produce eggs, and their flat and semi-transparent bodies permit observation of the egg assembly process with the microscope. Linton (1901, 1908) appreciated the virtues of capsalid monogeneans for observations of this kind, and he provided a detailed account of the process of egg assembly in *E. bumpusii*; Jahn and Kuhn (1932) published a shorter account of egg assembly in *Epibdella* (= *Benedenia*) *melleni*. More recently, these observations have been extended by Kearn (1985) on *Entobdella soleae*.

The tetrahedral egg of *E. soleae* (Fig. 11) is made in an ootype chamber with a corresponding tetrahedral shape (Fig. 36). The corner of the tetrahedral egg that is made in the distal corner of the tetrahedral ootype chamber, where it communicates with the uterus, forms a detachable lid or operculum, which permits the fully developed larva to escape from the eggshell. The egg appendage (Fig. 29) is assembled in a proximal tube that is constricted at intervals to give a beaded appearance and opens ventrally into the proximal end of the tetrahedral chamber. Since there are glands that are considered to be involved in egg assembly (see above) opening into the proximal end of this tube, and since the egg appendage consists of the same material as the eggshell and appears to be made by a similar process (see

below), the whole of this proximal tube and the tetrahedral chamber will be regarded as the ootype. The adhesive droplets arranged at intervals along the appendage are produced in the proximal tube, and the chambers accommodating these droplets give the tube its beaded appearance.

The first major event in the process of egg assembly in *E. soleae* is the release by the germarium of a single oocyte (or possibly zygote, see below) into the oviduct and its passage into the ovo-vitelline duct, closely followed by numerous vitelline cells. No special anatomical specializations (ovicapts or sphincters) that might control release of the oocyte from the germarium or release of the vitelline cells from the vitelline reservoir have been observed in *E. soleae* with the light microscope. Structures of this kind at the germarium exit have been described in other monogeneans, particularly in polyopisthocotyleans (e.g. Dawes, 1940; Williams, 1960a,b; Rohde and Ebrahimzadeh, 1969).

Sperms first gain access to oocytes in the germarium of *E. soleae* (Kearn 1971a) but it is not known when fertilization takes place. In other monogeneans fertilization is reputed to occur in the germarium (Wright and Macallum 1887), in the ovo-vitelline duct (Ktari, 1971), in the ootype (Kulo, 1980) or even after formation of the shell (Goldschmidt, 1902).

In *E. soleae* the oocyte and the vitelline cells, following closely behind, proceed rapidly along the ovo-vitelline duct and enter and fill the proximal tubular region and the distal tetrahedral chamber of the ootype, the oocyte coming to lie in the distal corner of the tetrahedral chamber where the operculum is formed. As soon as the ootype is full, peristaltic and anti-peristaltic movements of considerable vigour begin in the tetrahedral chamber. At this stage in egg assembly the proximal tubular region of the ootype does not have a beaded appearance; the tube is relatively short with a wide lumen and straight sides and tapers slightly in a proximal direction. This tubular region of the ootype does not undergo vigorous, peristaltic movements like the tetrahedral chamber, but vitelline cells in the former are periodically squeezed in a distal direction so that at an early stage in egg assembly all the vitelline cells come to lie in the distal chamber. The appendage is laid down as a hyaline lining to this tube. It seems likely that the appendage material is derived from vitelline droplets released by the vitelline cells as they pass in a distal direction along the tube, although the release of these droplets has not been observed. Vitelline droplets may also reach the tube by back-flow from the tetrahedral chamber but no evidence of this was found. The diameter of the lumen of the tube gradually decreases and the appendage material is compressed and eventually fuses to form a solid appendage. During this process, pockets in which the adhesive droplets will be secreted appear in the wall of the tube. At first the pockets are collapsed and empty, but they soon begin to fill up with adhesive material. It

seems that as the appendage is formed the tube increases in length, and it is likely that the newly formed appendage is stretched, presumably while it is still soft.

Ktari (1971) proposed a somewhat different mechanism for the formation of the short proximal appendage of the egg of *Microcotyle mormyri*. He claimed that the proximal end of the fusiform egg is firmly attached at the proximal end of the ootype. As the egg is pushed from the ootype into the uterus, the proximal tip of the egg is pulled out to form the appendage. Presumably the proximal tip of the appendage is released as soon as the egg leaves the ootype since the proximal appendage is about the same length as the long axis of the ootype. Ktari noted that the hook-like termination of the proximal appendage is fashioned where the gland ducts enter the ootype entrance, but he was unable to state how this is brought about. A soft terminal blob of shell material gripped by contraction of the ootype entrance would be moulded to the shape of the entrance. This may be the way in which the button-like termination of the proximal appendage of *Diclidophora denticulata* is moulded, since the edge of the button is notched, forming projecting teeth (ten according to Cerfontaine (1896) and nine according to Frankland (1955)) and these teeth may correspond with the duct openings of the ootype entrance glands. However, this proximal appendage is about four times the length of the ootype (Frankland, 1955) and if it is fashioned in the same way as the proximal appendage of *M. mormyri* then the egg must be pushed a long way along the uterus before the termination of the proximal appendage is released by the ootype entrance.

According to Ktari, the distal appendage of *M. mormyri* is formed in a different way from the proximal appendage. While the egg is in the ootype some eggshell material that is still soft is forced into the uterus by contractions of the ootype. This material is drawn out by the beating of the cilia lining the uterus to form a thread that becomes progressively finer. The distal appendage of *D. paradoxum* appears to be made in the same way (Bovet, 1967) and a similar mechanism may fashion those proximal egg appendages that are assembled in a proximal tubular part of the ootype and are much longer than this proximal tube (for example *Squalotrema llewellyni*, see Kearn and Green (1983)).

In *E. soleae*, the contractions of the tetrahedral chamber drive the vitelline cells and their released droplets to and fro. The eggshell is laid down as a lining to the tetrahedral chamber and is continuous with the appendage material. As time passes, the ootype movements become less vigorous and less frequent until they cease altogether. The duration of the egg assembly process in *E. soleae* measured from the entry of the oocyte and vitelline cells into the ovo-vitelline duct to the time when ootype contractions were judged

to have ceased, ranges from 4 to 6 min at 12°C, irrespective of adult parasite size.

There are many other references to vigorous peristaltic and anti-peristaltic contractions of the ootype in monogeneans, for example Heath (1902), Linton (1908) and Jahn and Kuhn (1932) in capsalids, Kearn (unpublished observation) in *Diplectanum aequans*, Dr. S. Macdonald (personal communication) in acanthocotylids, and Remley (1942), Tinsley and Owen (1975) and Radha (1975) in polyopisthocotyleans. Llewellyn (personal communication) has observed the ootype movements in some polyopisthocotyleans as a kneading process produced by local contractions of circular muscle fibres along the length of the ootype rather than as a co-ordinated peristaltic wave.

The possible role of the genito-intestinal canal in egg assembly in polyopisthocotyleans will be discussed below.

B. SOME FUNCTIONAL CONSIDERATIONS

The classical account of the assembly of eggs with tanned protein shells in platyhelminths is that of Smyth and Clegg (1959). The description was based mainly on *Fasciola hepatica* but although the authors claimed that they gave a generalized picture of the process in digeneans and in monogeneans there is no mention of ootype contractions. However, such movements have been described during egg assembly in digeneans (Guilford, 1961; Llewellyn 1965; Rees, 1968). Rees (1968) believed that these movements serve to stimulate the release of vitelline droplets, and a similar suggestion was made for *Polystoma integerrimum* by Kohlmann (1961). However, in *Entobdella soleae*, the proximal tubular region of the ootype where the appendage is formed does not experience the vigorous contractions that occur in the tetrahedral chamber (Kearn, 1985) and yet the vitelline droplets, which fuse within the tube to form the appendage, appear to be released within the tube. No evidence was found to indicate that these droplets in the tube are derived by back-flow from the tetrahedral chamber. This suggests that the release of vitelline droplets may be stimulated by glandular secretions entering the ootype rather than by ootype contractions.

Another possible function of the churning or kneading movements of the ootype wall appears to have been overlooked by previous workers. When the vitelline droplets are first released in the ootype they are likely to be widely dispersed throughout the vitelline cell mass. The vigorous mixing of this cell mass by the ootype contractions seems to be the only way in which vitelline droplets among the vitelline cells are likely to be transported in a peripheral direction to make contact with the ootype wall. Provided that the

ootype wall has some kind of adhesive property, these droplets will remain at the periphery and further consolidation of the eggshell would be aided by some means of promoting fusion of the droplets when they reach the ootype wall. Peristaltic contractions of the proximal tubular part of the ootype of *E. soleae* where the appendage is made are possibly unnecessary because the tube has a narrow lumen.

Oxygen may be required not only for the chemical process of tanning (see below), which may begin when the egg is still in the ootype, but also for consumption by the ootype muscles. It is interesting that Rohde and Ebrahimzadeh (1969) recorded in *P. malayi* that the gland surrounding the ootype is blood red in colour *in vivo*, and Thurston (1970) found that the anterior two-thirds of the body of mature, living specimens of the polystomatid *Oculotrema hippopotami* are dark red. Thurston (1970) was able to establish that the pigment is haemoglobin and that the red colour appears during development and is concentrated around the ootype. Respiratory pigments may be necessary in the vicinity of the rapidly contracting ootype muscles in order to facilitate the transport of oxygen and possibly to act as an oxygen store during intervals in egg production, but it is curious that such pigment has been reported in so few monogeneans. Haemoglobin associated with the ootype may be important only in those monogeneans that maintain a high rate of egg output, particularly in an environment where the ambient oxygen concentration is likely to be low.

There have been many suggestions as to the possible functions of ootype secretions (Löser 1965b; summary by Erasmus, 1972) and these speculations will not be repeated here. Rennison (1953) and Stranock and Halton (1975) identified lipids associated with the ootype glands of *Diclidophora merlangi*, and histochemical evidence of the presence of phospholipids and lipoprotein in digenean ootype secretions (for example, Burton (1960), quoted by Burton (1963), Clegg (1965) and Clegg and Morgan (1966)) has led to suggestions that the ootype glands lay down a thin membrane which forms a basis for deposition of vitelline globules, but the presence of such a membrane was not confirmed with the electron microscope by Burton (1967). Observations on egg assembly in *E. soleae* indicate that vitelline droplet release is more likely to be initiated by glandular secretions (possibly from the ootype entrance glands) than by the vigorous muscular contractions of the ootype (see above).

Ramalingam (1970a,b) claimed that the secretion of glands associated with the ootype in monogeneans is responsible for unmasking phenolic material and for the activation of a prophenolase in the vitelline droplets. His evidence will be considered in more detail in Section VI.

C. THE OPERCULUM

The claim by Smyth and Clegg (1959) that most monogenean eggs lack an operculum probably originates from a similar statement made by Dawes (1946) and is incorrect. Llewellyn (1957a) examined 13 species of monogenean and found that they all produced operculate eggs. However, it has been established that the operculum is lacking in a few specialized eggs. Tinsley (1978) found no operculum in *Eupolystoma anterorchis* and described hatching as involving a random, often longitudinal, splitting of the shell. The eggs of *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis* also have no operculum and the larva emerges through an irregular tear (Tinsley and Earle, 1983). However, the eggshells of these three last-mentioned parasites are thin and flexible, and these features, together with the lack of an operculum, are associated with the retention of the eggs in the uterus, extensive embryonic development *in utero* and rapid hatching, possibly by an osmotic mechanism, when the eggs are laid (Section XI). It seems likely that the chemistry of the eggshell and possibly eggshell assembly in these parasites differs in important respects from those of other monogeneans.

In digeneans, the opercular discontinuity has been regarded as a region where the eggshell is particularly thin (Kremer and Chaker, 1983) but the work of Kearn (1975a) on the egg of *E. soleae* indicates that the operculum is attached to the rest of the eggshell by an extremely thin layer of opercular cement. This cement is greatly weakened when proteolytic enzymes are applied experimentally to the inner surface of the eggshell, but not when these enzymes are applied to the outside of the shell (Sections VI, XI A), suggesting that the inner part of the opercular cement is proteinaceous in nature. It was suggested by Kearn (1975a) that an outer layer of tanned shell protein continuous with the rest of the shell and covering the opercular seal might provide the necessary protection from external enzymatic attack, should the eggs be eaten by a predator, and that this might explain the apparent absence of any external evidence of the opercular joint in scanning electron microscope (SEM) studies conducted by Macdonald (quoted by Kearn, 1975a) of the eggs of *E. soleae* and *Acanthocotyle lobianchi*. However, the opercular seal is visible with the SEM on the outer surface of the eggs of *Diclidophora denticulata* (Fig. 34c), *Rajonchocotyle emarginata* (Fig. 38b) and certain diphyllobothriid cestodes (Hilliard, 1972).

It might be expected that the opercular cement differs in origin and in chemical composition from the shell material, but this is not necessarily the case since the cement might represent a thin layer of proteinaceous shell material, the inner part of which for some reason fails to undergo sclerotization and hence retains its vulnerability to proteolytic enzymes.

There is no information on the way in which the opercular discontinuity is

created in monogeneans, but in digeneans the oocyte (or zygote) has been implicated in its formation by Goldschmidt (1909), by Poche (1927), by Gönnert (1962) and by Ebrahimzadeh (1966). Gönnert (1962) showed that in *Fasciola hepatica* the zygote precedes the vitelline cells into the ootype and becomes located at the distal pole of the ovoid egg where the operculum is formed. He presented histological evidence of pseudopodia extending from the zygote to the region of the shell where the opercular discontinuity develops. Further evidence in support of the role of the zygote in the formation of the operculum is the observation by Goldschmidt (1909) that eggs of the digenean *Dicrocoelium lanceatum* lacking a zygote had no opercular discontinuity, and Löser (1965b) made a similar observation on the cestode *Bothridium pithonis*.

In monogeneans a limited amount of similar evidence implicates the oocyte or zygote in the formation of the operculum. The oocyte has been observed preceding the vitelline cells in the ovo-vitelline duct in the polyopisthocotyleans *Discocotyle sagittata* (see Seil, 1973) and *Protopolystoma xenopi* (see Thurston, 1964) as well as in *E. soleae* (Section IV A). In *Microcotyle mormyri*, Ktari (1971) observed that the zygote in the form of a truncated cone is pressed against the anterior wall of the ootype. Moreover, he found in the uterus some eggs containing only vitelline cells and no zygote, and these eggs lacked an opercular discontinuity. Kearn (1975a) found some eggs of *E. soleae* that appeared to lack the opercular discontinuity; these eggs failed to develop but it was not possible to say whether this failure was due to the absence of an egg cell.

The observations of Rees (1968) on the digenean *Macrolecithus papilliger* are not consistent with the involvement of the egg cell in operculum formation. She observed that, although the oocyte enters the ootype first, followed by the vitelline cells, the operculum forms at the proximal end of the ootype. This suggests the alternative possibility that the ootype wall may be involved in the formation of the opercular discontinuity in platyhelminths. If there is a structural feature in the ootype wall responsible for the opercular discontinuity it is likely to be precisely located and hard to resolve. I have found no trace of such a feature with the light microscope in *E. soleae* and further research demands the careful preparation of accurately positioned ultrathin sections for TEM.

D. THE ROLE OF THE GENITO-INTESTINAL CANAL

The limited observations that have been made on polyopisthocotyleans indicate that there may be minor differences between egg assembly in these parasites and in capsalids, these differences being mainly concerned with the role of the genito-intestinal canal.

Egg assembly appears to demand that the oocyte or zygote should precede the vitelline cells into the ootype, and there is evidence that this is related to production of the operculum (see above). Any vitelline cells remaining in the ovo-vitelline duct after the ootype has been filled to capacity would prevent the next oocyte from attaining this leading position, and the genito-intestinal canal would provide an exit route for such obstructing vitelline cells. The observations of Ktari (1971) on *Microcotyle mormyri* suggest that the genito-intestinal canal does perform this role; cilia reported by Ktari (1971) in the ovo-vitelline duct and genito-intestinal canal of *M. mormyri* and described in other polyopisthocotyleans (e.g. Rohde and Ebrahimzadeh, 1969) would permit rapid removal of obstructing cells from the female reproductive tract. The transport of these excess cells to the intestine provides the opportunity to digest and recycle the vitelline material. Those monogeneans such as *E. soleae* that lack such an exit route are obliged to exercise greater control over the volume of vitelline cells released by the vitelline reservoir, so that the ootype is able to accept all the vitelline cells released into the ovo-vitelline duct, leaving an unobstructed pathway for the next oocyte.

The genito-intestinal canal may be a specialization relieving the vitelline reservoir of the need for precise control, but the presence of such canals in many turbellarians (Gremigni, 1983) and the fact that turbellarians and monogeneans are believed to share a common ancestry (Llewellyn, 1965) suggest that the genito-intestinal canal in polyopisthocotyleans may be a primitive feature retained from turbellarian-like ancestors rather than a specialized development. The acquisition of better control over release of vitelline cells into the ovo-vitelline duct in other monogeneans may have led to the loss of the genito-intestinal canal.

There have been other suggestions as to the function of the canal. Combes (1966, 1968) regarded the canal as a seminal receptacle in neotenic adults of *Polystoma integerrimum*. Llewellyn (1983) suggested that the genito-intestinal canal might provide a route to the oviduct for the sperms of *Gastrocotyle trachuri* introduced by non-localized hypodermic impregnation into a gut caecum. Sperms injected into the vitelline system would face a less hazardous journey. An earlier suggestion by Llewellyn (1957a,b) was that the canal might serve as a means of introducing symbiotic bacteria, which might assist digestion of host blood by the parasite, from the intestine of the parasite into the eggs. However, experiments by Bovet (1967) and TEM observations by Bovet and by Morris and Halton (1975) did not provide support for this suggestion.

E. THE ROLE OF THE UTERUS

Some monogeneans, for example monocotylids, lack a uterus, whereas in other monogeneans the uterus may be so extensive as to require repositioning of the other reproductive organs (see below). In some monogeneans, for example in *Diclidophora luscae*, the uterus may be used as a temporary store for eggs which may be attached to each other in some way and ejected as egg bundles (see Section VII B). In others, for example in some polystomatids, prolonged egg storage may permit extensive embryonic development, and this may be followed by storage of fully developed eggs for long periods or by hatching *in utero* (Section VII C 2).

Erasmus (1972) suggested that the uterus is much more than a tube carrying eggs to the exterior, and this suggestion was based partly on the finding by Halton (1967) that alkaline phosphatase was present in the uterus of seven species of monogeneans tested. According to Tinsley (1983), eggshell assembly and shell-hardening occur in the ootype in those monogeneans lacking a uterus, whereas in those monogeneans with a uterus this provides a site where shell-hardening can proceed without impeding the assembly of successive eggs in the ootype, thereby leading to substantial increases in egg output. However, in some monogeneans there is clear evidence that the uterus is little more than a passageway for the eggs to reach the outside world. In *Entobdella soleae*, some eggs pass through the uterus in less than 5 s and the eggshell tanning process may be completed after laying (Kearn, 1985), and Finlayson (1982) observed that the egg of *Kuhnia scombri* spends less than 2 min passing through the uterus.

The possibility that the uterus of some digeneans may be supplied with secretory material has been discussed by Gönnert (1962) and by Ebrahimzadeh (1966), and there are indications that in some digeneans and cestodes eggshell assembly may be completed in the uterus rather than in the ootype (Section V A p. 211).

There is evidence that shelled monogenean eggs that undergo embryonic development *in utero*, or that are stored for extensive periods after development is completed, may receive nutrients from the ambient uterine fluid (Section VI p. 219) and the uterine wall might be the source of these nutrients.

Llewellyn and Tully (1969) have pointed out that there has been a posterior shift of the germarium in four species of the genus *Diclidophora* that store eggs temporarily in an enlarged uterus; this repositioning of the germarium may be a consequence of the enlargement of the uterus to accommodate the large egg bunch. An additional feature of these four species of *Diclidophora* is the separation of the uterine opening from the opening of the male system; this separation may be related in some way to

the need for great dilatation of the uterine aperture during laying of the egg bunch. A similar rearrangement of internal organs to accommodate an enlarged uterus occurs in some polystomes (Tinsley, 1983).

The uterus is ciliated in *Discocotyle sagittata* according to Seil (1963), and in *Microcotyle mormyri* according to Ktari (1971). In the latter, the cilia appear to play a part in the production of distal egg appendages (see above).

In gyrodactylids there is no eggshell, and extensive development and growth of the embryo occurs inside the body of the parent parasite. It is generally assumed that this growth takes place inside the uterus but the discovery by Harris (1983) of *Oögyrodactylus farlowellae*, in which a shelled egg is assembled and retained for some time within the ootype, raises the possibility that the sac in which the embryo develops in the viviparous gyrodactylids may be a modified ootype rather than a uterus.

F. CONTROL OF EGG ASSEMBLY

Egg assembly in monogeneans is a complicated series of events that is initiated at regular intervals, but there has been little speculation on the way in which such a cycle might be initiated and controlled. The control of seasonal changes in egg output will be considered later (Section V B).

Kearn (1985) suggested that, in *E. soleae*, the vitelline reservoir might exercise a controlling influence over the egg assembly process, perhaps by way of a stretch receptor that sets in motion the events of egg assembly when sufficient vitelline cells to make an egg have accumulated in the reservoir. In *E. soleae* the egg assembly process is inhibited by the presence of an egg in the ootype but not by the presence of an egg appendage originating from an egg stored in the uterus, and there is no evidence of any feedback control from the uterus since egg assembly proceeds whether there are eggs in the uterus or not. However, situations have been observed in *E. soleae* in which egg production is delayed in spite of the fact that the ootype is empty and that the vitelline reservoir appears to contain an adequate supply of vitelline cells. Such an interruption in egg assembly might be brought about by the abnormal conditions to which the parasite was subjected by the observer, or might indicate that factors other than an empty ootype and a full vitelline reservoir are necessary for egg assembly to proceed.

Tinsley (1978) found some evidence to suggest that egg production in *Eupolystoma anterorchis* may slow down or cease when the uterus is full of eggs, indicating possible feedback inhibition of egg production by the uterus.

Nerves have been reported in association with the reproductive ducts of

monogeneans (e.g. Williams, 1960a) and these are likely to be important in co-ordinating successive events of egg assembly.

More intriguing problems of control are posed by those monogeneans that produce more than one kind of egg, such as *Choricotyle australiensis* which assembles eggs with different appendages (Section VII C 2), *Eupolystoma alluaudi*, which assembles eggs that produce different kinds of larvae (Section VII C 2) and *Dactylogyrus vastator*, which assembles eggs some of which may enter a state of diapause (Section X).

V. Egg Output

A. Rates of egg output

There have been many determinations of egg output in monogeneans, some of them based on egg production by parasites detached from the host (e.g. Llewellyn, 1957a; Anderson, 1981; Finlayson, 1982) and others based on egg production by parasites still attached to the host (e.g. Tinsley and Owen, 1975; Jackson, 1982). Tinsley (1983) has summarized these determinations with the general statement that most monogeneans deposit fewer than 100 eggs per parasite per 24 h and many deposit fewer than 25. However, Combes (1972) collected eggs of *Polystoma integerrimum* from isolated frogs parasitized by single parasites, and the maximum number of eggs recovered on one day for each parasite ranged from 1000 to 2500. Since the parasite has a relatively short uterus, it is unlikely that more than about 20 of these eggs could have been assembled on the previous day and stored in the uterus. Provided that some of these eggs were not detained in some way in the bladder of the host, then most of these eggs were probably assembled in a single day and this represents the highest daily output so far recorded for a monogenean. In contrast, the polystomatids *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis* probably produce fewer eggs than other monogeneans, since the annual output of these parasites appears not to exceed 250 eggs (see Tinsley (1983), Section IX p. 240, and Section XI A p. 257).

The time taken to assemble a single egg has been obtained by direct observation of the egg assembly process in a few monogeneans. Jahn and Kuhn (1932) quoted an average time of 5 min (range 2–10 min) for production of a single egg in *Epibdella* (= *Benedenia*) *melleni* and Kearn (1985) gave a figure of 4–6 min at 12°C in the related *E. soleae*. However, although it is theoretically possible for these capsalid monogeneans to produce eggs at the rate of one egg every 5 min, in practice the maximum

rate of egg production in detached specimens of *E. soleae* was found to be about one egg every 21 min. Delays in the egg assembly process in *E. soleae* include retention of the fully formed egg in the ootype after cessation of ootype movements and failure of the parasite to initiate immediate assembly of a new egg after expulsion of the previous egg from the ootype; the possible reasons for these delays have been discussed by Kearn (1985).

Estimates of the egg output of dactylogyrid monogeneans differ depending on whether such estimates were made on parasites attached to or parasites detached from the host (see work by Lyaiman and Iziumova, quoted by Paperna (1963a) and Prost (1963)). Prost (1963) experimentally infected fishes with oncomiracidia of *Dactylogyrus anchoratus* and commenced to count eggs after 12–15 days, when all parasites were likely to be mature and of the same age (see Table 1). Detached parasites, unable to renew their resources by feeding, might be expected to produce fewer eggs than attached parasites, but, surprisingly, Prost (1963) found that attached parasites laid fewer eggs than detached parasites. The reasons for this are not clear although Prost (1963) observed that the size of eggs laid by detached specimens of *D. anchoratus* diminishes during the course of the laying period, suggesting that production of larger numbers of smaller eggs may make a contribution to the increased egg output of detached parasites. Oxygen availability is also known to have a significant effect on egg production in some dactylogyrids (Iziumova, 1958) and on polyopisthocotylean gill parasites (see work by Houlihan and Macdonald (1979), discussed below).

Iziumova (1956) studied egg-laying in *D. vastator* while attached to the host and showed that, in general, egg output increased with the age of the parasite over a period of about 10 days. It has been known for some time that growth in *E. soleae* continues after sexual maturity is reached (Kearn, 1963b) and more recent work (Kearn, 1985) has shown that egg production increases as the adult parasite increases in size (Fig. 39). Very large adults may produce more than 60 eggs per day. Kearn (1985) found little difference in the time taken to assemble an egg in small and in large adults, and suggested that the storage capacity of the vitelline reservoir or the rate of vitelline cell production may limit egg output in small adults, there being some evidence for the latter from observations on the growth of the vitellarium. These egg output estimates in *E. soleae* were made on parasites detached from the host. A comparison with the egg output of attached parasites has not been made but there are no reasons to suspect differences, because specimens of *E. soleae* separated from the host live for a few days and continue to behave in the same way as parasites attached to the host (Kearn, 1985).

TABLE 1 *Egg output (number of eggs laid per parasite per day while attached to the host) of selected species of the genus* Dactylogyrus *at various temperatures*

Temperature (°C)	*D. anchoratus* (from Prost, 1963)	*D. vastator* (from Iziumova, quoted by Paperna, 1963a)	*D. vastator* (from Paperna, 1963a)	*D. lamellatus* (from Molnár, 1971)	*D. amphibothrium* (from Kashkovskii, 1982)
0.5					0.08
1.0					0.2
4– 5	0.045 (5°C)				0.6
8– 9					3.6
10–12		4.5 (12°C)	1.5 (12°C)	2.5 (12°C)	4.6
14	1.8				
16					10.7
17–19		9.3 (18°C)		8.5	
20–22				9.0	
23	3.87				
24			25.0	10.3	
28	2.13		29.0	15.0	
37			2.5		

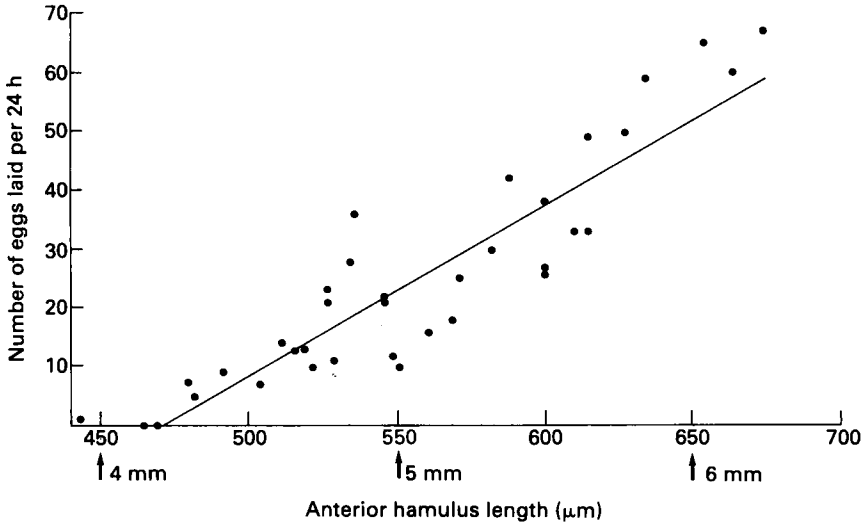


FIG. 39. The relationship between the length of the anterior hamulus and the rate of egg production in *Entobdella soleae*. The arrows indicate approximate total body length measurements. Reproduced with permission from Kearn (1985) *International Journal for Parasitology* **15**, 188.

It was suggested by Kearn (1985) that continued growth after sexual maturity, a strategy that is adopted by many poikilotherms, may have special significance for a parasitic animal like *E. soleae*. The hazards of host finding may be accompanied by high mortality, which may be offset by maintaining a high reproductive capacity.

Temperature has a profound influence on egg output. Figures for the egg output at various temperatures of selected dactylogyrid monogeneans attached to the host are given in Table 1. These figures illustrate the tendency in monogeneans to produce more eggs as the temperature increases (but see below); however, at temperatures at the upper end of the range, egg output may fall. The relatively wide range of temperatures at which eggs are produced in some dactylogyrids may reflect their freshwater habitat, which experiences a corresponding wide range of environmental temperatures. Some dactylogyrids, e.g. *D. amphibothrium*, appear to continue to produce eggs, albeit slowly, at temperatures between 0°C and 5°C, but Prost (1963) was of the opinion that egg production in *D. anchoratus* ceases below 5°C.

Egg production in monogeneans appears to be sufficiently flexible to adapt in some circumstances to particularly low environmental temperatures. For example, in *P. integerrimum* from the bladder of *Rana tempor-*

aria, egg production is higher at 4°C or at 8°C than at higher temperatures in the range 12–20°C (Combes, 1972) and this corresponds with an environmental temperature of 5–9°C when frogs and parasites lay their eggs. Egg assembly may proceed at a temperature just below −1°C in *Pseudobenedenoides branchicola* which, according to Szidat (1969), is found on a fish inhabiting perpetually cold Antarctic bottom waters (Section X p. 243).

Tinsley and Owen (1975) offered evidence of a relationship between the individual parasite egg output in *Protopolystoma xenopodis* and the number of parasites present in the toad host (*Xenopus laevis*). The number of eggs produced per parasite per day increased from eight in a two-parasite infection to more than 50 in five- or six-parasite infections. This contrasts with the findings of Combes (1972) for egg production in the bladder-inhabiting adult of *P. integerrimum*; he found that egg output per mg of parasite fell as the number of parasites in the bladder increased.

Oxygen availability may have a substantial influence on egg production. This is well illustrated by a comparative study of the skin parasite *E. soleae*

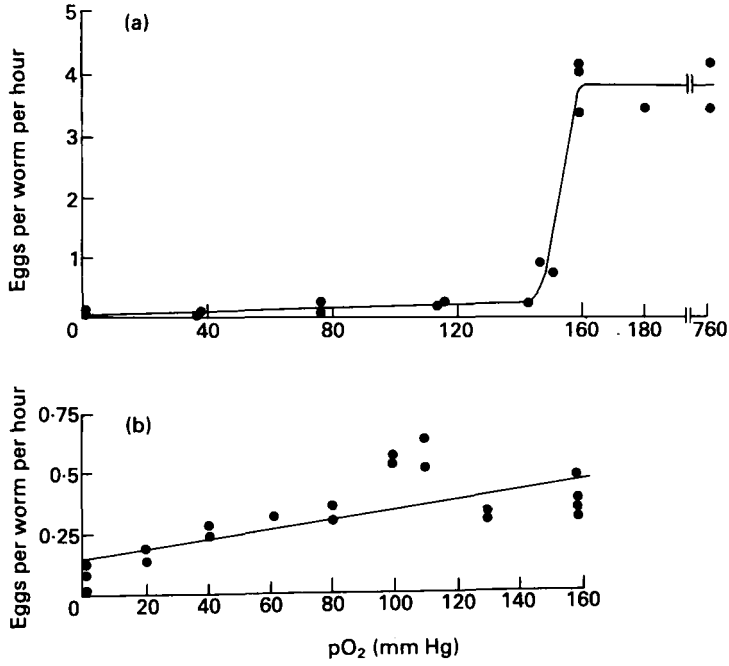


FIG. 40. Egg production of (a) *Diclidophora merlangi* and (b) *Entobdella soleae* at different partial pressures of oxygen. Reproduced with permission from Houlihan and Macdonald (1979) *Experimental Parasitology* **48**, pp. 111, 113.

and the gill-inhabiting polyopisthocotylean *Diclidophora merlangi*, made by Houlihan and Macdonald (1979). For *E. soleae* they found that, in general, egg production declined relatively slowly with declining ambient partial pressure of oxygen (Fig. 40b) whereas in *D. merlangi* egg production virtually ceased at oxygen partial pressures little below air saturation (Fig. 40a). Adults of *E. soleae* live on the lower surface of a flat-fish host that spends a lot of time buried in sediment that is likely to be partially deoxygenated (Kearn, 1962). The ability of *E. soleae* to decrease its body thickness, increase its surface area, and increase body ventilatory movements probably allows the parasite to maintain and even increase its oxygen consumption and hence egg production in situations in the natural environment where the ambient oxygen partial pressures fall. On the other hand, *D. merlangi* might be expected to be well oxygenated, living as it does in the continuous gill-ventilating current of its host. However, Houlihan and Macdonald (1979) quoted figures that suggest that the oxygen partial pressures of the gill current may be substantially reduced as a result of extraction of oxygen by the gills to a level at which egg production of *D. merlangi* would almost cease. The answer to this paradox may be that the bulk of the body of the parasite may disrupt the normal flow of water over the gills, so that the partial pressure of oxygen of the respiratory water reaching the parasite may not be depleted, or it may be that the parasite augments its oxygen supply by extracting oxygen from blood in the secondary gill lamellae (the parasite is a blood feeder). Curiously, Iziumova (1958) reported an increase in egg production in the gill parasite *Dactylogyrus solidus* as the oxygen content of the water decreased.

Anderson (1981) studied the effects of salinity on egg production in ancyrocephaline parasites of the mullet, *Chelon labrosus*. Young mullets up to 2 years of age live in tidal (brackish) pools and are infected exclusively by *Ergenstrema labrosi*, whereas fishes aged 4 years and older live in the open sea and are infected exclusively by *Ligophorus angustus*. Fishes aged between 2 and 4 years have both parasites. Anderson (1981) found evidence to suggest that this change in monogenean species composition is related to salinity changes between the habitats of young and old fishes. The rate of egg production in parasites separated from the host was proportional to salinity in *L. angustus* such that most eggs were laid in 100% sea water (Fig. 41), whereas in *E. labrosi* maximum egg production occurred in 50% sea water, egg output being adversely affected by higher salinities and ceasing in 100% sea water. However, observations made on parasites attached to the host suggest that some egg production does take place in *E. labrosi* in 100% sea water. *E. labrosi* lays more eggs when detached from the host than *L. angustus* at all salinities between 0 and 80%, and Anderson (1981) suggested that the higher rate of egg production by *E. labrosi* may be an adaptation to

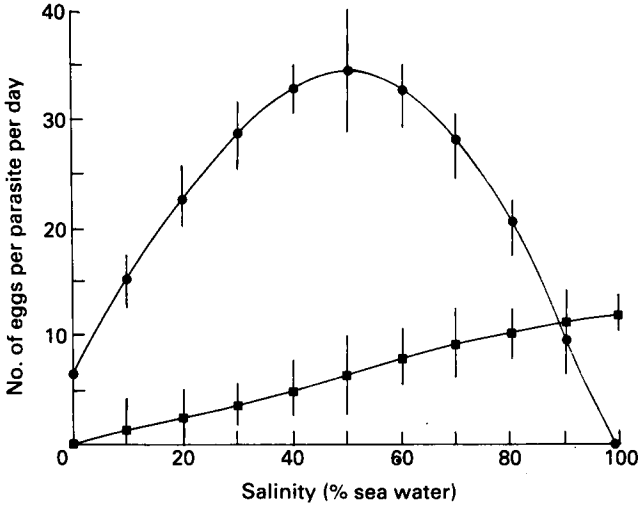


FIG. 41. The effect of salinity on the rate of egg-laying in *Ergenstrema labrosi* (●) and *Ligophorus angustus* (■). Reproduced with permission from Anderson (1981) *Journal of the Marine Biological Association of the United Kingdom* **61**, p. 837.

the hazardous conditions in the variable environment of a tidal pool compared with the more stable conditions in the open sea.

Winch (1983) studied egg production in *Atrispinum labracis* at various salinities ranging from 100% sea water to fresh water. With the exception of fresh water, egg production continued at all these salinities although the parasite is rarely encountered on hosts living in estuaries.

A study of the effects of host starvation on egg production in the blood feeder *P. xenopodis* has been made by Jackson (1984). She detected a 54% mean reduction of egg output when parasites were transferred from well fed toad hosts to toads starved for 6 months. She found no change in parasite egg production after experimental induction of host anaemia (a 50% reduction in packed cell volume), and came to the conclusion that any effect of host starvation on egg production is not the result of reduced availability of erythrocytes.

Llewellyn (1965) compared egg production rates in monogeneans, digeneans and cestodes. He pointed out that since the reproductive apparatus of members of the parasitic groups of platyhelminths is similar it seems very likely that the basic process of egg assembly is also similar. He estimated that an egg production rate of 1 egg per min (based on work by Nasir on a digenean) is probably somewhere near the limit of capacity for an assembly system with a single ootype. Llewellyn (1965) regarded the ootype as a bottleneck in the assembly line and considered that an egg production rate of

1 egg per min is unlikely to be exceeded unless the ootype bottleneck is eliminated by substituting many assembly units, each with its own separate ootype. He pointed out that such a step has been taken by the cestodes and he estimated that the pseudophyllidean cestode *Schistocephalus solidus* achieves by this means of an egg production rate of about 1 egg every 4 s, which he regarded as being impossible to achieve with a single ootype system like that of a monogenean.

Llewellyn's (1965) proposed upper limit for egg production in parasites with a single ootype of about 1 egg per min probably needs to be adjusted, since Guilford (1961) found by direct observation of egg assembly in four living specimens of the digenean *Halipegus eccentricus* that 20–30 s elapsed between release of an oocyte into the oviduct and expulsion of the egg into the uterus, and the work of Combes (1972) indicates that the monogenean *Polystoma integerrimum* is capable of assembling an egg in little more than 30 s. There have also been claims that fasciolid digeneans are capable of producing eggs at the rate of 1 egg every 4 s, and these figures have led Tinsley (1983) to challenge Llewellyn's (1965) arguments concerning the possible limitations imposed on egg production by the ootype. Tinsley (1983) suggested that the limitation is more likely to be related to the supply of components.

The egg assembly process in the monogenean *E. soleae* takes about 5 min (Kearn, 1985). There is some evidence that the length of delays between the assembly of successive eggs may be related to the availability of components (especially vitelline cells) but it seems unlikely that provision of an unlimited supply of components would lead to an increase in the speed of individual egg assembly. In fact, the egg assembly time is approximately the same in small and in large adults, even though the supply of vitelline cells is probably greater in large adults. It is conceivable that the egg assembly time in parasitic platyhelminths could be shortened by accelerating the major steps of egg assembly, such as discharge of vitelline droplets, transport of droplets to the ootype wall, coalescence of the droplets to form the shell, partial hardening of the shell and emptying and filling of the ootype. In ootypes of small volume, like those of many digeneans, vitelline droplets are likely to reach the ootype wall as a result of ootype movements more quickly than in parasites with a large ootype. It remains to be seen how a monogenean such as *P. integerrimum* with a relatively large egg is able to achieve an egg production rate of about 1 egg every 30 s.

It is even more difficult to imagine how such savings could achieve a reduction in egg assembly time to 4 s as claimed for fasciolid digeneans. One possibility is that their egg assembly process is specialized and differs in some aspects from that of monogeneans and possibly from that of some other digeneans. In fact, as long ago as 1906, Henneguy (1906) expressed the

opinion that the eggshell of *Distomum hepaticum* (= *Fasciola hepatica*) is laid down in the proximal part of the uterus, and he contrasted this with the situation in monogeneans in which eggs are made one at a time in the ootype. Gönnert (1962) also believed that the coalescence of vitelline globules to form the shell is not completed in *F. hepatica* until after the egg has entered the uterus. The completion of eggshell assembly in the uterus has since been reported in some other digeneans by Ebrahimzadeh (1966), and he made a distinction between these parasites and those digeneans in which eggshell assembly is completed before the egg leaves the ootype. The completion of eggshell deposition in the uterus has also been described by Löser (1965b) in some tapeworms that assemble tanned eggs. Premature expulsion of the egg from the ootype in digeneans such as *F. hepatica* might permit some increase in egg production in a system with a single ootype and might contribute to the high egg output of this animal. Egg production rates as high as those claimed for *Fasciola* or even higher could be achieved by the alternative strategy of replication of ootypes, which appears to have been adopted by ancestral cestodes (Llewellyn, 1965).

B. SEASONAL CHANGES AND CONTROLLING FACTORS

At moderate depths in the sea, even in temperate regions, the annual range of environmental temperatures may be relatively narrow. For example, the annual range of bottom temperature at a depth of about 70 m at Hydrographic Station E1 near Plymouth, England, is 9–14°C (Maddock and Swann, 1977). The common sole (*Solea solea*) is found at these depths and it is likely that egg output by *Entobdella soleae* continues throughout the year in the natural environment, since egg production, egg development and hatching proceed at these temperatures in the laboratory (Kearn, unpublished observation). Although small seasonal changes in egg output seem likely to occur in *E. soleae*, direct evidence of this is not easy to obtain. Finlayson (1982) found little difference in the percentage of egg-laying specimens of the marine monogenean *Kuhnia scombri* in seasonal samples taken in October, January, April and July.

Sproston (1946) introduced the idea that *Kuhnia scombri* undergoes an alternation of sexual phases, including at least two female phases, during its lifetime but Finlayson (1982) has found no evidence for this phenomenon.

Opportunities for host infection may be favourable all the year round in some marine monogeneans (possibly in *E. soleae*) but events such as annual host migrations may create circumstances which are highly unfavourable for infection, although, if such movements are concerned with spawning, then aggregations of hosts at the end of migration may provide especially

favourable conditions for infection. There is evidence of strong cyclical changes in egg production rates in some marine monogeneans, periods of maximum egg output apparently occurring at times when opportunities for host infection are particularly favourable. For example, Bychowsky (1957) reported intensive egg production in *Microcotyle gotoi*, coinciding with seasonal inshore spawning migrations of the host.

An adaptation to a seasonal change in the feeding habits of the host has been reported by Llewellyn (1962). The scad (*Trachurus trachurus*) leaves the bottom in the summer in pursuit of pelagic food organisms. The gill parasites *Gastrocotyle trachuri* and *Pseudaxine trachuri* cease to infect bottom-living scad prior to the summer feeding migration, but it is not known whether this is brought about by an anticipatory shutdown of egg output or by the induction of a state of diapause in the eggs.

In neither of these examples in which there might be a relationship between seasonal changes in parasite egg output and migratory habits of the host is there any evidence to indicate what factor (or factors) controls egg output. However, in *Protoancyrocephalus strelkowi*, Bychowsky (1957) found evidence for intensive egg output over a short period of time in the summer months, and this parasite is almost entirely confined to young hosts (*Limanda aspera*) living in the littoral zone where seasonal temperature change in the sea might be significant. Temperature changes seem less likely to influence parasites living at greater depths in the sea.

The temperatures of bodies of fresh water in tropical and sub-tropical environments may remain high throughout the year and may show little annual variation. Seasonal fluctuations of egg output may be small in these circumstances. Thurston (1964) collected the fully aquatic toad *Xenopus mülleri*, infected with *Protopolystoma xenopi*, from a fish farm near Kampala, Uganda and claimed that the parasites were shedding eggs at all times of the year. However, there is always the danger that parasites that are not producing eggs in the natural environment may be stimulated to do so when they are removed from the natural environment to the laboratory.

Situations may arise in these warmer latitudes that do not favour continuous infection of hosts, a striking example of this being the restriction imposed upon the breeding period of spadefoot toads by their harsh desert environment. The brief summer rains create temporary pools which provide the opportunity for hosts to breed and for their polystomatid parasites to lay eggs and infect new hosts (Tinsley, 1983; Tinsley and Earle, 1983). However, this breeding opportunity is so brief (1–3 nights in the year) and so unpredictable that there would be insufficient time for a monogenean to mobilize an inactive egg assembly line and for the eggs produced to develop, hatch and successfully invade the host. These polystomes have adopted an alternative strategy whereby eggs are assembled and stockpiled inside the

uterus, presumably during the period of time spent underground by the hibernating toads; these eggs complete their development *in utero* and are ready to hatch immediately when they enter water. A similar strategy appears to be adopted by *Eupolystoma anterorchis*; the tropical toad host has an annual breeding season but the parasites appear to produce eggs throughout the year, storing eggs in the large uterus if there is no opportunity to deposit them in water (Tinsley, 1978).

The temperate freshwater environment may experience a wide annual range of temperature. Seasonal patterns of population change in monogeneans parasitizing freshwater fishes have been thoroughly documented by Chubb (1977), who demonstrated that many parasites are scarce in the winter and show peaks of abundance in the summer months. Such a pattern might be expected if seasonal temperature fluctuations are largely responsible, with egg output increasing with temperature. Since many freshwater fishes spawn in the spring or early summer this provides an opportunity for infection of a new generation of hosts. However, some parasitologists have challenged the concept that seasonal temperature changes are the only factors responsible for spring and summer growth of monogenean populations. For example, Mashtakov (1977) showed that certain species of *Dactylogyrus* increased in numbers in the spring of two consecutive years in which the spring temperatures were widely different. Mashtakov (1977) noted the correspondence between the spring increase of dactylogyrids and the pre-spawning and spawning periods of the host, and suggested that parasite egg production might be stimulated by host hormonal activity. This suggestion was challenged by Kashkovskii (1982). He observed a decrease in numbers of *D. amphibothrium* in the host pre-spawning period and found that parasite egg production was not stimulated by injection of hosts with hypophysis extract. In addition, reports of delays in infection peaks when conditions in spring and summer are cold have been given by Rumyantsev (1972).

Zeller (quoted by Bychowsky, 1957, p. 112) believed that the reproductive system of *D. paradoxum* is not functional in the winter and that the reproductive products are not fully developed. On the contrary, Bychowsky (1957) found the reproductive system of *D. paradoxum* to be fully developed in the winter, requiring no period of accelerated development of reproductive products in the spring. This is confirmed by the observation of Bovet (1967) that, when infected hosts were captured in December and maintained in an aquarium at 20°C, their parasites commenced egg production after 48 h.

Bauer (1959) reported an apparently paradoxical situation in *Diclybothrium armatum* in which the main period of infection of its sturgeon hosts falls in the cold winter months. Bauer (1959) attributes this phenomenon to

the habits of the hosts, which, when the temperature drops in autumn, accumulate in large numbers in depressions in the river bed, creating highly favourable conditions for infection, even though egg production and development are probably slow because of the low winter temperatures. Other freshwater monogeneans have been reported to have seasonal peaks of abundance corresponding with periods of low environmental temperatures, for example in ancyrocephalines studied in the USA by Rawson and Rogers (1972) and in *Dactylogyrus legionensis* studied in Spain by Gonzalez-Lanza and Alvarez-Pellitero (1982), but the reasons for these fluctuations are unknown.

Parasites of the genus *Polystoma* provide striking examples of severe seasonal limitation of egg production, but this period of egg output coincides not with the season when environmental temperatures are at their height, but with the brief spawning period spent in water by the otherwise terrestrial anuran hosts. Moreover, Gallien (1933, 1935) showed that the reproductive organs of *P. integerrimum* from the bladder of *Rana temporaria* undergo striking seasonal changes, and these observations were confirmed and extended by Williams (1960a). The striking correlation between the phases of the reproductive biology of host and parasite have attracted comment from Gallien (1935) and later from Combes (1968). Not only is there coincidence between egg-laying periods of host and parasite but there is also correspondence between the periods of recession and regeneration of the reproductive system. Combes pointed out that, although there are latitudinal differences in the dates of the host spawning period, egg-laying in *P. integerrimum* corresponds in general with these dates. Moreover, sexual maturity in both host and parasite is attained at the same time, at an age of 3 years.

The frog *Pelobates cultripes* may have two egg-laying periods during the year, the first in spring and the other in autumn, and Combes (1968) made the interesting discovery that its monogenean parasite *Polystoma pelobatis* also has two egg-laying periods corresponding with these dates. However, in years of prolonged drought, the ponds in Combes' (1968) study area contain no water and the frogs do not undergo autumnal spawning. Combes (1968) showed that in spite of this the frogs and polystomes are mature and if the frogs are placed in contact with water at 20°C the polystomes begin to produce eggs, suggesting that there is a factor other than temperature that stimulates egg production.

The close correlation between the reproductive biologies of anuran hosts and their polystomatid parasites argues strongly for some kind of regulation of parasite reproduction by the host, and Gallien (1935) was the first to suggest that host hormonal changes may play a part in this regulation, the blood-feeding habits of the parasite offering a means of access to this

information. Miretski (1951) offered experimental evidence in support of this suggestion, claiming to have stimulated ovulation in *R. temporaria* and egg production in *P. integerrimum* by administering hypophysis extract or whole hypophyses to frogs collected while in hibernation in February. On the other hand, similar work conducted by Gorshkov (1964) suggested that the warmth of the laboratory was sufficient to precipitate polystome egg production, and these results led Gorshkov (1964) to reject the notion that host hormones have an influence on the reproductive cycle of polystomes. However, Combes (1968) was unable to accept this conclusion, pointing out that Gorshkov (1964) had demonstrated the role of warmth in the release of eggs and the uncertain action of hypophysial injection, but had failed to demonstrate that the frog's hormonal cycle had no influence on gametogenesis in the parasite.

The work of Stunkard (1959) is sometimes quoted (for example by Combes (1968)) as further evidence for control of egg-laying in polystomes by host hormones. However, Stunkard (1959) studied only a single specimen of *Polystoma stellai* and claimed to have induced egg production in the parasite and spawning in the host, *Hyla septentrionalis*, by implanting hypophyses from *Rana pipiens* in the dorsal lymphatic sacs of *Hyla*. In the absence of controls, this isolated experiment cannot be accepted as evidence of hormonal inducement of sexual maturity in the parasite.

Tinsley (1978) observed that egg production continued in the laboratory in *Eupolystoma anterorchis* even when the gonads of the host regressed, supporting field observations (see above) indicating that there is no coupling between the host reproductive cycle and egg production in this parasite.

VI. The Chemistry, Structure and Role of the Eggshell

Smyth and Clegg (1959) reviewed the evidence suggesting that the freshly formed eggshell of many "trematodes" (monogeneans and digeneans) consists of protein that undergoes a process of "tanning" or sclerotization leading to a gradual strengthening and darkening of the shell. They assembled evidence that the proteinaceous raw material of the shell and the precursors responsible for sclerotization are located in the vitelline cell globules. Tanned eggs (or cocoons) also occur in some cestodes and in turbellarians (Llewellyn, 1965). As pointed out by Barrington (1979, p. 182) the process of sclerotization is widespread in invertebrates, occurring in such diverse structures as the cuticle of insects, the chaetae of annelid worms and the byssus threads of *Mytilus*.

Smyth and Clegg (1959) followed proposals stemming from the work of Pryor (1940) on the cockroach ootheca and suggested that phenolic material

in the newly formed eggshell is enzymatically oxidized to quinone which then forms cross-linkages between the adjacent protein molecules. This concept of quinone-tanning has been widely accepted as an explanation of the sclerotization process in insect cuticle and in platyhelminth eggshells. However, the work of Vincent and Hillerton (1979) has thrown serious doubt on the basic concept of the sclerotization process in insects. Their experimental evidence suggests that covalent cross-linking, even if it exists in insect cuticle, is insufficient to account for the degree of stiffening of the cuticle occurring during sclerotization and that dehydration of the protein matrix, brought about in some way by the introduction of quinones, could account for the stiffness and insolubility of "tanned" cuticle in the complete absence of covalent cross-links. In the light of this evidence there is a need for a thorough re-examination of the mechanism of sclerotization in platyhelminth eggshells.

Whatever the mechanism of sclerotization may be, there is abundant histochemical evidence that the vitelline cell globules and the freshly formed eggshells of monogeneans contain phenolic substances and a phenolase involved in their oxidation. Positive results with the diazotechnique for phenolic substances and the catechol technique for phenolase (tests introduced by Johri and Smyth (1956)) have been obtained with many monogeneans, including *Gastrocotyle trachuri* (see Freeman and Llewellyn, 1958), *Entobdella soleae* and *Diclidophora luscae* (see Llewellyn, 1965), and *Oögyrodactylus farlowellae* (see Harris, 1983).

Smyth and Clegg (1959) pointed out that the red colour produced by incubation in catechol is localized in the shell globules, indicating that the phenolase actually occurs in the same globule as its substrate. They suggested that some sort of blocking system may be present, preventing premature sclerotization in the droplets before they coalesce to form the shell. This idea was taken up by Ramalingam (1970b) who claimed to have evidence that phenolase exists as an inactive precursor (prophenolase) in the vitellaria of monogeneans belonging to the genera *Pricea* and *Protomicrocotyle*. A similar claim was made for *Dionchus remorae* by Ramasamy (1984). Ramalingam (1970a, 1971a) also suggested that the phenolic material in the vitellaria of *Pricea multae* is masked by sulphated acid mucopolysaccharide. Premature interaction of components in *P. multae* is said to be further prevented by substrate specificity of the enzyme (Ramalingam, 1970a, 1971b) but Ramasamy (1984) claimed that the phenolase of *D. remorae* is not substrate-specific.

Ramalingam (1970b) reported that the vitellaria of *Pricea* and *Protomicrocotyle* failed to stain with catechol in the living animal or after treatment for 15 min with 5% neutral formalin, but stained well when living or formalin-preserved (15 min treatment) specimens were pretreated

either by immersion for 30 min in 0.2% sodium oleate or by inflicting mechanical damage. Ramalingam (1970b) found that *Pricea* and *Protomicrocotyle* also stained well after storage in 5% formalin or in 70% ethanol for 24 h. He interpreted these observations as evidence that the enzyme phenolase is in an inactive state in the vitellaria and that activation of the prophenolase is brought about by sodium oleate, by mechanical injury or by prolonged treatment with preservatives. He also pointed out that, in living parasites and in those preserved for 15 min in formalin, the eggshell in the uterus and shell precursors released from vitelline cells that had passed through the ootype stained positively after catechol treatment, and this was regarded as evidence for the role of ootype glands in the activation of prophenolase. However, catechol may fail to penetrate the living tegument or the tissues of briefly preserved parasites; trauma will permit direct access of catechol to vitelline tissue and catechol may penetrate more readily after treatment with detergents such as sodium oleate or after prolonged exposure to preservatives. Since the lumen of the uterus has direct access to the bathing medium, catechol may reach eggshells and other material in the lumen via the genital opening of living or briefly preserved parasites.

The evidence for masking of phenolic substances by acid mucopolysaccharides and for the liberation of phenolic substances from acid mucopolysaccharides by ootype gland secretion as suggested by Ramalingam (1970a, 1971a) is also unconvincing, being based on histochemical evidence for the presence of acid mucopolysaccharide in vitelline droplets and in vitelline cells "remaining as remnants in the proximal part of the uterus" (Ramalingam, 1971a).

Preliminary TEM observations by Kearn (unpublished) on the eggshell of *E. soleae* indicate that it is homogeneous, and in this respect it resembles the eggshell of the digenean *Fasciola hepatica* described by Wilson (1967). The opercular discontinuity has not yet been studied with TEM in monogeneans and Wilson (1967) failed to find it in TEM sections of the *F. hepatica* egg. In view of the suggestion made above (Section IV C p. 198) that the opercular discontinuity in the shell might represent shell protein that has not been sclerotized, it is of particular interest to determine with TEM whether or not the opercular cement is structurally different from the adjacent shell or whether there is structural similarity or even continuity.

There have been no studies of the permeability of the eggshell of monogeneans comparable with that of Wilson (1967) on *F. hepatica*. Wilson (1967) described a "vitelline membrane complex" underlying the eggshell of *F. hepatica*. Such a complex was not observed in the preliminary TEM work on the eggshell of *E. soleae* mentioned above, but this observation requires confirmation, since damage inflicted on the egg to permit the entry of preservative may have destroyed delicate layers underlying the shell.

The hatching that follows the addition of substances such as urea to eggs of some monogeneans (Section XI B 3) suggests that the shell or the opercular cement layer are freely permeable to small molecules. Kearn and Macdonald (1976) pointed out that the size of these molecules is such that specialized pores or channels in the shell, at the microscopic or ultrastructural levels, would be unnecessary to facilitate their passage, but the regular pits in the shell of *E. soleae* (Fig. 38a) may aid the rapid influx of small molecules by reducing the thickness of shell that must be penetrated. Paperna (1963a) found that the eggs of *Dactylogyrus vastator* will develop at a wide range of salinities but that oncomiracidia die soon after hatching at higher salinities, indicating that the eggshell (or possibly an underlying "vitelline membrane complex", if one exists) serves to protect the developing embryo and unhatched larva from detrimental osmotic effects.

One of the consequences of sclerotization is that the eggshells gradually darken, becoming yellow or brown. In spite of this, the eggshells of monogeneans are sufficiently translucent to permit the larva to be seen inside, and this translucent property is probably important because many monogenean larvae are either stimulated to hatch by changes in light intensity or have hatching rhythms that are controlled by the photoperiod (Section XI B).

The insolubility of tanned protein and its resistance to solvents are well known (Vincent and Hillerton, 1979). Llewellyn (1965) has shown that tanned platyhelminth eggshells are resistant to digestion by pancreatin. He pointed out that the relatively small eggs of parasitic platyhelminths are known to be eaten by microphagous animals and that a digestion-resistant shell would permit the eggs to pass unharmed through the gut of the predator. Kearn (1975a) found that the opercular cement of *E. soleae*, like the eggshell, is resistant to the attack of proteolytic and other enzymes applied to the outside of the egg. Paul (1938) observed in a laboratory situation that the eggs of *Polystoma integerrimum nearcticum* were occasionally swallowed by the tadpole host, but these eggs failed to develop after washing and incubation. On the other hand, Macdonald (unpublished observation quoted by Kearn (1975a)) found that eggs of *E. soleae* survived passage through the gut of various crustaceans, provided that the eggs were not damaged by the jaws of the predator, and, moreover, intact eggs subsequently produced oncomiracidia. Macdonald and Jones (1978) found that eggs of *Diplozoon homoion gracile* were able to continue their development and hatch after passing through the gut of their fish host, *Barbus meridionalis*.

It seems likely that some monogenean eggs habitually pass through the gut of their hosts. Llewellyn (1965) found eggs of what he regarded as a dactylogyrid monogenean in the intestine and in the faeces of the fish *Sebastes madurensis* and discovered the adult parasites inhabiting the

oesophagus of the host. The eggs of other gut-inhabiting monogeneans are likely to pass through all or part of the alimentary canals of their fish hosts. The ancyrocephaline *Enterogyrus cichlidarum* is known to inhabit the intestine (Paperna 1963b) and *E. papernai* the stomach (Gussev and Fernando 1973). *Montchadskyella intestinale*, described by Bychowsky *et al.* (1970), also occurs in the intestine and was regarded by these authors as having affinities with the monocotylids. Thus, the resistance of eggshells and opercular cement to attack by digestive enzymes must have been an important pre-adaptation that has made possible the colonization of the gut of fishes on more than one occasion. Moreover, Llewellyn (1965) suggested that a much older invasion of the gut of fishes by early monogeneans may have given rise to the gyrocotylideans and possibly also to the cestodes.

There has been a strong tendency in some monogeneans, notably in polystomatids, to develop ovoviviparity (Section VII C 2), and Tinsley (1983) has considered the possibility that embryos developing inside eggs retained in the uterus may receive supplies of nutrients through the shell from the parent. The eggs of polystomes such as *Pseudodiplorchis americanus* increase in size during development *in utero*. These eggs have little discernible vitelline content and the eggshell is thin and membraneous, and closely invests the contents of the newly formed egg, but expands to accommodate a large oncomiracidium (Section II p. 180). This implies that nutrients pass from the parent through the shell to the developing embryo within. The physical properties of these eggshells are strikingly different from those of the relatively rigid eggs of parasites like *E. soleae*, and may well reflect differences in eggshell chemistry that await further investigation. A further point is that the eggs of these polystomatid monogeneans may be retained in the uterus for long periods (over 6 months in *P. americanus*, see Section VII C 2 p. 230), until such time as external conditions are suitable for egg-laying. It is possible that fully developed larvae within the eggs may be supplied with nutrients via the shell during this period of prolonged storage.

Most gyrodactylideans have taken these developments further, since the eggshell appears to have been entirely lost and the embryo grows extensively in the uterus (or possibly in the ootype, see Section IV E) and reaches adulthood before birth (see also Section VII C 2 and Fig. 43d).

VII. The Fate of the Eggs

A. General considerations

Llewellyn (1965) has suggested that ancestral monogeneans parasitized slow-moving, bottom-dwelling, fish-like vertebrates. Many monogeneans

still parasitize bottom-dwelling fish hosts, but there has been a progressive tendency for fishes to colonize aquatic living space above the bottom and this trend has, in some instances, been associated with increases in swimming speeds and/or with the adoption of vertical and horizontal migrations. It is remarkable that the monogenean life cycle, based on a freely deposited egg and a relatively slow-swimming, ciliated larva, has shown sufficient adaptability and versatility to cope with the epipelagic, fast-swimming and sometimes migratory habits of hosts such as tunny, mackerel and squid. Moreover, although scarce, macrovalvitrematid monogeneans have been reported from mesopelagic fishes (Noble and Collard, 1970) and some other monogeneans have maintained a foothold on amphibian hosts, even those with infrequent and tenuous ties with water.

The freely deposited, passive egg, drifting at the mercy of currents, seems to be a particularly weak link in the transmission of monogeneans between active hosts. Gyrodactylid monogeneans have strengthened this weak link by suppressing egg-laying, acquiring a viviparous habit and relying on direct transfer of parasites from host to host, as for example in the genus *Isancistrum* parasitizing pelagic squid hosts (Llewellyn, 1984). Another way in which this weak link could be strengthened is by retention of eggs on the body of the host or by the parasite itself. A considerable body of evidence suggests that the former is a rare phenomenon (Llewellyn, 1957b, 1972) but the latter situation is not uncommon (see below).

Egg retention, either by the attachment of eggs to the outside of the parasite's body or by detaining them in the ootype or uterus, may be the consequence of a need to release eggs at specific times; such a periodic release might be favoured by the right environmental conditions or by situations where the host is vulnerable because it is resting or aggregated.

Egg retention also permits infection of the host of the parent parasite (auto-infection). According to Llewellyn (1981) the survival value of this phenomenon appears to be that of increasing the reproductive capacity of the parasite, since auto-infection is likely to be less hazardous than leaving the host to find a new one, but auto-infection also presents some problems. Llewellyn pointed out that, unless mortality on the host is abnormally high, this would lead to the build-up of a large and multiplying population. Such a build-up of parasites, if unchecked, would seem likely to have fatal consequences for host and parasites. Tinsley (1983) considered that the increased chances of inbreeding, which is a further consequence of auto-infection, may not necessarily be disadvantageous, and Llewellyn (1981) has suggested that mating between siblings might lead to the conservation of highly specialized characters.

B. FREELY DEPOSITED EGGS

There have been several studies of individual monogenean species that throw light on the fate of the eggs after they leave the host.

The eggs of the skin parasite *E. soleae* have appendages with sticky droplets (Fig. 29) but in spite of this there is no evidence that the eggs adhere to the bodies of their bottom-dwelling host, the common sole (Kearn, 1963a). On the other hand, a search of shell sand from the bottoms of tanks containing infected soles revealed eggs in groups of two or three attached to sand particles by their sticky droplets.

In addition to *E. soleae* there are other parasites that lay eggs with adhesive material either on the appendage, as in *A. lobianchi* (see Kearn, 1967) and *Entobdella australis* (see Fig. 27 and Kearn (1978b)), or on the egg surfaces, as in *Calicotyle kröyeri* (see Kearn, 1970a). All of these parasites are found on bottom-living, marine, teleost or elasmobranch flat-fishes, and sticky material may be of great importance for such parasites since attached sand particles would prevent the eggs being carried upwards, except by the strongest water currents, and would lead to rapid sedimentation of current-transported eggs when the current slackens. Turbulence might keep unweighted eggs in suspension where they could be carried away horizontally to areas of sea bottom not inhabited by their hosts.

The attachment of ballast to the eggs of parasites such as *E. soleae*, reducing the chances of such eggs being swept off the bottom by currents, is likely to favour prolonged close contact between the eggs and their hosts, and this provides an opportunity for the oncomiracidia to respond to chemical hatching factors originating from the host's body (Section XI B 3). Furthermore, the weighted eggs of *E. soleae* readily become buried in the sediment if it is disturbed by gentle shaking in the laboratory or, presumably, by currents in the sea (Kearn, 1980). Such eggs may make contact with the buried skin surfaces of soles which spend much time during the daylight hours concealed almost entirely in the sand (Kearn, 1971a). In *E. soleae* the oncomiracidium is free-swimming, and oncomiracidia hatching from eggs buried in the sediment are perhaps less likely to be swept away from any nearby potential hosts by bottom currents before they can attach themselves. Kearn (1980) has shown that hatching does take place from eggs just beneath the surface of the sediment and that such larvae are able to negotiate the channels between adjacent sand particles.

The eggs of the freshwater parasite *Discocotyle sagittata* have no appendages or adhesive material. Paling (1965) was of the opinion that the eggs would be carried downstream by water currents and deposited where the water is slow-moving. However, in his study area, he found that lake trout were infected whereas young trout in the tributary streams were rarely

parasitized, and there is no evidence that eggs are laid in the tributary streams. Thomas (1964) studied *D. sagittata* on trout living in rivers flowing into the sea. Eggs laid by parasites on these river trout are likely to be subjected to water currents, and Thomas (1964) made the interesting observation that the heaviest infections of the parasite occur in stretches of river having a sedimentary bottom; conditions favouring sedimentation of mineral particles would also favour settling of eggs.

Baer and Euzet (1961) were of the opinion that those eggs with long appendages that become entangled to form bunches would float in the sea but, so far, all the monogenean eggs that have been studied appear to be denser than sea water (Llewellyn, 1962; Kearn, 1963c; Paling, 1965; Bovet, 1967). Euzet (in Llewellyn, 1957b) reported finding monogenean eggs in the plankton near Sète on the Mediterranean coast of France, and Llewellyn (1972) has found eggs of *Kuhnia scombri* in the plankton at Plymouth, England, but it is not known how much time these eggs had spent in the plankton or for how long they remain at this level.

The spindle-shaped eggs of *K. scombri* have a needle-like, inflexible appendage at each end, and similar appendages project from each of the four corners of the tetrahedral eggs laid by capsalid monogeneans parasitizing epipelagic hosts such as sunfish and tunny (Euzet, 1958; Kearn, 1963c; Yamaguti, 1968). Such appendages increase the surface area of the eggs without adding appreciably to their mass and probably serve to reduce sinking rates. Similar structures are characteristic of most planktonic organisms, including marine invertebrate larvae (see review by Chia *et al.* (1984)). This retardation of sinking combined with water turbulence might keep such eggs in suspension for long periods of time. However, Llewellyn (1962) has presented evidence indicating that invasion of hosts by the oncomiracidia of *Pseudaxine trachuri* and *Gastrocotyle trachuri*, both of which produce eggs with needle-like appendages (Figs. 22, 23), takes place when the host is at the sea bottom. He calculated that, if eggs were laid at the surface off Plymouth, England, they would reach the bottom before embryonic development was complete. Moreover, eggs with needle-like appendages are laid by *Octoplectanum* (= *Neoheterobothrium*) *affine*, but this polyopisthocotylean parasitizes a teleost flat-fish (Linton, 1898). Needle-like appendages are discussed further in Section VIII p. 233.

Bovet (1967) observed that, in an aquarium containing the freshwater fish *Abramis brama* infected with *Diplozoon paradoxum*, some of the eggs of the parasite became trapped in the surface film and were difficult to reimmerse. He believed that eggs trapped in this way in the natural environment could be carried some distance by wind and he found that these surface-bound eggs would develop in the laboratory provided that a coat of bacteria did not develop upon them. It remains to be seen whether eggs laid in the natural

environment are transported in this way. According to Wheeler (1978), *A. brama* feeds on the river bed where opportunities for *Diplozoon* eggs to make contact with the surface would depend on transport of eggs by vertical currents. However, schools of *A. brama* are also said to feed in shallow water at night, often with their tails "dimpling" the surface as they move, and in this situation contact of freshly laid eggs with the surface may occur.

The eggs of some monogeneans, including those of *D. paradoxum* (Fig. 31a), have appendages that are long, slender and flexible. According to Bovet (1967) the coiled egg appendages of *D. paradoxum* are between 20 and 25 mm in length. Bovet (1959) observed that the coiled appendage uncoils during laying and becomes entangled with the appendages of other eggs (Fig. 31b) so that most of the eggs are held off the bottom. Bovet claimed that this is an advantage, a high proportion of eggs in such tangled heaps developing normally compared with eggs without appendages which rest on the bottom and become rapidly covered with micro-organisms or with detritus. However, micro-organisms may not develop to the same extent on the surfaces of eggs of *D. paradoxum* in the natural environment, and there are eggs of other monogeneans (e.g. *E. soleae*, see above) that undergo successful development while attached to sand particles at the bottom or even buried in the sediment.

A slender, spirally coiled appendage like that of *D. paradoxum* may have other functions. It is possible that such an appendage may serve to attach eggs to the substrate if suitable projections, such as plant material or hydroid colonies in the sea, were available to snare the appendages of eggs drifting in the current. Another possibility that has not been widely explored is that excessively long, slender appendages may promote the lifting of eggs off the bottom by water turbulence, in the same way that young spiders are carried aloft by the interaction of air currents with long threads of silk produced by the spinnerets. Such long appendages would also increase frictional resistance to sinking, so prolonging the time spent by the eggs in suspension. Turbulence sufficient to lift the eggs could be provided by the movements of host fishes swimming near the bottom, the advantage being that eggs containing fully developed larvae would be brought into close proximity to potential hosts. Similar events may facilitate infection of the dogfish, *Scyliorhinus canicula*, by the microbothriid skin parasite *Leptocotyle minor*. The eggs laid by this parasite each have a long, slender appendage, and sinking eggs are susceptible to even minor turbulence such as convection currents (Mr. I. Whittington, personal communication). Hatching in these eggs appears to depend on stimulation by a chemical factor from the host (Section XI B 3 p. 256) so that contact of the eggs with the host is probably advantageous if not essential.

In many monogeneans, eggs may be laid not singly but in bunches. In

Diclidophora luscae and *D. denticulata* the long, slender abopercular appendages become entangled in the uterus before laying (Fig. 34). Each of these eggs has a shorter, stronger, hook-shaped opercular appendage which is not attached to those of its neighbours but is free; these hooked appendages may serve to anchor the egg bunch as it is swept along the bottom to the algal mat or to hydroid or bryozoan colonies that encrust rocky surfaces.

Mr. I. Whittington (personal communication) has observed that the egg bunches of *D. luscae* sink more rapidly than single eggs removed from the bunch with as much of their abopercular appendage as possible. This may be related to the hydrodynamic properties of the relatively compact egg bunch such that frictional resistance to sinking is less than for the single egg.

Egg bunches bearing a striking similarity to those of *Diclidophora* are assembled by some species of the genus *Microcotyle* (for example by *M. gotoi*; see Fig. 24). However, in *Microcotyle*, the long, coiled appendage arises from the opercular pole and the hooked appendage from the abopercular pole. This seems to be a striking example of convergent evolution, comparable with the convergence between hexabothriid and mazocraeidean monogeneans in the phenomenon of egg-chain formation (see below).

Ktari (1969) suggested that the egg bunches of *M. salpae*, in which the free abopercular appendages terminate in a small swelling rather than a hook, may be drawn into the buccal cavity of the host (*Box salpa*) in the respiratory current or may enter the buccal cavity attached to the algae on which the host feeds. Inside the buccal cavity, specific chemical factors and mechanical stimulation induce hatching (Section XI B 3 and 4).

The further significance of this habit of laying bunches of eggs is discussed elsewhere (Section IX).

The long chains of eggs produced by certain hexabothriids (see Fig. 33), in which eggs are joined together by fusion of the opercular and abopercular appendages, may become entangled around projections on the sea bottom. According to Guberlet (1933) chains of between 10 and 65 eggs are produced by *Squalonchocotyle catenulata* and egg chains were observed by Dillon and Hargis (1968) in *Erpocotyle callorhynchi*. Egg chains of an unidentified hexabothriid from *Torpedo marmorata* were disentangled and found to measure as much as 5 cm in length (Kearn, unpublished observation). Some egg chains may exceed this length since they readily break when attempts are made to disentangle them.

Similar egg chains have been reported from some mazocraeideans, namely from *Microcotyle caudata* by Bychowsky (1957, p. 90) and from *Rhinecotyle crepitacula* by Euzet and Wahl (1970). This feature may have arisen as a perturbation in the timing of assembly of consecutive eggs such that the abopercular appendage of the first-formed egg is still sufficiently soft

to fuse with the newly formed opercular appendage of the next egg. Selective advantages of egg chains may then have led to incorporation of this phenomenon into the egg assembly process. These evolutionary events may have taken place independently in the hexabothriids and in the mazocraeideans, and possibly more than once in each of these groups.

Llewellyn (1965) recovered specimens of a dactylogyrid from the oesophagus of *Sebastes madurensis* and found eggs of the parasite in the intestine and in the faeces of the host. Unless the faecal matter is rapidly dispersed, it seems likely that eggs of these parasites and of other gut-inhabiting monogeneans (Section VI p. 218) undergo development in a faecal environment rich in bacteria and possibly low in oxygen.

C. EGG RETENTION PERMITTING EMBRYONIC DEVELOPMENT

1. *Attachment of eggs to the host*

There have been some specific references to the retention of monogenean eggs by attachment to the body of the host, but most of these have not stood up to closer examination.

There appear to be two well authenticated examples of eggs being attached to the host. Bychowsky (1957, p. 91) observed that the eggs of *Nitzschia sturionis* are firmly cemented by the appendages to the mucous membrane of the buccal cavity of the sturgeon. Hargis (1955) and Ktari (1971, 1977) found that the eggs of *Dionchus* spp. have appendages that are entwined with those of other eggs to form grape-like bunches which are attached to a loop of what appears to be shell material encircling the gill filament of the respective host (Fig. 42a). How the egg loop of *Dionchus* is made and implanted is not entirely clear. The shark-sucker hosts have the habit of attaching themselves to a shark; the day-to-day movements of the shark-sucker will be dictated by the "carrier" and if *Dionchus* eggs were shed into the water their chances of infecting a shark-sucker would seem remote. Ktari (1977) observed that some of the eggs in the bunch contained fully developed, unciliated larvae which would be able to infect the host of the parent parasite or possibly migrate to the skin of the carrier where they would be available to colonize newly arrived shark-suckers.

The loan by Dr. R. Bray (kindly arranged by Dr. D. Gibson) of permanently mounted, primary gill lamellae of *Rachycentron canadum* bearing attached egg bunches of *D. rachycentris* has permitted me to make a few additional observations on this interesting habit. Part of the loop opposite the point of attachment of the eggs is noticeably inflated and hollow (Fig. 42a) and in all five egg bunches available for study this inflated region

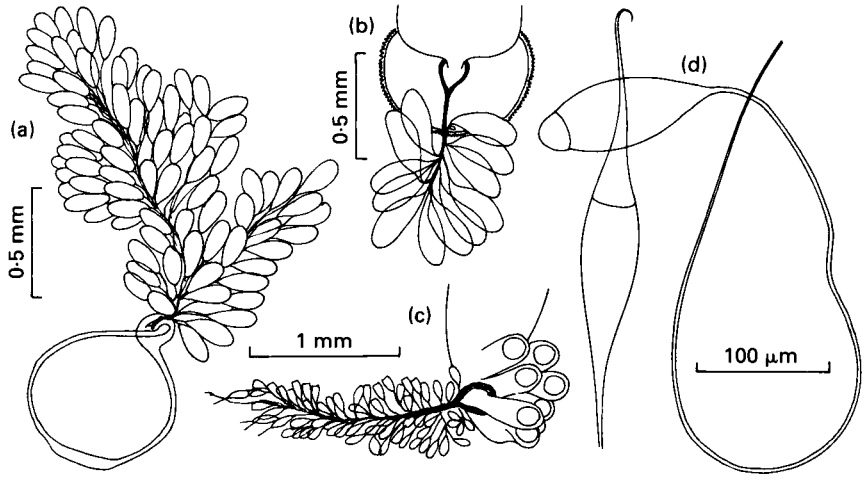


FIG. 42. Retention of eggs on the body of the host or by the parasite. (a) A bunch of eggs of *Dionchus rachycentris* anchored to a loop which serves to attach the bunch to the host primary gill lamella. Reproduced with permission from Hargis (1955) *Transactions of the American Microscopical Society* **74,** p. 215. (b) Eggs of *Pseudobenedenia shorti* attached to the peduncle joining the haptor of the parasite to the body. Reproduced with permission from Hargis and Dillon (1968) *Proceedings of the Biological Society of Washington* **81,** p. 408. (c) Egg bunch of *Choricotyle australiensis* attached to the peduncle of one of the haptor clamps. (d) Two kinds of egg of *C. australiensis*. (c) and (d) reproduced with permission from Roubal *et al.* (1983) *Australian Journal of Zoology* Supplementary Series No. 94, p. 41.

appeared to lie between adjacent secondary gill lamellae and may contribute in some way to anchorage of the egg bundle. Further examination of these preparations also indicated that part of the loop may be enclosed in gill tissue, a situation that could arise by hypertrophy of the gill epithelium, and it seems unlikely that these loops would slip off the end of the primary lamella as described by Hargis (1959). It is possible that the parasite provokes excessive growth of the gill epithelium and exploits it as a means of detaining egg bundles on the host.

Goto (1894) found that every specimen of *Tristomum* (= *Capsala*) *biparasiticum* that he examined was attached to a copepod (probably of the genus *Parapetalus*) from the gills of the tunny *Thynnus albacora*. He also observed eggs of the parasite attached by their appendages to the ventral side of the abdomen of the copepod. The recruitment of a parasitic copepod, able to swim strongly from one fish to another, would provide rapid transport of eggs and parasites from one epipelagic host to another. The relationship between this parasite and parasitic copepods has since been confirmed by

Iversen and Hoven (1958), by Yamaguti (1968) and by Sproston (1969) but none of these authors found monogenean eggs attached to copepods.

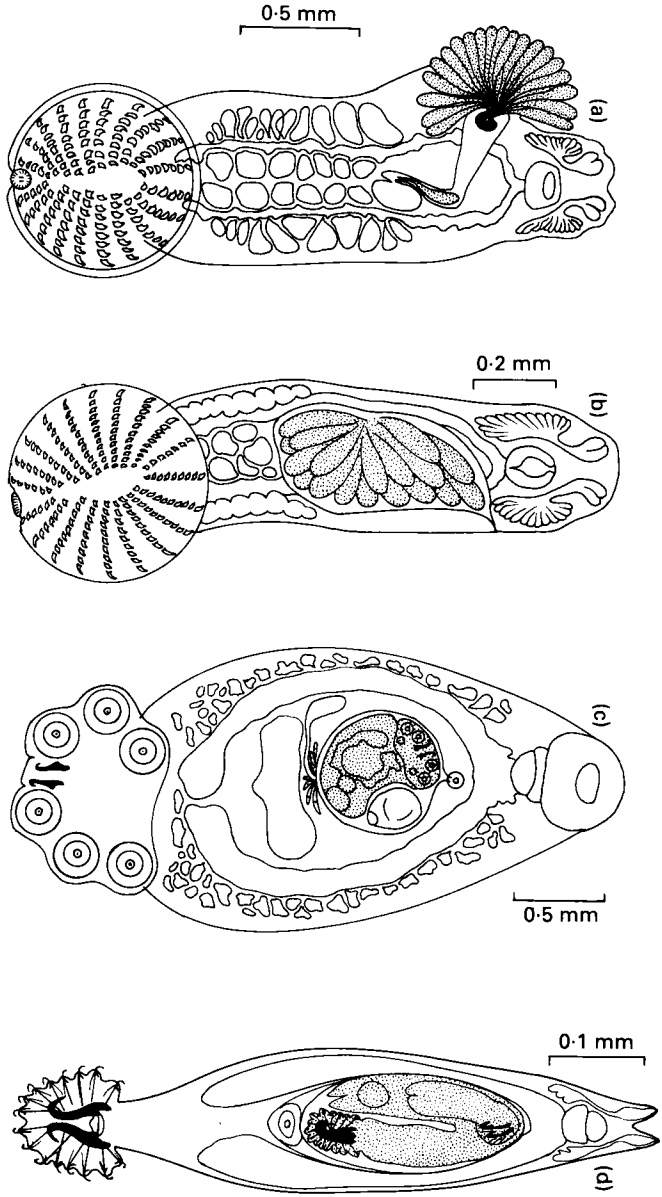
2. *Retention of eggs by the parasite*

Parona and Perugia (1895) observed that the egg appendages of *Placunella* (= *Ancyrocotyle*) *vallei* are wrapped around the peduncle joining the haptor to the body. This would seem unlikely to be accidental, since the same phenomenon has been described in other capsalids such as *Pseudobenedenia* (= *Pseudobenedenoides*) *shorti* (Fig. 42b) (Hargis and Dillon, 1968; Gibson, 1976), *Pseudobenedenoides branchicola* (see Szidat, 1969) and an undescribed benedeniine (Mr. Charles Hussey, personal communication). Moreover, a remarkably similar phenomenon was described in the polyopisthocotylean *Choricotyle australiensis* by Roubal *et al.* (1983) (Fig. 42c). In *Acanthocotyle greeni* the fused proximal discs of the appendages of up to about 80 eggs are clasped by the muscular distal region of the uterus (Macdonald and Llewellyn, 1980) (Fig. 43a).

In *P. shorti*, *P. branchicola* and *A. greeni* well developed larvae were found in some of the eggs and this would afford an opportunity for reinfection of the same host individual. Macdonald and Llewellyn (1980) found that some of the unciliated larvae of *A. greeni* hatched after treatment with host skin mucus. The way in which new hosts are infected by these parasites is not clear, but Macdonald and Llewellyn (1980) suggested that the egg bunch may be jettisoned by the parasite, so permitting younger eggs to continue their development on the sea bed, leading subsequently to infection of new hosts. Dr. K. Rohde (personal communication) observed that in some egg clusters of *C. australiensis* most eggs had lost their opercula, but this may have been due to preservation and he was unable to determine whether eggs in the attached clusters contained larvae. Dr. Rohde (personal communication) has no evidence that egg clusters can be shed by the parasite, although some individuals were found with one cluster attached to the haptor and another inside the uterus. It seems unlikely that individual eggs are able to drift away from the clusters, because all eggs are tightly held together, even in the uterus before laying, by the abopercular appendages.

A point of great interest in *C. australiensis* is that eggs of each cluster are of two types (Fig. 42d), some with and some without an additional hook-like opercular appendage. Dr. Rohde (personal communication) has observed relatively few eggs with intact opercula bearing hooked appendages, but those that he has observed are distally located in the clusters. The roles of these two kinds of egg remain to be determined.

A much more widespread method of prolonged egg retention in monogeneans is the habit of detaining eggs inside the body. This phenomenon


0·5 mm
(a)
0·2 mm
(b)
(c)
0·5 mm
0·1 mm
(d)

appears to have arisen independently several times. It occurs in gyrodactylideans (Harris, 1983), in acanthocotylids (Fig. 43b), in chimaericolid polyopisthocotyleans (Manter, 1955), in hexabothriids (Dillon and Hargis, 1968) in mazocraeideans (Ramalingam, 1969; Radha, 1975; Mamaev and Aleshkina, 1984) and in many polystomatids (Fig. 43c and see review by Tinsley (1983)).

In *Oögyrodactylus farlowellae* and in some polystomatids, for example in *Polystomoidella oblonga* (Fig. 43c), a single egg is retained in the ootype. In the former, embryonic development begins but apparently is not completed until after laying, whereas in the latter the single egg hatches inside the ootype and development is so extensive that primordia of the reproductive organs are recognizable. Harris (1983) has suggested that egg retention in *O. farlowellae* reduces the exposure time of the eggs to powerful water currents in the torrential streams inhabited by the host. In other gyrodactylideans, the eggshell appears to have been entirely lost and these viviparous parasites produce relatively large and well developed offspring (Fig. 43d).

In *Polystoma pelobatis* and in *P. integerrimum* a single egg is retained in the uterus at the end of the egg-laying season of the parasite (Combes, 1968). This egg hatches in the uterus and leads to infection of the same host (auto-infection) (Fig. 2).

Eggs can be retained in the uterus without interrupting egg assembly, and enlargement of the uterus permits the storage of large numbers of eggs in some polystomatids (up to 250 in *Pseudodiplorchis* and *Neodiplorchis*, 300 in *Eupolystoma* and in *Metapolystoma* and 350 in *Diplorchis*, according to Tinsley (1983)).

In *Metapolystoma cachani* some well developed eggs may hatch before leaving the uterus, leading to auto-infection, some may hatch soon after laying, and other less well developed eggs may continue to develop in the outside environment (Murith *et al.*, 1977).

Fournier and Combes (1979) have shown that *Eupolystoma alluaudi* produces two kinds of egg which share different fates. Some eggs hatch inside the uterus of the parasite and the emerging unciliated larvae find their way out of the uterine opening into the bladder of the toad host where they become established. Other eggs after laying are evacuated from the bladder

FIG. 43. Ovoviviparous and viviparous monogeneans. (a) *Acanthocotyle greeni.* Reproduced with permission from Macdonald and Llewellyn (1980) *Journal of the Marine Biological Association of the United Kingdom* **60,** p. 82. (b) *A. pugetensis.* From Bonham and Guberlet (1938) reproduced with permission from *American Midland Naturalist* **20,** p. 601. (c) *Polystomoidella oblonga.* From Oglesby (1961) reproduced with permission from *Journal of Parasitology* **47,** p. 243 (d) *Gyrodactylus* sp. Based on Sproston (1946) *Transactions of the Zoological Society of London* **25,** p. 195 (reproduced with permission).

in the urine; the larvae emerging from these eggs are ciliated and infect toads via the cloaca. Even more remarkable is the finding that there is some kind of density-dependent control of the ratio of eggs producing unciliated, auto-infecting larvae and those producing free-swimming, ciliated, cross-infecting larvae. Evidence of two kinds of larvae (with and without cilia) has been found also by Lambert and Kulo (1982) in *Polystomoides nabedei* from the bladder of a turtle.

The eggs of the polystomatids *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis*, which inhabit the bladders of desert-adapted toads, also complete their development *in utero* but it seems likely that these eggs spend longer *in utero* than those of any other monogenean. According to Tinsley (1983) the eggs of *Pseudodiplorchis* and *Neodiplorchis* remain *in utero* for over 6 months. Although the eggs are ready to hatch for 2–3 months, the laying of the eggs, their expulsion from the host and immediate hatching are delayed until the annual advent of the summer rains when the toad hosts emerge from hibernation and enter newly formed pools for spawning. This opportunity for the parasites to lay eggs *en masse* and for newly hatched oncomiracidia to infect new hosts via their nostrils is exceedingly brief, since male toads may visit the ponds on no more than three nights and females may spend only one night at the ponds (Tinsley and Earle, 1983; Tinsley, 1984).

VIII. The Significance of Egg Shape and Size

In spite of the surprising and fascinating diversity of egg shapes and sizes and the apparent uniqueness of the tetrahedral egg shape (Section II) there have been no previous attempts to assess the significance of shape and size in monogenean eggs.

In Section II it has been shown that monogenean egg shapes serve as a most unreliable guide to phylogenetic relationships. Eggs of markedly different shapes may be produced by different species of the same genus and the tetrahedral egg shape appears to have evolved independently in the microbothriids, monocotylids and dactylogyrideans and may well have evolved more than once in the latter. Since eggs of closely related monogenean species often develop in different environments depending on the way of life of their specific hosts, it is likely that the different shapes of these eggs are the consequence of different environmental selection pressures. The shape and size of the egg of any individual monogenean species is likely to be the result of a complex interaction of factors. The shape of the egg will determine the ratio between surface area and volume and also the minimum distance between the surface and centre, and these parameters are likely to

have a profound influence on the diffusion of gases and other materials into and out of the egg. Egg shape is also likely to influence the mechanical strength of the egg, which might be important in the event of eggs being eaten by small predators (Section VI p. 218) and in those eggs which tend to become buried in continuously shifting sandy sediment (Section VII B p. 221). Egg shapes are also likely to affect sedimentation rates of eggs, especially those laid by parasites of pelagic hosts (Section VII B p. 222).

The relationships between egg shapes and sizes and the diffusion of gases and other materials into and out of the egg have not been explored previously. It seems likely that the eggs of many monogeneans will spend some or all of their time developing on the bottom (Section VII) and since some sediments are known to be deficient in oxygen (Kearn, 1962) there may be strong selection pressure for egg shapes with high surface area/volume ratios and short distances between the surface and centre; large eggs will have more serious diffusion problems than small eggs and selection for such shapes is likely to increase with increase in egg volume. A theoretical consideration of the diffusion of gases into regular solids of various shapes will serve as a starting point for these explorations.

The area/volume ratio (A/V) and the minimum distance from surface to centre (d) have been calculated using simple geometry for various regular solids, in each of which the volume has been taken as unity. These figures are given in Table 2 and show that the highest values of A/V and the lowest values of d are achieved by the elongated spheroid and by the tetrahedron. Thus, both of these shapes would be expected to be distinctly advantageous for maintaining larger eggs at a given oxygen concentration or, conversely, for maintaining an egg of a given volume at a lower oxygen concentration.

Diffusion within tissues has been considered by Hill (1928). Assuming that the tissue is uniform, that the respiration coefficient is independent of

TABLE 2 *Geometrical and diffusional parameters of various regular solids (original data; see text for explanation)*

Parameter	Sphere	Prolate spheroid					
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 10	Tetrahedron
A/V	4.8	5.2	5.7	6.2	6.6	8.2	7.2
d	0.62	0.49	0.43	0.39	0.36	0.29	0.42
V_{max}	$62x$	$97x$	$137x$	$178x$	$220x$	$431x$	$204x$
$(C_o/Q)_{min}$	$0.064y$	$0.047y$	$0.038y$	$0.032y$	$0.027y$	$0.017y$	$0.029y$

N = elongation (ratio of major to minor axes); A/V = area to volume ratio for unit volume; d = minimum distance from surface to centre for unit volume; D = oxygen diffusion coefficient; C_o = external oxygen concentration; Q = respiratory (metabolic) coefficient; $x = (DC_o/Q)^{1.5}$; $y = V^{0.67}/D$.

oxygen concentration and that metabolism is obligate aerobic, it is possible to define a minimum distance (d) from surface to centre such that at a given external oxygen concentration (C_o), the oxygen concentration declines to zero precisely at the centre of the tissue. For a plate, cylinder or sphere of tissue, at maximum size, this minimum distance is given by:

$$d = \left(\frac{2kDC_o}{Q}\right)^{0.5},$$

where D is the oxygen diffusion coefficient, Q is the respiration coefficient and k is a geometrical parameter.

It is instructive to apply this treatment to some of the various geometrical shapes displayed by the eggs of monogeneans. The relation can be used to calculate the maximum volume possible (V_{max}) or, conversely, the minimum value of C_o/Q. This has been done for the sphere, for prolate spheroids of various elongations (elongation = ratio of major to minor axis) and for the tetrahedron. Many monogenean eggs are spindle-shaped but for the purpose of this exercise these can be regarded as prolate spheroids.

In the case of the sphere, the surfaces defined by equal oxygen concentrations within the sphere delineate similar, smaller, concentric spheres. In the other egg shapes that have been considered, approximations must be made. Within the prolate spheroid, it has been assumed that the surfaces defined by equal oxygen concentrations delineate smaller prolate spheroids with the same elongation. The tetrahedron has been treated as four sub-tetrahedra, the base of each being one of the four sides of the parent tetrahedron, and the surfaces defined by equal oxygen concentrations have been taken as planes parallel to the base of each sub-tetrahedron. The value of k for the prolate spheroid depends on the elongation and falls from 3 (the value for a sphere) to 2.36 at large elongations. For the tetrahedron the value of k is 3. Calculated values of V_{max} and $(C_o/Q)_{min}$ are given in Table 2. It can be seen that the elongated spheroid and the tetrahedral shape appear to have a considerable respiratory advantage. For example, if D is taken as 10^{-5} cm^2 s^{-1}, C_o as 5×10^{-3} cm^3 cm^{-3} and Q as 8.5×10^{-5} cm^3 cm^{-3} s^{-1} (planarian value fromHyman (1951), p. 207), then, for a sphere, V_{max} is about 0.9 mm^3, and for a tetrahedron, V_{max} is about 3 mm^3.

Since spheroidal monogenean eggs have values of N that do not exceed 4, it would seem that tetrahedral eggs of the same volume have the highest A/V ratios and V_{max} values. Such eggs would have a significant advantage in terms of obtaining oxygen for rapidly developing embryos in situations where the ambient oxygen concentration is low. The same properties of the tetrahedral egg would also promote the outward diffusion of carbon dioxide and other products of metabolism.

It is clear from the table that the prolate spheroids, in which the ratio N is high, are equal or superior to the tetrahedron in terms of A/V ratio and V_{max}. If it is assumed that the oxygen demand for the developing egg has had an influence on egg shape, then it needs to be asked why some monogeneans have adopted a tetrahedral egg shape rather than an elongated spheroid. The answer may be that the tetrahedral shape confers other advantages. For example, it is conceivable that the tetrahedral egg may be physically stronger and better able to resist damage when buried in shifting sand or when eaten by a small predator.

The tapering shape of the fusiform egg may provide a slightly higher surface area/volume ratio in comparison with a prolate spheroid, but it seems doubtful that the needle-like opercular and abopercular appendages possessed by some fusiform eggs (Figs. 22, 23, 28) would make any significant contribution to gaseous exchange. Although some of these appendages are hollow and contain fluid communicating with the egg contents, others are sealed off at or near the proximal end; moreover, when there is communication, the aperture is small and there is no obvious mechanism for the circulation of fluid from the narrow appendage to the embryo until the larva is sufficiently well developed to move. It has been suggested elsewhere (Section VII B p. 222) that these appendages may slow down the sinking rates of eggs in suspension.

It is particularly interesting that in two monogeneans the egg surface area has been substantially increased without changing the fusiform profile of the eggs. In *Dictyocotyle coeliaca* the sides of the fusiform egg are flattened so that the egg is triangular in cross-section (Fig. 15a,b) and in *Rajonchocotyle* spp. the egg surface has longitudinal corrugations (Fig. 13a,b 38b).

If there is a relationship between egg shape and the availability of oxygen in the environment of the egg, then it would be expected that eggs laid by parasites of bottom-dwelling hosts would tend to display shapes that enhance uptake of oxygen by diffusion. Eggs laid by parasites of fishes inhabiting bottom sediment such as mud or silt rich in decomposing organic material are likely to be exposed to an oxygen-poor environment as soon as they are laid, whereas eggs laid by parasites of pelagic hosts may take some time to sediment to the bottom, during which period embryonic development may proceed in an environment containing abundant oxygen. Eggs that have sticky droplets serving to attach them to sand grains at the sea bottom are likely to be buried beneath the surface of the sediment (Section VII B p. 221), where the ambient oxygen concentration may be particularly low and where stagnation may restrict diffusion of waste metabolites outwards from the egg. The egg of *E. soleae* is exposed to conditions of this kind and, furthermore, has a relatively large larva (about 250 μm in length

according to Kearn (1963b)). It is not surprising then that this large egg has a tetrahedral shape.

In Table 3, a list has been compiled of the shapes of eggs of various monogenean parasites of bottom-dwelling flat-fishes. This list includes representatives of most of the major groups of monogeneans. Many of the parasites included in this selection produce eggs that are roughly tetrahedral in shape or that, if not tetrahedral, have adaptations which may serve to increase their surface area, such as *D. coeliaca* and *Rajonchocotyle* spp. There are some notable exceptions, such as the eggs of *A. lobianchi* (Fig. 19), *Entobdella corona*, *Pseudodiplectanum kearni*, *Pseudocotyle squatinae* and *Octoplectanum* (= *Neoheterobothrium*) *affine*. However, the egg of *A. lobianchi* is relatively elongated ($N = 3.75$), tapers proximally and has a considerably smaller volume than that of *E. soleae*, and the spheroidal egg of *P. kearni* is small (length of major axis about 80 μm). It is noticeable that many monocotylids are associated with bottom-living flat-fishes and make tetrahedral eggs.

Round-bodied fishes range widely in habits and in habitats. This is reflected in the range of egg shapes produced by their monogenean parasites. Tetrahedral eggs are produced not only by parasites such as *Trochopus pini*, which inhabits the gills of a bottom-living gurnard (Kearn, 1971b) but also by *Capsala martinieri* (see Kearn 1963c), an inhabitant of the skin of the oceanic sunfish, which is regarded by Wheeler (1978) as pelagic. As mentioned above, other factors in addition to problems related to gaseous exchange may influence egg shape, and these factors may be particularly relevant for some eggs produced by parasites of round-bodied hosts. For example, the increased surface area/volume ratio of tetrahedral eggs or of fusiform eggs with high values of N, laid by parasites of pelagic hosts, may prolong the period of time spent by these eggs in suspension as a result of water turbulence. This would be further aided in both tetrahedral and fusiform eggs by appendages (Section VII B) and may increase the chances of oncomiracidia making contact with hosts that rarely visit the bottom.

There are some monogenean eggs that complete their development in the host's gill-ventilating current. Such eggs would be expected to be well aerated and it is interesting that the eggs of *Dionchus* spp., which are tethered to the host's gills (Fig. 42a), and those of a capsalid relative of *E. soleae*, *Pseudobenedenia shorti*, which are attached to the body of the gill parasite (Fig. 42b), are prolate spheroids with low N values (between 2 and 3). However, it must be said that the eggs of *Nitzschia sturionis*, which according to Bychowsky (1957, p. 91) are attached to the buccal lining of the host, are tetrahedral in shape.

It is particularly noticeable that polyopisthocotylean parasites, which parasitize mainly round-bodied fishes, produce eggs with a spheroidal or

TABLE 3 *Egg shapes of monogenean parasites of bottom-dwelling flat-fishes*

Parasite	Host	Egg shape (approximate description only)	Reference
Dactylogyrideans			
Acanthocotyle lobianchi	*Raja* spp.	Prolate spheroid	Figs. 19, 47
Entobdella soleae	*Solea solea*	Tetrahedron	Fig. 11
Entobdella corona	*Dasyatis* spp.	Prolate spheroid	Hargis (1955)
Bothitrema bothi	*Bothus maculatus* = *Scophthalmus maculatus*	"Flattened and triangular"	MacCallum (1913)
Amphibdella torpedinis	*Torpedo marmorata*	Tetrahedron	Euzet and Raibaut (1962)
Pseudodiplectanum kearni	*Microchirus variegatus*	Prolate spheroid	Kearn (unpublished)
Monocotylids			
Calicotyle kröyeri	*Raja naevus*	Tetrahedron	Fig. 10
Dictyocotyle coeliaca	*Raja naevus*	Prolate spheroid with flat sides	Fig. 15
Merizocotyle sp.	*Raja undulata*	Tetrahedron	Kearn (1968)
Horricauda rhinobatidis	*Rhinobatos batillum*	Tetrahedron	Fig. 12
Dendromonocotyle kuhlii	*Amphotistius kuhlii*	Tetrahedron	Fig. 16
Loimopapillosum dasyatis	*Dasyatis* spp.	"Piriform or angular"	Hargis (1955)
Microbothriids			
Pseudoleptobothrium aptychotremae	*Aptychotrema banksi*	Tetrahedron	Kearn (unpublished)
Pseudocotyle squatinae	*Squatina squatina*	Prolate spheroid	Palombi (1949)
Polyopisthocotyleans			
Rajonchocotyle spp.	*Raja* spp.	Ridged prolate spheroid	Figs. 13, 38b
Octoplectanum (= *Neoheterobothrium*) *affine*	*Paralichthys dentatus*	Fusiform	Linton (1898)

fusiform shape. None of these parasites has a tetrahedral egg shape, but the eggs of *Rajonchocotyle* spp., parasitizing the gills of bottom-dwelling rays, have a corrugated shell which undoubtedly increases their surface area, and the egg of *Plectanocotyle gurnardi*, which inhabits the gills of bottom-dwelling gurnards, is relatively small in volume (Fig. 3) and has a high N value (3.5).

Some of the largest monogenean eggs are assembled by polyopisthocotyleans (Section II); many of these eggs have a spheroidal shape, and this combination of large volume and relatively low A/V ratios demands comment. Unfortunately, in many instances the fate of these eggs and the environmental conditions in the micro-environment where they develop are not known. The relatively large spheroidal eggs of parasites such as *Discocotyle sagittata* (Fig. 9) may possibly develop in well aerated bottom water; in fact their salmonid hosts are known to prefer well oxygenated water (Frost and Brown, 1967). Many polystomes retain relatively large eggs in the uterus, and three factors may possibly influence the intra-uterine respiration of these eggs. Firstly, the parasites may tap the oxygen taken in by the host, since the parasites are blood feeders; secondly, egg-laying is infrequent (Section IX p. 240) and egg development may be relatively slow, so reducing the short-term demand but not the overall demand for oxygen; and thirdly, the eggshells of some of these parasites are known to be thin and flexible (Section VI p. 219) and may be less of a diffusion barrier than the shells of other monogenean eggs.

There appears to be no clear-cut relationship between the size of an egg and its shape; tetrahedral eggs may be small or large and there is a great range in the sizes of spheroidal eggs. Spheroidal eggs may present no disadvantages for gaseous diffusion even in an environment of low ambient oxygen concentration if the egg volume is small (for example *Dactylogyrus vastator*; Fig. 5) but, in terms of gaseous exchange, it is difficult to explain the tendency for some small eggs to display a tetrahedral shape, for example those of *D. chranilowi* (Fig. 4).

Factors other than gaseous exchange may play a part in determining egg size. There will clearly be a relatively low upper limit on the sizes of eggs produced by dactylogyrids, because the adult parasites are themselves small, although in relation to body size the eggs of these parasites may be proportionately large. The large size of the egg of *Polystomoides megaovum* in relation to the size of the adult parasite was commented upon by Ozaki (1936). Tinsley (1983) has made the suggestion that some polystomatid monogeneans may have increased the numbers of eggs that can be stored in the uterus by reducing the size of individual eggs. Host-finding may be better served in some instances by the production of large numbers of small larvae. It is interesting in this context that one of the largest monogeneans, *Capsala*

martinieri, with a circular body often exceeding 2 cm in diameter, produces tetrahedral eggs that are significantly smaller than, for example, those produced by *E. soleae*, which has an adult body length of about 5 mm (Kearn, 1963b, 1963c). In general, small eggs develop more quickly than large eggs (Section X) and rapid development may be advantageous in some circumstances. In contrast, polystomatids such as *Pseudodiplorchis americanus* appear to have adopted the opposite strategy of producing relatively small numbers of exceptionally large larvae (Section II, p. 180, Section V A p. 203). However, an assessment of the significance of the alternative strategies adopted by monogeneans in partitioning of their reproductive resources must await more detailed ecological studies of monogenean parasites.

In view of the diversity of shapes of monogenean eggs, the way in which the usually cylindrical larva is packed inside the egg deserves some attention. In spheroidal or fusiform eggs the body of the larva is straight or slightly curved as in *Protopolystoma xenopodis* (Fig. 44e) but, in *Eupolystoma anterorchis*, a relatively long larva (about 350 μm) is accommodated in a spheroidal egg of modest dimensions (maximum 185 × 105 μm) by the adoption of a "doubled-up" position inside the egg (Fig. 44b). The oncomiracidium of *E. soleae* is twisted inside the egg and is not arranged symmetrically with respect to the longitudinal plane of symmetry of the egg, although the head of the larva occupies the opercular corner (Fig. 44a). In contrast, the oncomiracidia of some monocotylids are symmetrically arranged inside the tetrahedral egg with the head located in the opercular corner (Fig. 44d). A previously published illustration (Kearn, 1970a, Fig. 1c) shows the larva of *C. kröyeri* in an atypical attitude. The unusual egg of the monocotylid *Squalotrema llewellyni* appears to be a partially flattened tetrahedron, and the fully developed larva adopts a U-shaped attitude with the head occupying the opercular corner and the haptor occupying one of the two lateral corners (Fig. 44c).

The head region of most fully developed oncomiracidia is located in the opercular corner of the egg whatever the egg shape, and the importance of this relationship for hatching will be considered later (Section XI A).

IX. Egg Laying

The eggs of monogeneans may be laid more or less continuously, each egg being voided as soon as assembly is complete, or they may be accumulated by the parasite and released at a specific time. Most monogeneans lacking a uterus presumably lay each egg as it is made, but some retain a single egg in the ootype (Section VII C 2). In *Entobdella soleae* and in *Acanthocotyle lobianchi*, brief retention of some eggs is practised, so that usually two or

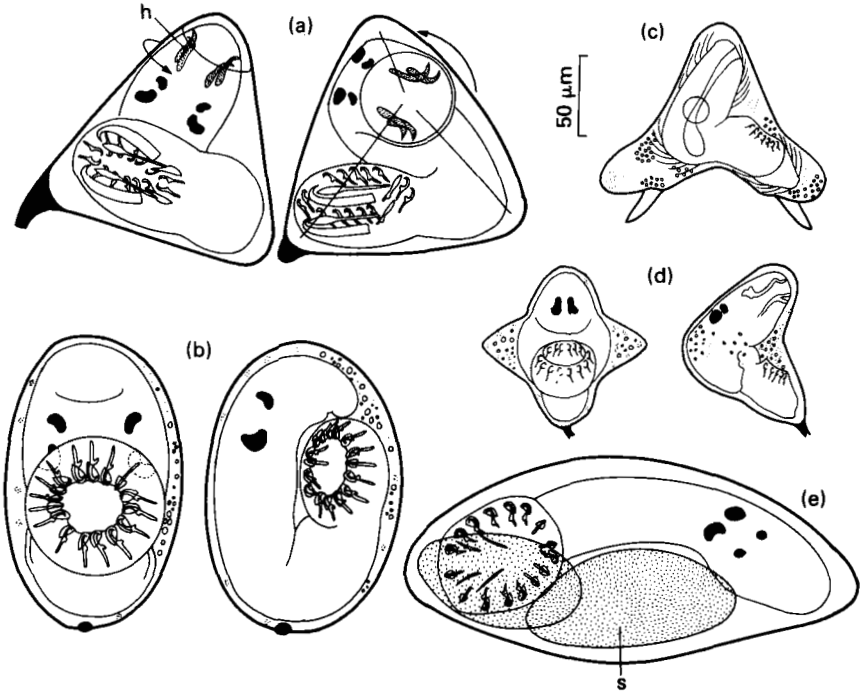


FIG. 44. The packing of the fully developed oncomiracidia inside the eggs of various monogeneans. (a) *Entobdella soleae* from Kearn (1975a) reproduced with permission from *Parasitology* **71,** p. 422. (b) *Eupolystoma anterorchis* reproduced with permission from Tinsley (1978) *Parasitology* **77,** p. 125. (c) *Squalotrema llewellyni* from Kearn and Green (1983) reproduced with permission from *Journal of the Marine Biological Association of the United Kingdom* **63,** p. 22. (d) *Dendromonocotyle kuhlii* (e) *Protopolystoma xenopodis*, drawn from photographs of eggs kindly supplied by Dr. R. Tinsley. Cilia have been omitted from all oncomiracidia except (c). h, Glands suspected of involvement in hatching; s, fluid-filled sac.

three eggs in *E. soleae* and a few more in *A. lobianchi* are attached to one another by sticky material on the appendages and are voided together (Kearn, 1963a, 1967).

Iziumova (1956) found no significant difference between the numbers of eggs laid by *Dactylogyrus vastator* during the day and during the night, but Macdonald and Jones (1978) found that *Diplozoon homoion gracile* lays significantly greater numbers of eggs at night, and they suggested that this might lead to the accumulation of eggs in sheltered, night-time resting areas sought out by the host, *Barbus meridionalis*. Their observations indicate that these differences may be due to a decrease in egg production

during the day rather than to an accumulation of eggs during the hours of daylight.

Daily egg-laying rhythms have not yet been demonstrated in other monogeneans, but situations could be envisaged in which it would be distinctly advantageous to restrict egg-laying to the period of daylight or darkness, or even further to a specific time of day or night. An example would be if the hosts repeatedly used the same locality for resting at the same time each day and dispersed at other times of day. The need to produce large numbers of eggs during a relatively short daily egg-laying period would be difficult to meet by changes in the rate of egg assembly but could be achieved by storing eggs temporarily. Those monogeneans such as *Diclidophora luscae* (Fig. 34) and *Oculotrema hippopotami* (see Thurston, 1968) may well lay their eggs once a day since they accumulate in the uterus approximately the same number of eggs (40–60) as the continuous egg-layer *E. soleae* produces in one day (Kearn, 1985). In fact, in *O. hippopotami*, the habit of the hippopotamus host of feeding mainly at night on land and spending the daylight hours in water (Vaughan, 1978) would seem likely to exert strong selection pressure on the parasite to restrict its egg-laying to the daylight hours.

According to Llewellyn (1981) the embryonic development of *D. luscae*, which retains eggs in the uterus and lays them in a bundle, begins as soon as the eggs are exposed to sea water. If this is so, then the temporary retention of eggs in the uterus might be an adaptation to delay the onset of embryonic development in individual eggs so that embryonic development may begin simultaneously in all retained eggs when the egg bunch is expelled into the water. According to Llewellyn (1981), simultaneous oviposition of eggs in a bundle, followed by synchronous embryonic development, would lead to simultaneous hatching and the tendency for larvae from the same egg bundle to invade the same host specimen. This in turn would lead to mating between siblings, the survival value of which might be the conservation of highly specialized characters. The egg groups laid by parasites such as *E. soleae* seem rather small to confer a significant advantage of this kind, and there is some evidence that the laying of eggs in groups in this parasite may be a consequence of more frequent egg assembly and greater provision for storage (longer uteri) in large adults (Kearn, 1985).

Llewellyn compared the situation in *D. luscae*, in which eggs are retained in the uterus, with the habit of retaining the eggs outside the body of the parent at the uterine aperture as in *Acanthocotyle greeni* (Section VII C 2 p. 227). Embryonic development of each egg of *A. greeni* is said to begin as soon as it is added to the bunch and thereby exposed to sea water. This would lead to sequential hatchings, in contrast with the relatively synchronous hatchings of *D. luscae*, and this would permit infection of the host of the

parent parasite while the egg bundle is retained by the parasite and other hosts after the bundle is shed.

There is evidence that embryonic cell division does not begin until after laying in polyopisthocotyleans (for example, in *Polystoma integerrimum* (see Goldschmidt, 1902; Halkin, 1902) and in *Diclidophora denticulata* (see Frankland, 1955)). However, there is not yet any evidence that cell division is initiated by exposure to water. According to Frankland (1955), the eggs of *D. denticulata* are laid in bundles of 100–200; she found that cleavage of the zygote began as early as 15 min after laying in some eggs of the bunch, but in other eggs cleavage did not begin until much later, most zygotes having begun to divide 5 h after laying. Frankland (1955) interpreted this as evidence that the onset of cleavage must be determined by the time of egg assembly or by sperm penetration of the egg cell rather than by a stimulus, such as contact with sea water, that affects the whole egg bunch at once. Frankland (1955) came to the conclusion that the longest time that an individual egg spends in the uterus is about 5 h, but there is reason to doubt this estimate since it indicates an egg assembly rate of 20–40 eggs per hour, which is exceptionally high compared with the egg assembly rates of most other monogeneans (Section V A).

If further studies confirm the suggestion that development is triggered by exposure to water, then those monogeneans in which eggs undergo partial or complete embryonic development inside the parent parasite must have abandoned this developmental stimulus and adopted a new one, perhaps provided by the parent parasite.

Some other features of egg bunches, like those produced by *D. luscae* and by *D. denticulata*, are considered elsewhere (Section VII B p. 224).

Eggs that complete their development in the uterus may be laid at infrequent, sometimes annual, intervals. For example, the main opportunity for egg-laying in the toad bladder parasite *Eupolystoma alluaudi* appears to be during visits by the host to water bodies for spawning (Salami-Cadoux, 1975). Tinsley (1975) recorded that egg deposition in *E. alluaudi* appears to be stimulated when the host is in water, and the general inference is that an osmotic stimulus leads to the mass deposition by the parasite of ready-to-hatch eggs. However, experiments on *E. anterorchis* performed by Tinsley (1978) led to the conclusion that, although an osmotic laying-stimulus seemed most likely in this parasite, other factors must be involved.

In desert spadefoot toads, opportunities for egg-laying by the polystomes *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis* are unpredictable and exceedingly brief (Tinsley, 1983; Tinsley and Earle, 1983; Tinsley, 1984). The toads respond quickly to the advent of the annual summer rains, emerging from their burrows and commencing to spawn in

temporary pools on the first night of heavy rains. Female toads leave the pools immediately after spawning whereas males may return on one or two further nights. The parasites are able to take advantage of this brief opportunity since eggs with fully developed larvae are maintained *in utero* in a ready-to-hatch condition for two to three months. When the hosts enter water the eggs are laid *en masse* and hatch immediately (Section XI A p. 251), rapidly infecting the aggregated toads. How such a precise timing of egg-laying is achieved in these parasites is still unknown.

The act of egg-laying in *E. soleae* appears to involve the expenditure of considerable muscular effort involving contraction of the general body musculature as well as of the muscles associated with the reproductive tract (Kearn, 1985). Similar energetic oviposition has been described in some polyopisthocotyleans, for example by Remley (1942) in *Microcotyle spinicirrus* and by Frankland (1955) in *D. denticulata*.

A distinct behaviour pattern observed in *E. soleae* appears to be related to egg-laying (Kearn, 1971a). Specimens are frequently found with two or three tetrahedral eggs hanging freely in the surrounding water but still attached to the parasite by the appendages which are lodged in the uterus. A parasite carrying eggs in this manner frequently lifts the head region above the substrate with the eggs uppermost (Fig. 45); the parasite then usually lowers and raises the head rapidly, perhaps two or three times. When the parasite is attached beneath its flat-fish host such movements may assist the separation of the eggs from the parasite and their attachment by the sticky droplets on the egg appendages to sand grains.

Monogeneans of the genus *Amphibdella* spend the early part of their lives

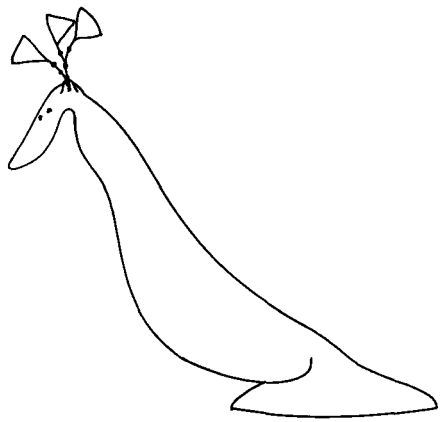


FIG. 45. The attitude adopted by *Entobdella soleae* when partly laid eggs are detained by egg appendages lodged in the uterus.

in the blood system of their hosts (*Torpedo* spp.) and evidence suggests that after mating the parasite migrates to the gill mucosa, which is penetrated by the anterior end of the parasite, thus exposing the uterine opening and permitting the eggs to be laid and carried away in the gill-ventilating current (Llewellyn, 1960). The skin-parasitic monogenean *E. soleae* is known to migrate from the upper to the lower surface of its flat-fish host and Kearn (1984) has suggested that one possible advantage of this migration might be to facilitate the attachment of freshly laid eggs to sand grains beneath the host.

X. Egg Development

Reference has already been made briefly (Section IX p. 239) to some early events of egg development. Extensive studies of the embryonic development of polyopisthocotylean monogeneans were conducted by Halkin (1902), by Goldschmidt (1902) and by Ktari (1971). Other workers have concentrated on particular aspects of embryology such as development of the tegument (Lyons, 1973) and the hooks (Kearn, 1963b; Lyons, 1966). In the contexts of comparative embryology and phylogeny these studies are of great interest, but they have less relevance to a study of the role of the egg in the general biology of monogeneans and will not be considered further here. However, the effects of environmental factors such as temperature on the rates of development of monogenean eggs are relevant to this survey.

Many monogenean eggs appear to hatch spontaneously when development is completed. The development times of eggs for a small selection of such parasites are given in Table 4. In general, most of these monogenean eggs take between 10 and 40 days to complete their development and hatch at environmental temperatures. However, it will be seen in Table 4 that the small egg of *Dactylogyrus lamellatus* is reputed to complete its development and hatch in the remarkably short time of 1.5 days at 26°C, and the duration of development in the marine monogenean *Dictyocotyle coeliaca* lies between 112 and 147 days at 10°C. The duration of development of most monogenean eggs shortens at higher incubation temperatures (see Fig. 46 and *Plectanocotyle gurnardi* in Table 4). Although development may proceed more rapidly at higher temperatures, a smaller proportion of these eggs may complete their development and hatch (see below).

In many monogeneans, egg development appears to cease at temperatures close to zero. For example, Prost (1963) found that eggs of *Dactylogyrus anchoratus* and *D. extensus* kept in the laboratory ceased to develop at 3°C, although development of some of these eggs continued when the temperature was raised (see below). The reports by Szidat (1969) of the

TABLE 4 *The egg development time (interval between laying and spontaneous hatching) in a selection of monogeneans*

Parasite	Temperature (°C)	Egg development time (days)	Authority
Dactylogyrus lamellatus	18	4	Molnár (1971)
	26	1.5	
	32	2.5–3	
Urocleidus adspectus	20±2	5–6	Cone (1979)
Pseudodactylogyrus microchirus	20	5–8	Imada and Muroga (1978)
	28–30	2–5	
Merizocotyle sp.	20	5–6	Kearn (1968)
Dictyocotyle coeliaca	10	112–147	Kearn (1970a)
Entobdella soleae	13	30 (approx).	Kearn (1973)
Capsala martinieri	13–17	14	Kearn (1963c)
Plectanocotyle gurnardi	13	21–30	Llewellyn (1957a)
	18	13–16	
	20	8–11	
Protopolystoma xenopodis	26	20–32	Tinsley and Owen (1975)
Diclidophora luscae	13	32–36	Macdonald (1977)
Diplozoon homoion gracile	22–27	5–6	Macdonald and Jones (1978)

monogenean *Pseudobenedenoides branchicola*, together with attached eggs containing fully developed larvae, on the gills of the Antarctic fish *Trematomus bernacchii*, are of special interest because this fish is known to experience exceptionally low environmental temperatures throughout the year. This fish is found in McMurdo Sound where the water temperature is said never to rise above −1.5°C (DeVries, 1971) but also occurs in the waters adjacent to the Antarctic Peninsula where water temperatures are a little higher, reaching +1°C. Thus, it is surprising but likely that this monogenean parasite is able to complete its development at these low temperatures.

Combes (1968) explored the complex interactions between environmental factors, host biology and rates of egg development in *Polystoma integerrimum*, which is found in the bladder of the common frog *Rana temporaria*. Eggs laid by adult polystomes in the bladders of frogs congregating in ponds to spawn, produce oncomiracidia which invade the gills of tadpoles (Fig. 2). These oncomiracidia have two options; on young tadpoles they undergo precocious sexual development producing neotenous, miniature adults, whereas on older tadpoles they are likely to continue to grow, migrating at metamorphosis of the frog to the bladder where they become egg-laying adults. Eggs produced by neotenic adults are said to produce a late generation of oncomiracidia which colonize the remaining old tadpoles

and give rise ultimately to bladder-dwelling adults. Combes (1968) quoted earlier opinions, based on work in more northerly latitudes, in which the neotenic generation is regarded as an essential part of the life cycle of *P. integerrimum*, but he pointed out that, in his Pyrenean study area, neotenic adults are rarely found in the natural environment. However, when young Pyrenean tadpoles were exposed in laboratory experiments to the larvae of their polystomes, neotenic adults were obtained and Combes (1968) turned his attention to factors that might account for the rarity of neotenics in the natural environment. He found that, over the range of environmental temperatures from 5°C to 25°C, the lower the incubation temperature the longer the eggs of the parasite and those of the host took to complete their development, but he also found that, at any given temperature, frog eggs completed their development more quickly than polystome eggs (Fig. 46). Moreover, the difference in time between the completion of development of host and parasite eggs increased from 4 days at 25°C to more than 50 days at 5°C. Combes (1968) showed that egg-laying in his Pyrenean frogs and their parasites was almost simultaneous, and came to the conclusion that at the low temperatures prevailing in his study area, provided that eggs of host and parasite develop in the same conditions, the oncomiracidia will hatch when the tadpoles are well developed, and neotenic adults are unlikely to occur.

Combes (1968) identified another factor that decreased the chances of neoteny in the Pyrenees. He pointed out that frog-spawn usually floats whereas polystome eggs sink to the bottom; during the daytime the sun warmed up the surface layers of the ponds while the bottom water remained cold. Combes (1968) noted that the warm water at the surface would accelerate the development of the frog-spawn while the development of the polystome eggs at the bottom would be retarded by the cold water, leading to a further separation in time between oncomiracidia and young tadpoles. He recorded a temperature difference of 10°C in 30 cm of water and observed that spawning occurred in water at least 20 cm deep so that polystome eggs were unlikely to be deposited in shallow warmer water at the edges of the pools. He made the additional observation that tadpoles tend to gather in shallow warmer water where their development would be accelerated. He went on to consider the possible reasons for the occasional appearance of neotenic polystomes in two of the Pyrenean pools he had chosen to study. He found that small, water temperature differences created by the presence of shade or snow on one side of the pond and not on the other, and migrations of tadpoles from the cool to the warmer sides of the ponds, provided conditions favourable for the production of some neotenic adults.

An important study of the effects of environmental factors on the develop-

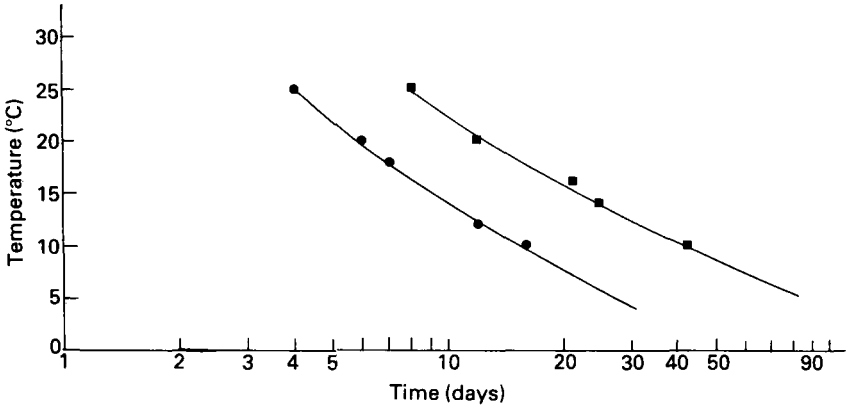


FIG. 46. Relationship between duration of development and temperature in the eggs of *Polystoma integerrimum* (■) and those of the host *Rana temporaria* (●). Reproduced with permission from Combes (1968) *Mémoires du Muséum National d'histoire naturelle* **51**, p. 133.

ment of the eggs of freshwater dactylogyrid monogeneans has been made by Prost (1963). She found that the eggs of *Dactylogyrus anchoratus* are unable to resist drying and that, if water containing eggs freezes and remains frozen for 2 h or more, no further development or hatching of the eggs occurs. However, after freezing for 30 min., eggs containing larvae survived and hatched whereas cleaving eggs died. Eggs failed to develop when incubated at 3°C but when eggs were kept for up to 8 weeks at 3°C and then transferred to an environment at 21 to 24°C, larvae became active in 80 to 85% of those eggs containing developed larvae and most of them hatched. In contrast, only 10 to 15% of the eggs containing cleaving embryos completed development and hatched. Similar results were obtained with *D. extensus*.

Prost (1963) found that in *D. anchoratus* at 22 to 23°C, 80 to 90% of the eggs developed whereas a smaller proportion of the eggs completed development at temperatures higher or lower than this optimum. The corresponding optimum temperature for development of *D. extensus* eggs was found by Prost (1963) to be lower (16–17°C). It is surprising that the optimal temperatures for the development of *D. extensus* are markedly lower than those for *D. anchoratus* (and for *D. vastator* according to Prost (1963)) since these parasites share the same host, but Prost (1963) pointed out that her observations were in agreement with data collected by earlier workers. She also found that, during the summer, the intensity of infection of carp with *D. anchoratus* increases more rapidly than that of *D. extensus*. She pointed out that the numbers of eggs laid by the two species of parasite and the periods of larval development at various temperatures are similar, and she sug-

gested that the differences between the optimum temperatures for egg development are responsible for this difference in intensity, more larvae of *D. anchoratus* developing from similar numbers of eggs during the warm summer period.

A field experiment of considerable significance was conducted by Prost (1963) during the winter of 1961. A method was devised for maintaining eggs of *D. anchoratus* in tubes throughout the winter at the bottom of a pond. When the eggs were recovered and incubated at 20 to 22°C (temperature of pond water 2°C) most of the eggs that contained developed larvae subsequently hatched and a small proportion of cleaving eggs continued their development and hatched, providing convincing evidence that some eggs of *D. anchoratus* can overwinter in the wild. The experiments of Bovet (1967) led him to conclude that overwintering eggs of *Diplozoon paradoxum* contribute little to the maintenance of the species.

Nybelin, Nørdquist and Wunder (all quoted by Bychowsky 1957, p. 107 *et seq.*) maintained that *Dactylogyrus vastator* produces small "summer" eggs which develop and hatch without interruption and larger "winter" eggs which enter a state of arrested development or diapause until the following spring or summer when development is completed. Nybelin also reported that *D. vastator* perishes in the autumn, so that diapausing winter eggs are the only source of parasites for the next season. However, considerable variability in the sizes of eggs laid by individual parasites was recorded by Kulwiec (quoted by Bychowsky (1957)) and by Bychowsky (1957) himself and the further development of all of these eggs is described as uniform with no differences observed in the duration of development. Lyaiman (quoted by Bychowsky (1957)) and Bychowsky (1957) reported that the development of the eggs of *D. vastator* is extended at lower temperatures, ceasing altogether at 4°C; Bychowsky (1957) came to the conclusion that the spring infection was a result of larvae emerging from eggs in which development had slowed down and stopped as temperatures gradually fell during the winter, and then had continued and completed their development as temperatures began to rise in the spring (as appears to be the case in *D. anchoratus*, see above). Bychowsky (1957) also quoted work by Lyaiman that showed that *D. vastator* persists, albeit in small numbers, throughout the winter on the gills of carp.

The introduction of infected carp into Israel, and the relatively warm conditions in this area throughout the year, have provided a natural experiment that has been exploited by Paperna (1963a,c, 1964). He found *D. vastator* only on carp less than 60 mm in length and claimed that immunity develops so that older carp are free from infection. Carp fry grow quickly in Israel, reaching 60 mm in length in late June early July, whereas carp do not reach this length until their second year of life in Europe and the USSR.

Consequently, in Israel, carp fry of a suitable size to sustain parasites are usually available only in April, June and July. The initial infection of fry takes place almost simultaneously in ponds widely removed from one another, and since there are no fry of a suitable size to maintain an infection in the autumn and winter, the inference is that overwintering eggs are the only source of infection in the following year. However, in Israel, winter temperatures in the fish ponds seldom drop below 10°C, temperatures below 12°C occurring usually only during December. Paperna (1963a) pointed out that development of the "summer" eggs continued at 12°C although prolonged to 15 days (compared with only 2–3 days at 24–28°C) and he found that only at a temperature as low as 5°C (well below Israeli winter temperatures) did development cease. Furthermore, when the parasite disappeared from the carp in late June, early July, water temperatures in the ponds were still high enough for optimal development of eggs and winter temperatures were not low enough to retard the development of eggs for more than one or two weeks, so the inescapable conclusion is that any overwintering eggs of *D. vastator*, at least in Israel, are likely to be in a state of diapause. Paperna (1963a) found little difference in sizes of eggs developing immediately and "retarded" eggs. He claimed that "retarded" eggs showed no development nor degeneration after more than one year, but his attempts to "break" diapause failed.

Paperna (1963c) stressed the simultaneous appearance of parasites on young carp at the beginning of the season in widely separated ponds and suggested that this may be due to the simultaneous hatching of diapausing eggs, that is eggs that have overwintered in pond mud. However, the wide practice of draining ponds before the onset of the host's spawning season failed to free the ponds of parasites. Even the addition of lime to ponds that had been left dry for about 1½ months did not prevent subsequent infestation of fishes, and Paperna (1963c) regarded the eggs as highly resistant and possibly buried deep in the mud. However, this appears to be inconsistent with the findings of Prost (1963) who showed that eggs of *D. anchoratus* are unable to survive drying.

It has been suggested by Llewellyn (1962) that deposition of diapausing eggs is one way in which the parasite *Gastrocotyle trachuri* might cope with the seasonal migration of its host away from the sea bottom where infection occurs.

There have been relatively few studies of the effects of environmental factors other than temperature on the development of monogenean eggs. The effects of salinity on egg development have been studied by Paperna (1963a) in *D. vastator* and by Lambert (1977) in *Ergenstrema mugilis*.

An increase in the size of eggs during development has been reported in certain polystomatids that retain eggs inside the uterus, and it is possible that

this increase in size is related to the passage of nutrients through the shell permitting extensive growth of the embryo (Section VI p. 219).

XI. Egg Hatching

A. The mechanism of hatching

Detachment of the operculum in the digenean *Fasciola hepatica* is known to be brought about by an increase in internal pressure caused by the swelling of a "viscous cushion" lying beneath the operculum (Wilson, 1968). A viscous cushion has not been reported in any monogenean egg, although there is evidence that internal fluid pressure does play a part in hatching in some species (see below).

The most detailed study of hatching in a monogenean is that of Kearn (1975a) on *Entobdella soleae*, where hatching tends to occur spontaneously early in the period of daylight or may be stimulated to occur at any time by host skin washings (see below). In both of these situations the surface cilia of the larva begin to beat and the head of the larva, which is located in the opercular corner of the egg, rotates in an anti-clockwise direction at a speed of 6 (2 – 8) s per revolution (at 17–20°C) until the operculum suddenly breaks loose. At this stage muscular activity may help the larva to squeeze through the opercular opening, but the cilia continue to impart rotation to the larva up to the moment when it escapes from the shell. Surprisingly, none of the cilia appears to be removed when the larva squeezes through the narrow opercular aperture. There is some evidence that the sticky appendage of the egg may assist some larvae to escape by anchoring the egg. Larvae may escape from the egg as soon as 4 or 5 min after stimulation by fish washings.

Oncomiracidia of *E. soleae* were occasionally found with the head occupying not the opercular corner but one of the other three corners of the egg and such larvae invariably failed to hatch. Successful hatching therefore appears to depend on the positioning of the head of the larva and Kearn (1975a) found a considerable body of circumstantial evidence suggesting that head glands (Fig. 44a) secrete a proteolytic hatching fluid that dissolves the opercular cement.

There is a little evidence to suggest that other monogenean larvae may hatch in the same way as *E. soleae*. Paul (1938) observed that when an egg containing a fully developed larva of *Polystoma nearcticum* is crushed it never breaks at the opercular line, and he suggested that the "cephalic cells" of the larva secrete a substance that unseals the egg. The orientation of

monogenean larvae with the head at the opercular pole of the egg has been described on many occasions and these references have been summarized by Kearn (1975a). Bychowsky (1957, p. 92) and Bovet (1967) have referred to the difficulty experienced by larvae in escaping from the egg when the larva is orientated with the head at the abopercular pole. However, Vande Vusse (1976) described the haptor as emerging first from the egg of *Parapolystoma crooki*.

There is no doubt, however, that some monogenean larvae hatch in a different way. The unciliated larvae of *Acanthocotyle lobianchi*, like those of *E. soleae*, respond to host skin washings but the larvae hatch in a few seconds (Macdonald 1974). The rapidity of hatching points to the rupture of the opercular joint by direct pressure exerted on the operculum by the vigorously extending body of the larva (Fig. 47). However, there was evidence that the opercular seal is already weakened in anticipation of the thrust of the larva.

It was observed by Kearn (1982) that, in terms of speed, the hatching of *Entobdella diadema* resembles that of *A. lobianchi* more closely than that of its close relative *E. soleae*. The oncomiracidia of *E. diadema* escaped from fully developed eggs between 3 and 5 s after the light was extinguished (see

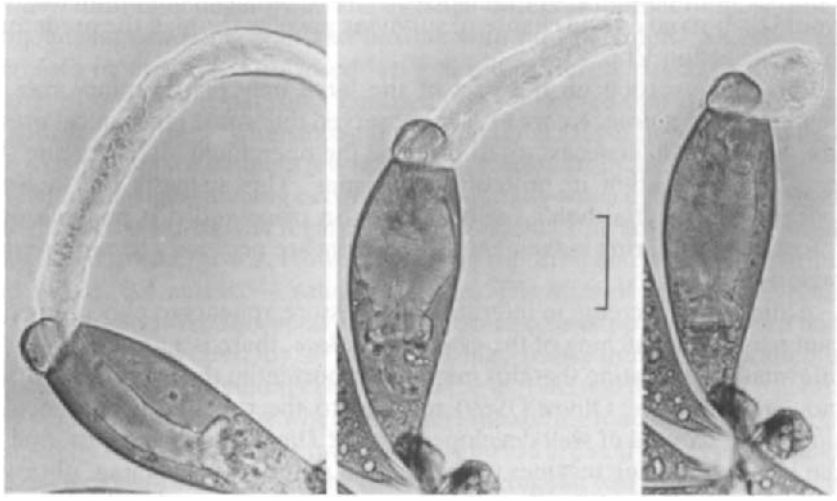


FIG. 47. Hatching in *Acanthocotyle lobianchi*. Successive positions of the living larva photographed with electronic flash. (a) Taken immediately after stimulation of the unhatched egg with host mucus. The larva responds by rapidly extending the body thereby pushing off the operculum. (b) (c) Taken successively as the larva withdrew into the shell after hatching. Scale bar = 50 μm.

below). This again suggests that, on receipt of the appropriate stimulus, the opercular cement is already weakened in readiness for rapid escape from the egg. Rapid hatching in response to a chemical hatching stimulus has been observed also by Whittington (in preparation) in the eggs of *Leptocotyle minor*.

Many workers have expressed the opinion that muscular exertion by the larva plays a part in dislodging the operculum (e.g. Prost, 1963; Owen, 1970; Tinsley and Owen 1975; Cone 1979), but it seems rather unlikely that the modest forces exerted by an oncomiracidium would be sufficiently strong to break an opercular seal which had not been pre-weakened. Cone (1979) described repeated thrusts by the larva of *Urocleidus adspectus* directed usually but not always against the operculum, but he found no evidence for a progressive weakening of the opercular joint during embryonic development. However, it seems possible that there is a "last-minute" softening of the cement, perhaps by release of a hatching fluid into the space between the larva and the shell rather than by direct application of the fluid to the cement, as seems to occur in *E. soleae*. It would seem to be advantageous for the parasite to delay weakening of the cement for as long as possible, since the operculum would be less likely to be dislodged if eggs were manipulated by or swallowed by a predator and the intact, digestion-resistant, eggshell would then stand a good chance of surviving passage through the predator's gut (see Section VI p. 218).

Muscular exertion on the part of the larva may play a minor role in hatching in *E. soleae*. Kearn (1975a) observed that some mechanical pressure, albeit small, is necessary to dislodge the operculum after piercing the eggs and incubation in proteolytic enzymes. This suggests that a little indigestible material holds the operculum in place and it is possible that forces exerted during movements of the larva are necessary to rupture this material.

Although an increase in internal fluid pressure appears to play no significant part in the hatching of the eggs of *E. soleae*, there is a growing body of information indicating that this might be important in the hatching of other monogenean eggs. Oliver (1969) referred to the rounded appearance of ridges and corners of well developed eggs of *Diplectanum aequans*, and to the fact that the egg resumes its tetrahedral shape after hatching, observations that are consistent with an increase in fluid pressure within the egg prior to hatching. In *U. adspectus*, Cone (1979) found that the larva is accompanied by two prominent, fluid-filled sacs within the egg. He observed that these sacs gradually swell after the operculum becomes detached and while the larva is emerging and, unless the activity of the larva ruptures one of the sacs, they continue to swell after the departure of the larva and eventually burst. Cone (1979) pointed out the similarity of these sacs to those found

inside the egg of *F. hepatica*. In the latter, these were regarded as the remnants of vitelline cells, and the swelling of the sacs after the loss of the operculum was thought to aid in ejecting the miracidium from the egg (Wilson, 1968). They may have a similar role in the egg of *Urocleidus* but there is a further possibility that the swelling prior to hatching may exert sufficient force to dislodge the operculum. Osmotic expansion of similar sacs in the egg of the digenean *Schistosoma mansoni* seems to be responsible for hatching (Smyth and Halton, 1983, p. 110). Similar sacs occur in the egg of *Protopolystoma xenopodis* (Fig. 44e and Thurston (1964)) and in the egg of *Oculotrema hippopotami* (see Thurston, 1968). Cone (1979) considered that the involvement of the sacs in the hatching of freshwater monogeneans may be more widespread than previously realized but such sacs are not confined to freshwater monogeneans. Kearn (1970a) observed similar sacs in the fully developed eggs of *Dictyocotyle coeliaca* and, more recently (Kearn, unpublished observation), sacs have been found alongside the larva in the egg of *Calicotyle kröyeri*.

The observations of Tinsley (1978) on the hatching of *Eupolystoma anterorchis*, a parasite of the bladder of the toad *Bufo pardalis*, indicate that there is a high internal pressure prior to hatching. These eggs are maintained *in utero* until fully developed. The eggshell differs from that of most other monogeneans in being thin and flexible with no operculum, and the egg increases in size as the enclosed larva develops during passage down the uterus of the parent parasite. Tinsley (1978) observed that hatching is an "explosive splitting", often along the longitudinal axis of the shell, and that this may take place within a few seconds of release from the uterus. Tinsley (1978) found that hatching occurred most rapidly when eggs were released into distilled water and he regarded this as evidence that an osmotic stimulus is involved in emergence. However, he also reported hatching in host urine and in 0.3, 0.4 and 0.5% saline solutions. The eggs of *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis* also develop fully *in utero* and each larva is invested by a loosely fitting, transparent, membraneous eggshell with no operculum (Tinsley and Earle, 1983 and Section VI p. 219). The eggs are laid *en masse* when the desert-inhabiting toad hosts enter pools formed by the brief summer rains, and the larvae are said to flex and extend themselves vigorously, emerging through an irregular tear in the shell within a few seconds of release from the uterus. It is probable that water uptake by the egg is partly responsible for rupture of the shell (Tinsley, personal communication). Hatching has been observed to occur in water, 0.3% saline, 0.6% saline and in toad urine.

B. HATCHING RHYTHMS AND HATCHING FACTORS

1. *Hatching rhythms*

There are some early references (Bychowsky, 1957, p. 92; Zeller in Bovet, 1967) that indicate that the hatching of the eggs of some monogeneans may not be continuous throughout the 24 h period. Kearn (1973) showed that, when eggs of *E. soleae* are subjected in the laboratory to alternating periods of light (matching in intensity and spectral quality the light at depths where the host, *Solea solea*, lives) and darkness (LD 12:12, LD 6:18 or LD 18:6) at constant temperature, hatching is rhythmical, most of the oncomiracidia emerging during the first 4 h of the period of illumination on each successive day (Fig. 48a). Rhythmicity persists when eggs are exposed to natural daylight in the laboratory and rhythmical hatching continues with a periodicity of about 24 h when eggs, previously exposed to LD 12:12 until hatching begins, are then maintained either in constant darkness or in constant light, suggesting that there is a strong endogenous component to the rhythm. The host is a marine fish common in middle latitudes (Wheeler, 1978) where it is likely to be exposed to daylight periods of varying length throughout the year. The experimental evidence suggests that the circadian hatching rhythm is responsive to cues from the light regime and that eggs laid by *E. soleae* at any time of the year are likely to hatch soon after dawn, whatever the natural day length may be. It is well established that *Solea solea* is inactive during the day, spending most of its time buried in the sediment where the eggs of the parasite lie (Section VII B p. 221). Because of the inactivity of the host during the hours of daylight, this period of the day seems likely to be particularly favourable for the relatively slow-swimming oncomiracidia to make contact with their hosts; at night the hosts may be actively swimming and a relatively fast-moving fish may be a difficult target. There is an added advantage in hatching soon after dawn, because this maximizes the period of exposure of the inactive soles to the free-swimming larvae. Alternating photopositive and photonegative swimming movements of the larvae are thought to enhance the chances of host location during the hours of daylight (Kearn, 1980). The timing of the emergence of the larvae might also minimize the capture of oncomiracidia by bottom-dwelling filter feeders, detritus feeders or other predators but the significance of predation on monogenean larvae, if it occurs at all, is hard to assess.

It is presumed that a photoreceptor in the larva of *E. soleae* is responsible for monitoring day length and for controlling hatching; the possible identity of this photoreceptor has been discussed by Kearn (1973). The translucent nature of the eggshell and of the larva takes on a new significance with the appreciation of the role of light in the control of hatching.

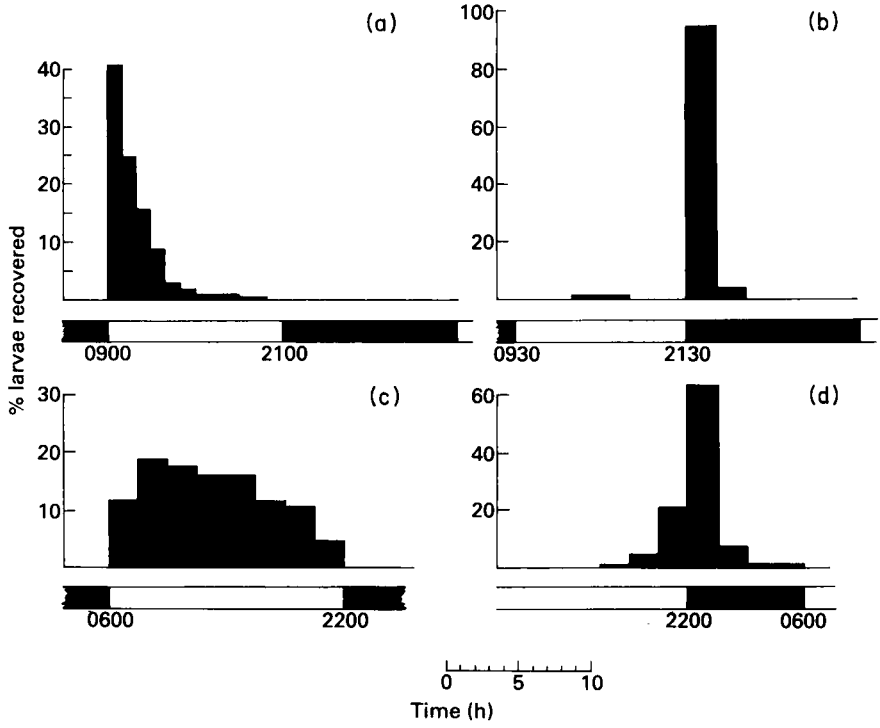


FIG. 48. The daily patterns of hatching in (a) *Entobdella soleae*, based on Kearn (1973) *Parasitology* **66,** Table 1, p. 108. (b) *Entobdella hippoglossi*, from Kearn (1974a), redrawn with permission from *Parasitology* **68,** p. 164. (c) *Polystoma integerrimum*, reproduced with permission from Macdonald and Combes (1978) *Chronobiologia* **5,** p. 279. (d) *Diplozoon homoion gracile*, reproduced with permission from Macdonald and Jones (1978) *Journal of Helminthology* **52,** p. 27. Each histogram summarizes observations made over a period of 3 days or more; in each case recoveries of larvae for each of 24 daily 1 h periods (a) or 12 daily 2 h periods (b,c,d) have been added together and given as percentage recoveries. (a) (b) Fluorescent lighting turned on and off abruptly; (c) (d) natural illumination.

In *E. hippoglossi* from the halibut, *Hippoglossus hippoglossus*, hatching occurs during the first few hours of darkness (Fig. 48b), in contrast with the "morning" hatching of its close relative *E. soleae*. Nothing is known of the behaviour of the halibut but a comparison of its sense organs and diet with those of the common sole point to diurnal feeding habits in the former, perhaps with a resting period at night, a conclusion which is consistent with the parasitological evidence (Kearn, 1974a). Hatching shortly before and after dusk was reported by Macdonald and Jones (1978) in *Diplozoon*

homoion gracile from the gills of the freshwater barbel, *Barbus meridionalis* (Fig. 48d).

Macdonald and Combes (1978) explored the hatching of the eggs of *Polystoma integerrimum* collected from the bladder of the frog *Rana temporaria*. Using natural daylight and a constant incubation temperature (10°C or 18°C) they found that hatching began during the 2 h span following dawn (0600h) and continued until dusk (2200h) (Fig. 48c). The adult frogs spawn in shallow water around the edges of the three lakes where the material was collected, and the parasite's eggs are also laid in these sites and undergo development there. During the day tadpoles congregate in this shallow warm water, whereas at night they move into deeper water; thus oncomiracidia hatching during the day are more likely to meet a host.

Rhythmical hatching has been reported in monogenean larvae without pigment-shielded eyes such as in species of *Diclidophora* by Macdonald (1975) and in *Rajonchocotyle emarginata* by Whittington and Kearn (1986). Macdonald (1975) found that hatching patterns of related species of *Diclidophora* differed, and she was able to relate these patterns to differences in the behaviour patterns of their specific hosts. Macdonald (1975) also found evidence of a seasonal difference in the hatching pattern of eggs of *D. merlangi* collected at Arbroath, Scotland and also differences between the Arbroath patterns and the hatching pattern of eggs of *D. merlangi* collected at Plymouth, England. She suggested that the former situation might reflect local seasonal differences in the behaviour of the host (*Merlangius merlangus*) at Arbroath and that the latter situation might reflect behavioural differences in the Arbroath and Plymouth stocks of *M. merlangus*. She also pointed out that pattern differences could arise by uneven sampling of a population of *D. merlangi* comprising some individuals tending to hatch at dawn and others tending to hatch at dusk. She argued that such a balanced polymorphism might give maximum chances of host location to the population as a whole.

Kearn (1975b) failed to find any evidence of a hatching rhythm in *Dictyocotyle coeliaca*, a parasite inhabiting the body cavity of *Raja naevus*.

Some hosts are parasitized by more than one species of monogenean, e.g. *Raja naevus* by the skin parasite *Acanthocotyle lobianchi*, the coelom-inhabiting parasite *Dictyocotyle coeliaca* and the gill parasite *Rajonchocotyle emarginata*. Each of these three parasites has adopted a different hatching strategy, *A. lobianchi* failing to hatch spontaneously and relying on a hatching stimulant in host skin mucus (see below), *D. coeliaca* failing to respond to skin mucus and hatching spontaneously and arhythmically (Kearn, 1975b) and *R. emarginata* also failing to respond to skin mucus but hatching rhythmically (Whittington and Kearn (1986)). Kearn (1975b) pointed out that the contrasting strategies adopted by *A. lobianchi* and by

D. coeliaca would each seem to be well adapted for the invasion of a host lacking a well marked activity rhythm, but the adaptive value of rhythmical hatching in *R. emarginata* is less clear. Whittington and Kearn (1986) suggested that monogenean larvae that rely on being drawn passively into the gill chamber of the host in the gill-ventilating current may sometimes fall victim to filter-feeding or predatory organisms, and there may be times of day when emerging larvae would be less likely to be drawn in by the feeding currents of filter-feeders or eaten by micro-predators.

It has been suggested that the activity rhythms of some hosts may be related to the tides (Kearn, 1975b). Monogeneans infecting hosts with such tidal activity patterns may have corresponding tidal hatching rhythms, but Kearn (1975b) has pointed out that such rhythms may not reveal themselves in monogenean eggs reared in an experimental environment lacking tidal cues.

2. *Hatching in response to light intensity fluctuations*

Experiments in which eggs of *Entobdella soleae* were maintained in total darkness revealed that embryonic development proceeds in these conditions and that there is no significant delay in the commencement of hatching (Kearn, 1973). Macdonald and Combes (1978) obtained similar results with eggs of *Polystoma integerrimum* incubated in total darkness at 18°C but when eggs were incubated in total darkness at 10°C hatching did not occur until one month after the expected date of hatching, unless the eggs were exposed to light. There are other indications that light intensity fluctuations might directly stimulate hatching in some monogeneans (Bychowsky, 1957, p. 93; Remley, 1942; Bovet, 1967; Tinsley and Owen, 1975) and in *E. diadema* there is good evidence that a sudden reduction in light intensity stimulates rapid hatching (within 3–5 s) (Kearn, 1982). The host of *E. diadema* is a stingray that prefers shallow, probably well illuminated water, and is likely to cast a strong shadow on the bottom when it rests or cruises slowly. A rapid, shadow-induced hatching response is likely to be distinctly advantageous for this parasite, especially if the host is diurnally active with the habit of resting intermittently on the bottom. The shadow-induced hatching stimulus will be non-specific but may still be advantageous if stingrays are abundant.

3. *Hatching in response to chemical stimulation*

Early references to hatching responses to chemical substances from the host are those of Euzet and Raibaut (1960) in *Squalonchocotyle torpedinis* and Ktari (1969) in *Microcotyle salpae*. Mechanical stimulation also seems to be

important in the hatching of *M. salpae* (see below). The way in which egg bundles of this parasite might enter the buccal cavity of the host has been referred to above (Section VII B p. 224).

Host skin mucus was found to induce rapid hatching (within 2–4 s; see Fig. 47) in the unciliated larva of *Acanthocotyle lobianchi* by Macdonald (1974), and the eggs of *A. greeni* respond in a similar way (Macdonald and Llewellyn, 1980). Macdonald (1974) found that the susceptibility to host mucus increased with the age of the eggs in *A. lobianchi* and that some unstimulated oncomiracidia survived without hatching for up to 83 days; lipid droplets may serve as a food store for resting larvae. Thus *A. lobianchi*, *A. greeni* and possibly also *S. torpedinis* and *M. salpae*, have adopted a sit-and-wait strategy relying on direct contact with the host and stimulation by host hatching factors to induce hatching, the consequence being that surface cilia are no longer required for free-swimming and have been lost in all of these parasites.

The hatching of some ciliated oncomiracidia is known to be stimulated by chemical substances from the host. Hatching in *E. soleae*, in the absence of the host (*Solea solea*), is controlled by an endogenous rhythmical process which receives its cues from the light regime, so that small numbers of larvae hatch each day soon after dawn (see above). A second strategy adopted by *E. soleae* appears to involve the maintenance of a reserve of unhatched larvae which do not respond immediately to photoperiodic cues but do respond readily when stimulated by chemical substances from a nearby host (Kearn, 1974b). It is possible that the response to host hatching factors in *E. soleae* is a relatively new development which has arisen because of the adoption by the host of sedentary habits which have increased the chances of prolonged contact between the eggs of the parasite and the host. This would explain the retention by the parasite of the parallel strategy of spontaneous hatching controlled by an endogenous rhythm, and the need to be able to swim using epidermal ciliated cells. In parasites such as *A. lobianchi*, the original strategy of spontaneous hatching may have been lost together with the ciliated epidermis of the larva, successful invasion of the host depending entirely on the sit-and-wait strategy. However, Whittington (in preparation) has found that the ciliated larva of *Leptocotyle minor*, a skin parasite of the dogfish *Scyliorhinus canicula*, hatches in response to chemical stimulation from the host but appears to have no second strategy, eggs failing to hatch if kept in sea water free from host contamination. The retention of the ability to swim in this parasite may be a reflection of the special problems involved in infecting the skin of a relatively active, round-bodied host (Section VII B p. 223). Stimulation of hatching in the freshwater monogenean *Pseudodactylogyrus bini* by mucus from the eel host has been reported by Chan and Wu (1984).

The chemical stimulus in *E. soleae* is non-specific in that skin washings from a variety of fishes that are not hosts for *E. soleae* prompt hatching (Kearn 1974b). Kearn (1974b) suggested that such a lack of specificity may not be as disadvantageous as it may seem since soles may exercise a preference for certain kinds of sea bottom and consequently may be more common than other fishes in these areas, so that a hatching stimulus is more likely to be provided by a sole than by any other non-host fish (cf. non-specific hatching of *E. diadema* eggs by shadows discussed above). Moreover, eggs of *E. soleae* stimulated to hatch by non-host fishes may still survive by exercising their preference for attachment to sole skin rather than to the skin of the alien hosts.

Kearn and Macdonald (1976) reported that eggs of *E. soleae* hatched when treated with dilute solutions of urea or ammonium chloride in sea water, and there is some evidence that arginine is effective, but no response was obtained when eggs were treated with glutamine or with trimethylamine oxide. Tests showed that the urea concentrations in sole skin mucus are comparable with the urea concentrations in the experimental solutions that stimulated hatching, but with ammonium chloride this is only so after the host had recently fed. It was suggested that ammonia may have a synergistic role whereby the effectiveness of urea as a hatching stimulant is enhanced by its presence, or that fish urine may contain effective concentrations of ammonia.

Whittington (in preparation) has made the interesting observation that hatching rarely occurs in *E. soleae* when test solutions are made up using extra-pure "Aristar" urea from BDH chemicals, England (analytical grade urea was employed by Kearn and Macdonald (1976)). This suggests either that the hatching factor is a contaminant present in the urea used by Kearn and Macdonald (1976) or that a trace substance had a synergistic effect upon the urea used by Kearn and Macdonald (1976). Tests performed by Kearn and Macdonald (1976) in which sole mucus was treated with urease also gave inconsistent results and it is clear that the identity of the chemical substances that stimulate hatching in *E. soleae* requires further study.

Using solutions of analytical grade urea in sea water Kearn and Macdonald (1976) achieved rapid hatching in *A. lobianchi* but the eggs did not respond to tests with trimethylamine oxide, with ammonium chloride or with some of the amino acids found in ray skin mucus. Tests with urease-treated ray mucus gave consistent results, the enzyme readily destroying the stimulatory properties of the mucus which were restored by addition of a small crystal of urea. Whittington (work in progress) has found that the eggs of *A. lobianchi* readily hatch when stimulated by test solutions containing "Aristar" urea. Kearn and Macdonald (1976) found that in *A. lobianchi* the hatching threshold with urea solutions was higher than in *E. soleae*; this

corresponds with their finding that the levels of urea in ray skin mucus are significantly higher than in sole mucus.

Observations on the eggs of *L. minor* made by Whittington (in preparation) indicate that dogfish skin washings, urea ("Aristar" grade) and ammonia may be effective as host hatching factors.

4. *Hatching in response to mechanical and other stimuli*

Bovet (1967) claimed that water turbulence promoted hatching in *Diplozoon paradoxum* and Ktari (1969) found that several larvae of *Microcotyle salpae* hatched when the eggs were disturbed by a jet of water from a pipette; a chemical hatching stimulus was also implicated in the hatching of *M. salpae* (see above). Agitation of the water was found to induce the hatching of the unciliated larvae of *Neonchocotyle pastinacae*, a gill parasite of the stingray *Dasyatis pastinaca*, by Ktari and Maillard (1972) but the eggs did not respond when placed with host ventral skin mucus and gill fragments.

Hatching was found to be rapid in the monocotylid *Dendromonocotyle kuhlii* when fully developed eggs were transferred to a glass slide and observed with the microscope, some larvae emerging before the eggs were focused (Kearn, unpublished observation). On some but not on all occasions, when eggs were disturbed with a needle, larvae emerged immediately, but further experiments are required to determine whether mechanical disturbance is the only effective hatching stimulant or whether heat or light generated by the microscope lamp play a part in hatching. Tinsley and Owen (1975) also described the almost simultaneous hatching of large numbers of eggs of *Protopolystoma xenopodis* when transferred to a microscope stage and they suggested that the eggs might have responded to the range of "shock" stimuli including heat, light and mechanical disturbance to which the eggs were subjected during the transfer. Such a range of stimuli might be experienced in the natural environment since the aquatic toad host is likely to stir up pond sediments and propel the eggs into warmer, better illuminated waters.

It is possible that an osmotic hatching stimulus may play a part in the activation of the larvae of polystomatids, such as *Pseudodiplorchis americanus*, that hatch immediately when the eggs enter rain-water pools (p. 251).

XII. Summary and Conclusions

An attempt has been made for the first time to review the work that has been done, especially during the last 30 years, on the eggs of monogeneans. Most monogeneans assemble operculate eggs with rigid shells made of tanned

protein and, in general, these eggs undergo embryonic development for varying periods outside the host and on hatching liberate a ciliated, free-swimming larva.

A survey of the whole spectrum of the eggs of monogeneans reveals a surprising range of sizes and shapes. Some of the latter, such as the tetrahedron, appear to be unique in the parasitic platyhelminths and possibly in all the invertebrates. The significance of some of these shapes in relation to phenomena such as gaseous exchange and egg sedimentation has been considered for the first time in this review. A mathematical analysis of egg shapes indicates that the tetrahedral egg and the elongated spheroidal egg may have advantages in terms of gaseous exchange which may be significant in those eggs developing in micro-environments low in oxygen; similar egg shapes may serve to prolong the time spent in suspension by eggs laid by parasites of pelagic hosts. Egg appendages are also more common in monogeneans than in their parasitic platyhelminth relatives; these appendages include short stout hooks, appendages of medium length sometimes supplied with adhesive droplets, and long, slender threads. Other features of monogenean eggs that have been identified include possible daily egg-laying rhythms, egg-hatching rhythms, responses to hatching stimulants from the host, seasonal restriction of egg production, egg retention by the parasite and seasonal egg-laying, and possibly the production of diapausing eggs.

All of these features, together with the variation in the shapes and sizes of eggs and in their appendages, appear to be more closely related to the diversity of the habits and habitats of their vertebrate hosts (mainly fishes and amphibians) than to the phylogeny of the parasites. This is well illustrated by the genus *Entobdella*. Some species produce tetrahedral eggs and others spheroidal eggs and the appendages vary in length considerably from species to species, having adhesive droplets at intervals in *E. soleae* or at the end of the appendage in *E. australis*; there are also differences between the species in the timing of their hatching rhythms in relation to the daily cycle of illumination and in the responses of the eggs to stimuli from the host such as chemical substances and shadows.

Some polystomatid monogeneans produce eggs which have relatively thin, flexible shells. This feature appears to be associated with ovoviviparity, and an investigation of the way in which these eggs are assembled and the chemistry of the eggshell is awaited with interest.

Tanned eggs (or cocoons) are of widespread occurrence in platyhelminths, having been found not only in monogeneans but also in free-living turbellarians, most digeneans and some cestodes, and there is evidence that monogeneans and cestodes share a common ancestry, possibly from rhabdocoel-like turbellarians (Llewellyn, 1965). The direction of cestode

evolution has led to different selection pressures on their eggs. Perhaps the most significant development is the acquisition of intermediate hosts which has led to the requirement for tapeworm larvae to gain access to the gut of these intermediate hosts. Some fish tapeworms produce tanned, operculate eggs but in many cestodes (for example in cyclophyllideans) tanning and the operculum have been lost and additional new external coverings for the larva have been derived from the external layers of the embryo itself. Without the protection provided by a tanned shell, these external layers are removed by the digestive enzymes of the intermediate host when the eggs are eaten, thereby releasing the larva. A parallel development in tapeworms has been an increase in egg output, achieved by the multiplication of genitalia and the provision of many ootypes.

The changes that have taken place in the role of the tapeworm egg in infection of the intermediate hosts appear to have demanded little adaptive change in terms of egg shapes and appendages. Most tapeworm eggs are ovoid or spherical and lack appendages. However, Jarecka (1961) has described some cyclophyllidean eggs that have departed from the ovoid or spherical shape, and these changes appear to enhance their chances of reaching and being eaten by specific, aquatic intermediate hosts. The relatively small size of tapeworm eggs may be a contributing factor to the uniformity of their egg shapes; small, ovoid or spherical eggs have a relatively large surface area per unit volume for exchange of gases and other solutes, and there may be no requirement to increase the surface area by departing from these shapes.

Digeneans are most probably more distantly related to the monogeneans than the latter are to the cestodes (Llewellyn, 1965) but nevertheless most digeneans resemble the monogeneans in producing tanned, operculate eggs which release ciliated free-swimming larvae. These digenean larvae usually penetrate the soft, external surface of their molluscan hosts. However, most digenean eggs are relatively small, lack variety in shape (mostly ovoid) and rarely have appendages. This conservatism of shape may again be related partly to the small size of the eggs but may also be the consequence of the lack of diversity in the habits and habitats of their slow-moving mollusc hosts. Claims have been made that the rate of egg production in some digeneans is significantly higher than in monogeneans, but it is hard to imagine how this is achieved because, like the monogeneans, digeneans have a single ootype. A phenomenon which may contribute to increased egg production in digeneans is the reported ability of some species to complete egg assembly in the uterus.

There have been extensive comparative studies at the light microscope level of the ootype glands and their deployment in the cestodes and in the digeneans. However, although monogeneans are probably the least-

specialized of the three major groups of parasitic platyhelminths and may have diverged least of all in terms of ootype architecture and function from their free-living platyhelminth ancestors, there has been no similar study of monogeneans. In fact, in the few monogeneans that have been examined, the picture is confused and in some groups of monogeneans such as the microbothriids the ootype and its glands have received little attention. The possession of a genito-intestinal canal in polyopisthocotyleans is of special interest and may possibly be a primitive feature.

It is well established that in those platyhelminths assembling tanned eggs, components of a quinone-tanning system are present in the vitelline droplets which fuse to form the eggshell, but the old concept of quinone-tanning in insect cuticle has been challenged recently and there is a need for a re-examination of this process in the platyhelminths. It has emerged also from this review that our knowledge of the events of eggshell assembly is still incomplete, and our understanding of the roles of the secretions produced by the ootype glands has made little progress since speculations about their function were summarized and discussed by Dawes in 1940. Living monogeneans may provide a way forward for research, because the clarity with which the stages of egg assembly can be viewed in some living monogeneans (particularly capsalids), offers an opportunity to "freeze" the ootype by the application of fast-acting preservatives at selected times and to compare, using the electron microscope, successive events in egg assembly. The lack of attention paid to living material in the past is probably reflected in the absence of references to rhythmical ootype contractions in many accounts of egg assembly in parasitic platyhelminths, and yet these movements seem likely to be of fundamental importance in transporting vitelline droplets to the ootype wall.

Acknowledgements

This review was prepared during sabbatical leave kindly granted by the University of East Anglia. I am particularly grateful to Dr. P. C. Croghan of the University of East Anglia for his interest in the curious shapes of monogenean eggs and for his help with the mathematical analysis of egg shapes. Critical comments on the manuscript by Professor Jack Llewellyn of the University of Birmingham were much appreciated and have greatly improved this review. I also wish to record my special thanks to Mrs. Audrey Cox who typed the final draft efficiently and quickly in her own time.

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Biochemical Strain Variation in Parasitic Helminths

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ADVANCES IN PARASITOLOGY VOL. 25
ISBN 0-12-031725-7

I. INTRODUCTION

A. GENERAL

There is little dispute over what constitutes a genus of organisms, but there is considerable debate about the nature of a species. Probably the most useful definition is the one that approaches real life most closely. Species are reproductively isolated populations that do not (not "cannot") exchange genes with neighbouring populations. However, the process of speciation is continuous, so several adjacent populations may exist between which there are different levels of gene exchange. At high levels there is little chance that the populations will diverge sufficiently to become separate entities. At very low levels there is every chance that separation will occur. Somewhere in this continuum of gene flow lies that elusive point at which an observer might say that the populations are sufficiently different to warrant their description as variants or strains.

Darwin (1859) clearly recognized that definition depends on authority. He wrote that: "No one has drawn any clear distinction between individual differences and slight varieties; or between more plainly marked varieties and sub-species . . . what a multitude of forms exist, which some experienced naturalists rank as varieties, others as geographical races or sub-species and others as distinct, though closely allied species." And later, he commented ". . . species are only strongly marked and permanent varieties . . ."

Given this ambiguity, it is perhaps best to agree with Simpson (1961) that an evolutionary species is a lineage that evolves separately from others.

B. VARIATION AND POLYMORPHISM

A taxonomist, using so-called objective data, will have little difficulty in identifying strains on the basis of morphological information. Linear dimensions, relative positions of internal and external organs, and numbers of repeating parts are all criteria that can be called upon to enable an assessment of systematic status, as Kumaratilake and Thompson (1982) have done for *Echinococcus granulosus*. However, underneath morphological changes there are the first causes. Schopf (1982) comments that palaeontologists recognize that skeletal change in fossil "hard parts" may underrate total mutational change by a factor of ten. Assuming that a proportion of the remaining 90% is expressed as morphological changes in "soft parts", there surely remains a substantial residue of mutations that are concerned with

function rather than morphology as perceived by the taxonomist. Neither is it wholly clear where the morphologist stops and the physiologist or biochemist begins. Is a change in the mitochondrial architecture a morphological change or a functional one? Is a change in the tertiary structure of an enzyme morphological or functional—or both? In the end, it appears that the decision depends upon the nature of the technique used to detect the variation: a point discussed in more detail below.

The first causes of variation may be changes in that part of the genome of the organism that is expressed, or in that part that regulates expression. Variation may result from changes in the interrelationships of the secondary products of gene activity, the proteins, perhaps brought about by a subtle alteration of the cellular environment. However, the most important criterion in the establishment of a variant is that, whatever the source of the difference between the two populations of organisms under scrutiny, those populations must be stable. That is, if one population is removed from the possibility of any gene exchange with neighbouring populations, it will retain the characteristics that distinguish it from the other populations over an indefinite number of generations. It may even diverge further from the parent population under the triple influences of restricted gene exchange, genetic drift and unique selection pressures.

The phenomenon of the existence of a true variant of a species must be clearly distinguished from that of genetic polymorphism. Balanced polymorphism is the condition that occurs when an isolated population continues consistently to produce two or more morphs, the frequency of occurrence of those morphs being in Hardy-Weinberg equilibrium. With unrestricted gene flow any subgroup of that population will be capable of producing the full array of different types. A famous example is the polymorphism of banding patterns in snails of the genus *Cepaea* (Cain and Sheppard, 1954). In this example, there is differential survival of morphs under seasonal environmental changes and so they are maintained in the population. Polymorphisms may become unstable, however, so that a single phenotype goes to fixation. Such a phenomenon provides the basis for the formation of new, permanent strains. If a subpopulation becomes isolated and its gene pool is heavily selected—bottlenecked—its capacity for producing all the morphs of the parent population may become severely restricted, and a single morph may become established as a variant in its own right. There is some evidence for believing that this is the mechanism by which anthelmintic resistant strains of *Ostertagia circumcincta* become established (Le Jambre *et al.*, 1978). They are sustained in their hosts by virtue of a change in their environment—regular or routine anthelmintic challenge—but are normally present in the "wild" population in small numbers only.

These polymorphisms must also be distinguished from environmentally

induced polymorphism, in which there is a direct interaction between an environmental parameter and a physiological process, such as that observed in rotifers and in some lake-dwelling *Daphnia* species that undergo a process known as cyclomorphism. In the cooler seasons, spring and autumn, some *Daphnia* are normally round-headed but in the summer, as the water temperature increases, their heads elongate to assume a helmet-like shape. There is little doubt that both water temperature and internal, physiological factors are involved; genetic factors are involved only in the sense that genetic factors underlie all biological processes (Pennak, 1978). A parallel example is to be found among the trematodes. Dinnick and Dinnick (1964) report that, in Kenya, the life cycle of *Fasciola gigantica* includes only rediae in the molluscan host when the temperature is held below 16°C. At a constant 26°C, however, first-generation rediae produce rediae and cercariae alternately. Normally, this trematode also possesses a sporocyst generation. It is not difficult to imagine, in the light of the foregoing, how such a variant became established, and this further testifies to the flexibility of parasite life cycles. The *Daphnia* example nicely illustrates morphological variation, whereas that of *Fasciola* shows variation in the timing of development, a non-morphological characteristic.

C. ADAPTIVENESS

It is not clear that strain variations in parasitic helminths are necessarily adaptive. Certainly they may be if they arise in direct response to some identifiable ecological change, so that a speciation event can be recognized. However, it is indisputable that many variations arise stochastically, by random isolating events in the environment. These variants are the raw material upon which selection acts if a speciation event subsequently does occur. Naturally, for two populations genetically separated, events that induce speciation may have different end-products given that the gene pools are even ever so slightly different. A good example is *Echinoccoccus granulosus* and the separation of horse and sheep strains in the UK (Thompson, 1977). The stochastic event is its first establishment in a sheep or a horse; the "speciation" event is the contrived separation of sheep and sheep-dogs and horses and hounds.

The adaptive nature of strain variation is thus especially problematical for the growing group of systematists who exploit the phenomenon of enzyme polymorphism as an aid to distinguishing between species. This is discussed in more detail in Section II.

Another important point is that it is unlikely that organisms are precisely adapted. Precision of final form and regulatory system of any organism may

have to be sacrificed to the physical constraints of geometry or molecular interaction. Further, an organism is subject to many simultaneous selection pressures which may interact in conflict. The final organism is a compromise between precise adaptation and what is possible, given the starting materials at that particular time. This is clearly recognized by such authorities as Ernst Mayr (1983): "Evolutionary change falls far short of being a perfect optimization process. Stochastic processes and other constraints upon selection prevent the achievement of perfect adaptedness. Evolutionists must pay more attention to these constraints than they have in the past. However, as already stressed by Darwin (1859, p. 201) there is no selective premium on perfect adaptation."

It is a truism worth repeating that organisms are adapted to their environments at all stages of their life cycles. However, helminth parasites, like insects, often have a number of discrete stages in their life histories. A life cycle may require that successive life stages are adapted to very different environmental conditions or hosts. For example, the free-living larvae of many nematodes are adapted to life on pasture where oxygen is plentiful, while the adults are parasitic in the rather more anoxic intestinal environment in a ruminant. *Hymenolepis diminuta* survives as a larva in the tissues of a great number of Coleoptera, while the adult is adapted to the intestine of rodents. Each stage requires its own, specific developmental program, and each is, of course, acted on by forces that may induce speciation. However, as the child is father of the man so the larva is parent of the adult, and adaptation at one developmental stage cannot be independent of adaptation at another.

D. THE FOUNDER EFFECT

The term "founder effect" was first used by Mayr (1940) to explain the peculiar properties of isolated populations whose genetic constitutions did not reflect those of the parent populations but rather the small subset of organisms that founded them. It is extremely apposite when applied to parasites, for parasites live in discontinuous environments. Each host is effectively an island, and is subject to the same constraints of colonization. Very often infection is by a relatively small number of organisms that cannot possibly represent all the possible genetic combinations of the population at large, and so a founder effect operates with a very high probability of producing a distinct strain. This will not happen if the progeny of the isolated population becomes commixed with the parent population at the next generation, nor if the sample of the population in a single "island", or host, is sufficiently great to be representative of the whole population. However,

the reverse often occurs. Many parasites incorporate asexual amplification stages into their life cycles, so that colonization by a single isolated individual is often possible, and leads to the establishment of a separate clone within an individual host. It may be this process that is operating when a parasite, such as *Echinococcus granulosus*, extends its range, establishes in a new host and produces a clone which is capable of surviving the novel conditions provided by that host.

A rapid artificial means of bringing about strain differentiation is by cultivation. This is, of course, the commonly accepted way of establishing any parasite in a laboratory. In effect, it involves taking a small sample of organisms from a wild population, in which only a tiny fraction of the total genetic variability of the population can be represented. There is therefore a high probability that strain differences will emerge sooner or later.

E. METABOLIC VARIATION

Although up to now we have discussed primarily the widely accepted morphological variation that enables taxonomists to identify strains and species, other modes of variation must not be ignored. As we have already remarked, morphological variation implies that a whole host of physiological, developmental and biochemical characteristics also vary between strains. From here, it is a simple conceptual leap to the view that many of the variations present at this level of organization need not be represented in morphological features at all, but may be expressed in other ways—for example, in host preference, behaviour, development, or biochemistry. This review is concerned with the last of these.

The possible causes of variation between helminth parasites are legion, and the possible sources of variation with individuals of a population scarcely less so. As remarked earlier, strain variation may originate in changes in the genome or in the way that the genome is regulated. Thus, although the strains may have identical genomic information, changes in the milieu in which that information is expressed may give rise to differences. Another possibility that may give rise to the establishment of variants is change in the interactions between secondary gene products. Then there are the effects of hosts on parasites. The host may constrain phenotypic expression by imposing limits upon the development and metabolism of the parasite, as, for example, in the extreme case of a large parasite such as *Fasciola hepatica* in a small host, the rat (Rajasekariah and Howell, 1977). The constraints can be imposed in at least three ways. The first depends directly on the physical and chemical nature of the environment provided by the host and the capacity of the parasite to respond to those environmental

cues. The second depends on the capacity of the host to respond to cues offered by the parasite. This includes all aspects of the immune response, and few would deny that they must generate enormous selection pressures on helminths. Finally, it is possible that the host selects only those parasites that are capable of surviving in the particular environment that it offers. It is part of the adaptive programme of parasites to be opportunistic, and doubtless all these selection pressures are frequently encountered by helminths when they find themselves in new and unusual hosts.

At first sight, it might appear that metabolic pathways, the mechanisms by which the gene products do their work, should be invariant, or at any rate vary only in the sense that their output varies when more or less of the products of their activities are required by the organism. Much of the traditional biochemistry is in fact carried out on metazoan organisms that appear to function in this way. Thus, a liver possesses, among a myriad of functions, pathways for the synthesis or degradation of glycogen, for respiration and for maintaining redox balance, which are finely tuned between limits that can be exceeded only to the detriment of the organism. A mammalian biochemist will confidently expect that rat livers—even rodent livers—will conform to the same general pattern of components and functions. Such a person will expect to work on a metabolic pathway and will not seriously entertain the possibility that Wistar rats and Hooded rats are sufficiently different to possess generically different metabolic pathways. This is the more surprising because other workers on mammals, the immunologists, have long since recognized strain variation in their favourite organisms as a valuable research tool (Mitchell, 1979).

Yet those who work on helminth metabolism have only to turn to the biochemistry of prokaryote organisms for inspiration. It is well known to microbiologists that closely related bacteria may have widely different metabolic pathways. These pathways may not always be detectable in an individual cell, but may be expressed at different times or in response to different nutrients or inhibitors. A microbiologist would have no *a priori* inhibitions to prevent the consideration of alternative pathways, even for a process that, on the face of it, seems as conservative as respiration.

There are two concepts that biologists have invoked in the past but that are, in their operation, contradictory. The first is the so-called law of parsimony (e.g. Weatherly, 1965), which suggests that an organism will perform any function in the most economical way consistent with the limitations put upon it by the properties of its component parts. This implies that once a thermodynamically efficient way of carrying out a process has evolved, then that process will be conserved. Unfortunately, there is no justification for the view that evolution is parsimonious (Johnson, 1982). So while some aspects of biology may exhibit parsimony other aspects may be

spectacularly extravagant. Or, and perhaps this is the most important qualification of all, they seem that way because we are not in possession of all the facts. Parsimony, then, cannot be generally recruited to help us understand organisms. Some phenomena are parsimonious; for example, the active centres on enzymes such as cytochrome oxidase may be conserved through epochs (Chapman and Schopf, 1983). Others, such as the haemoglobins in human populations, of which there are hundreds of variants, are certainly not (Cavalli-Sforza and Bodmer, 1971).

Another principle often invoked in an attempt to understand variation in organisms, and a favoured view of those who regard organisms as regulatory control systems, is that of optimization. An optimized system is one of compromise; perhaps a less efficient mechanism is utilized because other functions impose demands which it alone can satisfy. For example, other as yet unclear functions of lipids in parasitic helminths are presumably more important than their use as an energy source, even though a thermodynamic "imperative" demands that lipids be used immediately as the most abundant and highest yielding source of ATP (Frayha and Smyth, 1983). Optimization is often confused with maximization. A maximized system is one in which the most thermodynamically efficient process is always turned full on, consistent with the resources available. It is clearly not an advantageous strategy for any organism. Successful survivorship requires husbanding of resources and their optimal allocation to essential functions. Strategic compromise is characteristic of organisms and determines the limits of what is possible rather than what is thermodynamically elegant. Flexibility, the capacity to adapt to the widest possible range of variable environments, resides in compromise.

II. Protein Polymorphism

Webster's Third New International Dictionary defines a parasite as "an organism living in or on another living organism, obtaining from it part or all of its organic nutriment, commonly exhibiting some degree of structural modification and causing some degree of damage to its host". Structural modification pervades all levels of the helminth organization, and this section discusses its occurrence at the molecular level.

Harris (1980) described polymorphism as "a term used to specify a situation in which members of a naturally occurring population can be categorized into sharply distinct phenotypes which are determined by two or more alleles at a given gene locus and in which the different phenotypes are each relatively common in the population". The idea that the possession of high levels of polymorphism is usual in natural populations was not firmly

established until 1966, when surveys of allozymic variation yielded direct estimates of genic heterozygosity for *Drosophila pseudoobscura* (Lewontin and Hubby, 1966) and for human populations (Harris, 1966). Extrapolation from these electrophoretic surveys of protein variation led to the conclusion that most diploid organisms are polymorphic at thousands of structural gene loci and that every individual has a unique protein complement (Harris, 1970). However, it was acknowledged that levels of genic heterozygosity, as measured by surveys of allozymic variation, are much lower in populations of large mobile animals, such as most vertebrates, than in those of small, relatively immobile animals (most invertebrates). The differences are not consistent with hypotheses relating to population size and species number nor to dispersal ability and gene flow, but are predicted by Levins' theory (1968) of adaptive strategies in the face of environmental uncertainty. Mobility and a degree of homeostatic control apparently are important factors that influence the levels of genic heterozygosity in natural populations, and observations argue indirectly that at least a major proportion of allozymic variation is maintained by natural selection (Selander and Kaufman, 1973).

A. THE DETECTION OF POLYMORPHISM

For taxonomic purposes, such as solving systematic or evolutionary problems, most polymorphisms are detected by initial electrophoretic separation of proteins and subsequent staining, either for proteins generally or for specific enzyme activities. Electrophoresis has become a popular technique because it is easy to use, large numbers of samples can be analysed comparatively quickly, it is relatively inexpensive and the results are quite easy to interpret. Other methods in use are isoelectric focusing, immunological techniques and now the various methods of recombinant DNA technology that are available.

Electrophoresis has some limitations, as it only detects certain types of molecular differences—that is, those which have resulted in a net change in the charge of a protein molecule, for amino acid substitutions that cause no change remain undetected. Neither will it detect synonymous mutations, where a single base change in a nucleic acid has simply altered one codon to another that specifies the same amino acid in the protein molecule.

Ability to detect enzyme polymorphisms is dependent on the techniques available, and over the past 20 years they have improved considerably with the development of better supporting media for electrophoretic separation and new methods of staining enzymes. However, it is probable that many variants remain undetected because of the inadequacy of existing

techniques, and even the new staining techniques will not detect those enzyme variants whose catalytic function has been lost because of intrinsic or extrinsic factors beyond the control of the experimenter.

B. ASSESSMENT OF TAXONOMIC SIGNIFICANCE

Many enzymes are genetically polymorphic and so individuals within a population differ from one another. However, the differences between populations, and therefore frequency distributions of the various morphs, are what is taxonomically significant, not just differences between individuals (Ayala, 1983). In outcrossing sexual organisms, genotypic frequencies can be estimated from the allelic frequencies by means of the Hardy-Weinberg equation, to which these populations generally approximate closely. The degree to which the distribution of genotypic frequencies of any two populations overlap can then be determined for each gene locus. The average of all the loci studied provides an estimate of the amount of genetic differentiation between populations, and a large number of statistical methods have been devised to quantify it.

Genetic differentiation in organisms may arise either because many loci have a moderate amount of differentiation or because there is complete differentiation at only a few loci, with the remaining ones showing great similarity. For taxonomic purposes, these two situations are quite distinct. A locus at which complete differentiation between two populations exists can be used to determine the population from which an individual is derived, but a locus at which only partial differentiation occurs cannot be so used. It is possible to use jointly several loci at which two populations are partially different, but the statistical manipulations are more complex. These considerations obviously have practical significance in, say, the correct diagnosis of human helminthiases in the field.

C. MAINTENANCE OF POLYMORPHISM

There are two arguments that attempt to explain how enzyme polymorphisms survive the selection process. They were originally formulated to accommodate the view that the maintenance of variation in a population should entail some sort of evolutionary cost (Kimura and Crow, 1964). Firstly, there is the hypothesis of selective neutrality, in which electrophoretically different alleles are considered to be functionally identical, having an equal effect on the fitness of the organism, so that natural selection does not discriminate between them. Secondly, there is the more general

argument that degrees of enzyme polymorphism reflect physiological function, some classes of enzyme being more variable than others. The two points of view are incompatible and there are problems with both of them.

For example, a number of requirements must be satisfied before a judgement can be made that the existence of a particular enzyme polymorphism is due to selective processes and therefore adaptive (Clarke, 1975; Koehne, 1978).

They are:

(a) The genotypes of the assemblage under consideration must display phenotypic diversity which can be described in terms of the differing molecular functions of the enzyme polymorphs. This is a particularly difficult criterion to satisfy as, for most animal populations, the best that can be hoped for is a strong correlation between phenotype and the presence of a given isozyme.

(b) The different molecular functions must be relevant to the metabolic economy of the organisms. In other words, polymorphism in an enzyme that serves a peripheral purpose, such as that of detoxifying a class of compounds, some of which may never be encountered, is neither adaptive except in a very general sense, nor useful in assigning systematic status, as the enzyme will lack the conservatism imposed by relevant selection pressure.

(c) A specific locus on the genome must interact with a specific ecological component by means of the enzyme produced at that locus. There are a number of instances of this in the literature, but it must be said that they are exceptions rather than the rule. Good examples are those enzymes, such as pyruvate kinase in *Mytilus*, whose polymorphisms vary with degree of anaerobiosis and temperate acclimation (Holwerda *et al.*, 1983). Unfortunately, even in these cases, the relationship is one of correlation and not necessarily cause and effect.

(d) The phenotypic differences must confer some measure of fitness. Fitness is so difficult to define, let alone determine, that we feel a discussion of it here would be out of place and the reader is referred to chapter 10 (An Agony in Five Fits) in Dawkins (1982).

The great difficulty in meeting these criteria has maintained the gulf between those, like Kimura (1983), who continue to argue that enzyme polymorphisms are not adaptive (neutralists) and those, like Cain (1979), who are adaptationists. The neutralist position is now supported by the

concept of genetic drift, and by the idea that the genome of an isolated population suffers stochastic changes and that the sum total of those changes will be different from those experienced in an adjacent but isolated population of similar organisms.

A sensible view is that both neutral and adaptational changes take place in the molecular evolution of organisms. However, it is interesting to note that the literature is beginning to extend beyond laboratory models. Recently, Lavie and Nevo (1982) sampled two species of marine gastropods and showed that the polymorphism of phosphoglucose isomerase was related to survivorship in the presence of heavy metal pollution.

D. FUNCTIONAL SIGNIFICANCE

Although most enzyme polymorphisms are discovered by their electrophoretic characteristics, it should be noted that, although the differences indicate a modified protein structure, such characteristics do not provide information about possible functional changes in enzyme activity. The search for functional differences between enzyme phenotypes which make up the different polymorphisms is obviously important, but information is scant because it is a difficult problem to address.

One type of variation which has been reported is in the total amount of a given enzyme activity in different phenotypes, but these differences are not necessarily associated with differences in electrophoretic mobility. There is evidence for such variation in about half the known enzyme polymorphisms in humans but, of course, enzyme assays *in vitro* may be a poor indicator of function and activity *in vivo*. However, in the human condition reduction in enzyme activity may result in a functional abnormality that can be detected clinically (Harris, 1980).

There are several hypotheses proposed for the physiological role of enzyme polymorphism (Johnson, 1974). Gillespie and Kojima (1968) proposed that the levels of enzyme polymorphism reflected environmental variation in substrate availability. Their work on natural populations of *Drosophila* indicated that greater variability occurred in "non-glucose" metabolizing enzymes. This, they suggested, might reflect the greater range of substrates, many of which originate in the external environment. Indeed, enzymes of broad specificity have been found to be far more variable than those enzymes utilizing specific, metabolically produced substrates.

Polymorphisms have also been associated with regulatory reactions in metabolism. Johnson (1971) suggested that those enzymes that exert control over the flow through metabolic pathways should be most sensitive to

selective forces. He argued that selection must act ultimately upon the reproductive fitness of the individual and the contribution of particular metabolic sequences to that fitness, so that selective advantage must be considered in terms of overall pathway output rather than in terms of the specific reactions. Changes at loci whose enzymes regulate flow through metabolic pathways would be expected to produce far greater alteration of fitness than changes affecting enzymes which do not regulate metabolic flow. Regulatory enzymes can be of several types; non-equilibrium enzymes that initiate metabolic pathways, enzymes at branch points in metabolism and multifunctional enzymes. There are many factors which may complicate the regulatory patterns, such as intracellular compartition of metabolites, metabolic differences between tissues, developmental alterations of metabolism, species or strain differences, and response to divergent physiological conditions.

Various surveys of the incidence of polymorphism in different types of enzymes have been conducted (Johnson, 1974; Harris, 1980). It would appear that increased levels of heterozygosity do, in fact, occur in enzymes which have either a regulatory function or a wide substrate specificity. These observations suggest that enzyme polymorphisms provide a means of compensating metabolically for a varying environment and therefore confer genetic advantage on the organism. Individuals with multiple molecular forms of an enzyme may be capable of minimizing the effects of changed reaction conditions. It is well documented that isozymes can differ in a number of ways apart from electrophoretic mobility; for example, their kinetic properties may differ, their concentrations may differ from one tissue to another, and they may change during development. Other metabolically significant differences include changes in pH optima and isoelectric point. Our understanding of the details of isozyme function is yet at an elementary level.

III. Helminth Strains Detected by Electrophoresis

A. TREMATODES

The most widely investigated genus of trematodes is *Schistosoma*. Some ten species and 19 enzyme systems have been described (see Table 1, which also gives the list of abbreviations of enzyme names that are used in the text). Most of the work has been concerned with species identification in an attempt to solve epidemiological problems, such as that of distinguishing between cercariae of different species which cycle in the same snail host, as

S. mansoni and *S. rodhaini* in *Biomphalaria* (Fletcher *et al.*, 1981a), or that of unravelling species complexes (Ross *et al.*, 1978).

1. Schistosoma mansoni

The early papers by Conde-del Pino *et al.* (1966, 1968) and Coles (1970, 1971a,b) on *S. mansoni* from Puerto Rico and East Africa, respectively, reported differences in intensity of staining patterns, differences between

TABLE 1 *Enzyme electrophoretic studies of schistosomes*

S. mansoni		***S. haematobium***		***S. margrebowiei***		***S. japonicum***	
ALP	1,2,4	ES	5,10	LDH	16	MDH	8,18,19
ICD	1	ACP	5,15	MDH	16	LDH	19
ASAT	1	PEP	5	ACP	16	G6PDH	8,19
G6PDH	1–5,7,8,10	GDH	5	***S. mattheei***		GAPDH	19
MDH	2–12	6PGDH	5	MDH	10,15,16	GDH	19
LDH	2,4–6,9,10	***S. bovis***		LDH	10,15,16	HK	8,19
GDH	2–5	GPI	14	G6PDH	10	PGM	8,19
ES	5,6,10	MDH	12,15,16	ES	10	ACP	19
PEP	2–5	LDH	15,16	ACP	15,16	GPI	8,19
ACP	2–5	ACP	15,16	***S. rodhaini***		6PGDH	8
6PGDH	5,8	***S. intercalatum***		MDH	3,7,10	***S. mekongi***	
FK	7	GPI	15,17	LDH	10	LDH	19
PGM	2–4,7,8	PGM	17	G6PDH	3,7,10	MDH	19
DIA	2,4	G6PDH	17,	ES	10	G6PDH	19
HK	2–4,7,8	LDH	15,17	GPI	3,7	GAPDH	19
ALD	2–4	MDH	15,17	MPI	3,7	GDH	19
AK	2–4	ACP	15,17	HK	3,7	HK	19
GPI	2–4,7,8,13	HK	17	AK	3,7	PGM	19
MPI	2–4,7	***S. leiperi***		FK	7	ACP	19
S. haematobium		GPI	14	PGM	3,7	ALD	19
GPI	14	LDH	16	ALD	3	GPI	19
LDH	5,10,15	MDH	16	ACP	3		
MDH	5,10,12,15	ACP	16	GDH	3		
G6PDH	5,10			PEP	3		

Key ACP, acid phosphatase; AK, adenylate kinase; ALD, aldolase; ALP, alkaline phosphatase; ASAT, aspartate aminotransferase; DIA, diaphorase; ES, esterase; FK, fructokinase; GAPDH, glyceraldehyde phosphate dehydrogenase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; GPI, glucose phosphate isomerase; HK, hexokinase; ICD, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MPI, mannose phosphate isomerase; PEP, peptidase; 6PGDH, 6-phosphogluconate dehydrogenase; PGM, phosphoglucomutase.

References 1, Conde-del Pino *et al.* (1968); 2,3,4, Fletcher *et al.* (1981c,a,b); 5,6, Coles (1970,1971a); 7, de Boissezon and Jelnes (1982); 8, Agatsuma and Suzuki (1983); 9, Conde-del Pino *et al.* (1966); 10, Coles (1971b); 11,12, Rotmans (1978a,b); 13, Jelnes (1983); 14, Ross (1977); 15, Ross (1976); 16, Ross *et al.* (1978); 17, Wright *et al.* (1979); 18, Yan *et al.* (1976); 19, Fletcher *et al.* (1980).

cercariae and adults, and strain variation within the East African isolates. However, Coles (1971b) reported that the patterns changed after passaging and concluded that the isozyme staining technique would probably prove to be of no use in characterizing field strains. Unfortunately, pooled samples of worms were used for these experiments and so little of relevance to strain variation can be attached to the results.

Fletcher *et al.* (1981c) were the first to carry out a detailed survey of polymorphisms in *S. mansoni*. They used individual worms so that they could detect differences between individuals in a population and obtain an accurate estimate of the amount of variation. They studied 14 enzyme systems, representing approximately 18 gene loci, and found that genetic variation, average heterozygosity at a single locus and the proportions of polymorphic loci were low for most of the 22 populations, Old and New World, that were examined. The genetic divergence between the geographical strains was slight, which supports the hypothesis that the American parasite was a recent introduction from Africa by way of the slave trade. In evolutionary terms, the American parasite has been isolated from the parent population for a very short time. de Boissezon and Jelnes (1982) also found very little intraspecific variation in *S. mansoni* populations from different geographical areas, and none of their isolates was characterized by a unique phenotype. A comparison of recent isolates with those maintained for many generations in the laboratory showed that they had only slightly higher values of polymorphism and heterozygosity (Fletcher *et al.*, 1981c). This is probably due to several factors—a founder effect, because they had been isolated from only one human or just a few snails; a population bottleneck because the strain had been passaged through a small number of snails, thereby restricting the gene pool; and the possible selection against certain alleles by cycling through laboratory hosts. Thus, an important point raised by these authors is that laboratory strains may indeed lack the complete genetic variability found in the natural population and are therefore atypical.

Schistosomes are particularly interesting digenetic trematodes because they are dioecious. Sexual differences have been seen in some enzymes, for example, LDH and MDH-2 and PEP (Fletcher *et al.*, 1981c). Differences in staining intensity are likely to be due to the lower concentrations of enzymes in samples from females and not necessarily to differences in the activities of the isozymes themselves. LDH is such an example; it had a very low activity and could only be detected in pooled samples, but in this case the enzyme from females did have a different mobility. So, too, did MDH-2, while PEP had an extra band. The extra band may be sex-linked or it may represent a PEP specific to eggs. Work by de Boissezon and Jelnes (1982) and Jelnes (1983) has shown that the gene coding for GPI is sex-linked.

In *S. mansoni*, the investigation of the possible use of genetic markers for such characters as infectivity or drug susceptibility has only recently begun. Fletcher *et al.*, (1981b) attempted to correlate phenotype frequencies for polymorphic enzymes with their degree of infectivity to snails. Two strains were selected from each of three isolates of the Puerto Rican parasite—one for high infectivity and one for low—and tested after several generations. Four loci were found to be polymorphic and one of them, that for LDH, had phenotypic frequencies that correlated with infectivity to snails.

2. Schistosoma japonicum

As with *S. mansoni*, earlier workers used pooled samples of worms for their experiments but, even so, they detected strain differences (Yoshimura, 1968). Ruff *et al.* (1971; 1973) reported a considerable amount of variation in the protein profiles of the two sexes, as well as qualitative and quantitative differences between Philippine, Japanese and Formosan strains. They concluded that the latter pair were more closely related to each other than to the Philippine strain. Yan *et al.* (1976) investigated strain variation in MDH patterns in *S. japonicum* isolates from Formosa, Philippines and Indonesia, and were able to separate the Formosan strain from the other two, in both sexes.

A recently described parasite, *S. mekongi* (Davis *et al.*, 1976; Voge *et al.*, 1978), is morphologically very similar to *S. japonicum*. This similarity prompted a study into the speciation of the two parasites, in which ten enzymes (13 loci) from individual worms were used (Fletcher *et al.*, 1980). Differences between four geographical strains of *S. japonicum* affected 17% to 36% of the loci, and were invariant. Genetic distances between the strains were small. The lack of polymorphism at the loci studied was possibly due to a founder effect, as the strains had been established in the laboratory for a long time. By contrast, *S. mekongi* and *S. japonicum* diverged at 82% to 91% of loci; calculations of genetic distance suggest that they have been diverging for 8.5–12 million years.

3. *The terminal-spined group*

This group of African schistosomes contains important parasites of humans and animals, including *S. haematobium*, *S. intercalatum*, *S. bovis* and *S. mattheei*. The taxonomic relationships between the members of the group are extremely complex, because different species are found in the same geographical area and hybridization can occur. Further, the different species can infect the same snail intermediate hosts. Initial work on unravelling the complex was done with pooled worm samples but, even so, the

results gave indications that there were intraspecific variations detectable by isoelectric focusing and enzyme staining (Ross, 1976, 1977; Ross *et al.*, 1978). Other evidence supporting the idea of intraspecific variation was already available from the fieldwork; for instance, it is known that geographically separate forms of a parasite develop in different snails. This is the case with *S. intercalatum* from Guinea and Zaire (Wright *et al.*, 1979). Individual worms showed very clear strain differences in three (LDH, G6PDH and PGM) out of seven enzymes, thus supporting other observations of strain variation: the lack of cross-infectivity between the parasites from the two areas and their respective snail hosts, and decreased hybrid viability (Frandsen, 1978). Another example of strain variation in *S. intercalatum* was noted by Brown *et al.* (1984). They found that a strain from S. E. Gabon resembled another from Cameroun rather than that from Zaire. The similarity included egg shape and infectivity to the snail host, *Bulinus forskalii*.

Wright and Ross (1983) compared 22 isolates of *S. haematobium* from 13 countries. They noted polymorphisms in G6PDH and PGM, and found that certain forms were restricted to particular geographical areas. Where they occur outside their usual area it is suggested that movements of human populations mix the strains.

The question of the occurrence of natural hybridization has been addressed in a number of studies, reviewed by Wright and Southgate (1976). Electrophoretic techniques have been used to show that this is the case with *S. haematobium* and *S. mattheei* in South Africa (Wright and Ross, 1980). This observation may have important implications for control measures, because the poor response of *S. mattheei* infections to oxamniquine treatment may be due to hybridization with *S. haematobium*, which is not susceptible to the drug. It is also thought that heterosis is exhibited, as the hybrid has increased infectivity for snail hosts and experimental animals, as well as a greater growth rate and reproductive potential.

4. *Purified schistosome isozymes*

There is very little information about purification and characterization of schistosome isozymes. Rotmans (1978a,b) has purified two MDH isozymes from *S. mansoni*, mitochondrial and cytoplasmic, and found that they could be distinguished by their susceptibility to substrate inhibition. It is of some interest that only the mitochondrial isozyme forms an MDH-active precipitation line against mouse sera. No comparison has been made between strains, but *S. mansoni*, *S. haematobium* and *S. bovis* MDH antigens are immunologically indistinguishable. Similarly, the three major species of

Schistosoma, *S. mansoni*, *S. haematobium* and *S. japonicum*, show extensive cross-reactivity in serological assays. Aronstein and Strand (1983) analysed species- and gender-specific polypeptides by two-dimensional gel electrophoresis of metabolically labelled (^{35}S-methionine) proteins and glycoproteins. Whole worm total protein extracts contained approximately 100 polypeptides, whereas released (shed or secreted) proteins had 60 polypeptides and different protein patterns. They contained more species- and gender-specific polypeptides than the worm extracts. The most striking differences were seen in the released glycoproteins.

5. Fasciola hepatica

In Japan, the population of the common liver fluke has been found to consist of individuals with different chromosome numbers ($2n = 20$; $2n = 30$), and of individuals with mixed cell populations. Agatsuma and Suzuki (1980) found that the enzymes PGM and AK were polymorphic but not 6PGD and ES, and neither were GPI, GDH nor ME (Agatsuma, 1981c). The amount of variation detected in PGM and AK was very small but the sample sizes were only about 50 worms.

Polymorphism has been reported in an esterase of *F. hepatica* which, when subjected to isoelectric focusing and staining, revealed no fewer than seven isozymes that hydrolysed α-naphthyl acetate (Alcaino *et al.*, 1976). Combinations of the isozymes revealed that there were seven distinct phenotypes, the frequency of which varied among local populations of flukes obtained from individual sheep of different origin.

Three isozymes of MDH have been reported from whole extracts of *F. hepatica* by Probert and Lwin (1977). All three were found in cell-sap fractions, whereas only one was present in the mitochondrial fraction. It was suggested that two types of MDH existed, one in the cytoplasm, which had three isozymes, was more heat stable and was inhibited to a greater extent by high concentrations of malate, and one in the mitochondria, which existed in only one form and was inhibited to a greater extent by high concentrations of oxaloacetate.

6. Paragonimus *species*

Polymorphisms have been detected in natural populations of a number of species of *Paragonimus*. For example, PEP is polymorphic in *P. ecuadorensis* (Zillman and Voelker, 1980), and GPI is polymorphic in *P. miyazakii* and *P. iloktuenensis* (Agatsuma, 1981a,b). In *P. miyazakii*, GPI was found to have the typical dimeric structure seen in a number of other animals and to be controlled by a single locus with two codominant alleles.

Similarly, the enzyme from *P. iloktuenensis* was a dimer and controlled by a single locus with three codominant alleles which were completely different from those in *P. miyazakii*.

B. CESTODES

1. Echinococcus *species*

Within the genus *Echinococcus* 16 species have been described, of which only four are generally accepted as valid. They are *E. granulosus*, *E. multilocularis*, *E. oligarthus* and *E. vogeli*. The reader is referred to Kumaratilake and Thompson (1982) for a detailed review of taxonomy and speciation. There has been much discussion about the taxonomic status of cestodes, as they cannot easily be made to correspond with the accepted definitions of species. They do not interbreed, they are hermaphroditic, undergo self-fertilization and polyembryony and are sympatric. Unfortunately, the situation below the species level is even more complicated. The occurrence of strains is well documented and the extent of the intraspecific variation has been investigated by a variety of techniques—those of morphology, developmental biology, physiology, biochemistry, epidemiology and the study of host specificity. The importance of strain identification is particularly associated with the epidemiology of human hydatidosis; over 50 intermediate hosts can harbour *E. granulosus* (Smyth and Smyth, 1964) and there is evidence that each form in a different host may represent a separate strain which varies from other strains in its infectivity to man and domestic animals. In order to implement an effective hydatid control programme, therefore, characterization of strains is recognized as being an important consideration (McManus and Macpherson, 1984).

In the UK, *in vitro* culture (Smyth and Davis, 1974) and biochemical studies (McManus and Smyth, 1978) have identified a "horse" and a "sheep" strain of *E. granulosus*. Biochemical characterization by enzyme electrophoresis of *E. granulosus* isolates from horse and sheep cysts and *E. multilocularis* from cotton rats, for "out group" comparison, showed intraspecific differences in all the enzymes studied except AK; these were ACP, LDH, MDH, GPI, ME, ICD, ALD and α-GPDH. Material was pooled from several cysts to make one extract, but extracts prepared separately gave the same results. The consistent absence of enzyme polymorphisms in either of the two forms of *E. granulosus* suggests that each exists as a monomorphic strain. The cause of this lack of genetic diversity could be the self-fertilizing hermaphroditism, mentioned earlier, of these parasites. Inbreeding causes a reduction of genetic variability within strains and an

increase of diversity between strains. There is evidence to show that there may be a barrier between the sheep and horse strain, with neither able to develop in the heterologous host (Smyth, 1977). McManus and Smyth (1979) discuss the point that inbreeding increases uniformity by increasing homozygosity and hence allows strains to achieve closer adaptation to their hosts. It could also be argued that such a gain in adaptation to one host may carry with it the penalty of diminished capacity to infect other hosts.

In Australia, as in the UK, strains of *E. granulosus* have been identified. There appear to be at least three. Two are domestic, maintained in a typical sheep/dog cycle, one on mainland Australia and the other in Tasmania. The third is maintained in a sylvatic cycle, with dingoes as definitive hosts and macropods as intermediates (Thompson and Kumaratilake, 1982). The important epidemiological question is whether the sylvatic parasite is infective to sheep (Thompson, 1982).

Kenya offers a unique opportunity for studying hydatidosis, as it contains a wide range of animal hosts that harbour the parasite (Macpherson, 1981) and, in the Turkana district, the disease has the highest human endemicity (10–15%) in the world (French *et al.*, 1982). Macpherson and McManus (1982) compared protoscoleces from individual cysts from different animals, organs and geographical locations in Kenya and revealed an extremely complex epidemiology. They found no polymorphic variation in PGM and GPI in material removed from humans, camel or sheep, but GPI varied in samples from cattle, and both enzymes varied in samples from goats. The major banding patterns were very similar for both enzymes in human, cattle, sheep and goat (type A) isolates, as were the banding patterns for camel and goat (type B) material. The location of the cysts had no effect, and no significant differences were seen in the patterns obtained from larvae and homologous adults. Analysis of the adult worms from 26 naturally infected dogs from one area showed that most of them acquired the infection from sheep, human or goat origins.

In Argentina, Le Riche *et al.* (1982) compared GPI patterns of cyst material from two sheep and 34 cattle, and found that the cattle isolates were identical and different from the sheep isolates. Unfortunately, no comparison has been made between this material and parasites from other locations.

2. Hymenolepis diminuta

The kinetic analyses of Walkey and Fairbairn (1973) suggested that there were two LDH isozymes present in protein extracts of *H. diminuta*, one form predominating in eggs and cysticercoids, and the other in the anterior proglottides and prepatent worms. This was confirmed by Logan *et al.* (1977) who demonstrated the two isozymes by starch-gel electrophoresis. They

postulated the presence of two loci encoding LDH-A and LDH-B subunits, respectively. The LDH-A locus is expressed in all tissues, including the ovary, throughout the length of the adult, whereas the LDH-B locus is expressed predominantly in ovarian tissues, and the level of its expression depends on the maturation of the ovaries. It is at its greatest in mature eggs, where it almost equals that of LDH-B.

3. Moniezia expansa

This parasite, like *Echinococcus*, occurs in several different hosts. Hermoso *et al.* (1982) compared adult worms from cattle and sheep. Of four enzymes, MDH, LDH, GPI and G6PDH, only MDH varied in its mobility and number of isozymes between the two strains. The number of isozymes was also different in gravid and mature proglottides.

C. NEMATODES

1. *Ascaridoidea*

Enzyme electrophoresis has been used extensively on this group to attempt to solve a number of taxonomic problems, including the identification of sibling species (*Parascaris equorum* and *P. univalens*—Biocca *et al.*, 1979; Bullini *et al.*, 1979; Nascetti and Bullini, 1982), the allocation of parasites to the correct genera, as in *Toxascaris leonina* and *Baylascaris transfuga*, and the confirmation of other genera as in *Neoascaris* (Nascetti and Bullini, 1982). A considerable amount of biochemical work has been done on *Ascaris*, as it is a large worm, is readily available and can be dissected into individual tissues for study. The classification of human and porcine worms has been a disputed point, since they have been variously described as belonging to a single species with two subspecies (*A. suum lumbricoides* and *A. s. suum*, respectively) or to two species (*A. lumbricoides* and *A. suum*, respectively). The worms are similar morphologically and immunologically, and infective eggs have some success in surviving in the heterologous host. Nascetti *et al.* (1980) and Nascetti and Bullini (1982) have used enzyme electrophoresis to investigate the genetics of the two parasites, and conclude that they are two genetically well differentiated species. They examined 15 enzymes (21 loci), four of which were found to be fully diagnostic. These were MDH-3, G6PDH, ES and ALD. The observed mean heterozygosity per locus was 2.69% for *A. lumbricoides* and 3.81% for *A. suum*. It is interesting to note that these values are significantly higher than those for species within the closely related genus *Parascaris*. The average genetic

distance indicates that the two species are the product of a relatively recent speciation event about 1.6 million years ago.

Leslie *et al.* (1982) investigated polymorphism in *A. s. suum* from different geographical locations in the USA. They examined 18 enzymes (38 loci), seven of which (12 loci) had at least one polymorphic locus. They were 6-PGDH, MPI, ES, PEP, LDH, LAP and MDH, and their gene frequencies fitted the Hardy-Weinberg distribution. The level of genetic variation was low, the percentages of polymorphic loci in the samples from two areas were 20.7% and 16.7%, and the mean percentages of heterozygous loci per individual were 6.6% and 5.3% respectively, below the usual values for invertebrates. Comparisons between worms from two geographical areas failed to reveal any marked differences, and major alleles at the polymorphic loci were the same.

2. *Anisakidae*

Human anisakiasis is caused by larvae of a number of genera belonging to this family. Seventeen genera can be distinguished by the morphological characteristics of the adults but the larvae are difficult to identify, so biochemical characterization has been found useful.

Cain and Raj (1980) reported that, for a given larval type, the electrophoretic patterns and thermostability properties of ADH were the same, regardless of host species, position within the host or geographic location. In contrast, ES showed a high degree of intraspecific variation. Further, MDH from *Phocanema* was found to have apparent quantitative isozymic differences that depended upon whether parasites were derived from populations in the North Pacific or the North Atlantic. Like ADH, PGM was found to be a monomorphic enzyme in two types of *Anisakis* larvae, but the enzyme from each type possessed a different mobility under the same conditions (Agatsuma, 1982).

3. Trichinella *species*

The controversy regarding the speciation of *Trichinella* is well known and a good review of the subject is given by Dick (1983). Once again, the problem of identification is brought about by the lack of reliable morphological characters, but substantial evidence is available from other sources, such as infectivity studies, cross-breeding experiments and geographical location, which indicates that intraspecific variants of *T. spiralis* do exist. A preliminary study by Flockhart *et al.* (1982) on the muscle-stage larvae of five isolates, in which only four enzyme systems were examined, revealed distinct differences between the north-temperate "domestic" *T. spiralis* and

the tropical and arctic "sylvatic" isolates of *T. nelsoni* and *T. nativa*. This work has now been extended to 21 isolates from as many hosts and geographic locations as possible, and 12 enzyme systems have been studied. The results have provided three markers for *T. pseudospiralis*—GPI, ES and LDH. GPI identified all the separate populations—*T. nativa*, *T. nelsoni* and *T. pseudospiralis*—very well, but also distinguished between domestic and sylvatic *T. spiralis*. Coefficients of similarity were calculated to compare the populations from different geographical areas, with a domestic *T. spiralis* strain as the reference. They showed that all the pig, human and rat isolates were closely related, irrespective of origin or number of passages. The status of *T. nelsoni* and *T. nativa* is still in question, as only GPI appears to be a marker for these two and their genetic relationship remains to be calculated (Flockhart, unpublished observations).

4. Brugia

The majority of reports on these parasites has been concerned with interspecific differentiation of morphologically similar worms where the species are sympatric and have the same vector (Lim *et al.*, 1981; Oothuman *et al.*, 1983; Flockhart and Denham, 1984). However, Lim *et al.* (1981) have reported intraspecific variation in GPI and LDH patterns with subperiodic *B. malayi* from different hosts.

5. Onchocerca volvulus

There has been a great deal of interest in strains of the human pathogen, *O. volvulus*. It has become clear, with the many new descriptions of the disease and the parasite vector complexes, that more than one form of onchocerciasis is presented by the different clinical pictures seen in the various areas where the disease is endemic. The important strain difference is the greater pathogenicity of the African "savannah" parasite compared with the forest strain. An electrophoretic survey was carried out by Flockhart *et al.* (1986) on *O. volvulus* worms collected from forest and savannah areas of subsaharan Africa. Polymorphisms were detected in four out of seven enzymes, LDH, MDH, PGM and MPI. Comparisons of allele frequencies showed that the populations from the two forest countries (Liberia and the Ivory Coast) had a similar composition, but that there was divergence between these and populations from the two savannah areas (Sudan and Burkina Faso). Genetic distances were calculated, even though the number of loci was small and it was clear that the observed differences between the countries were slight (Fig. 1), although they may increase if more loci are examined.

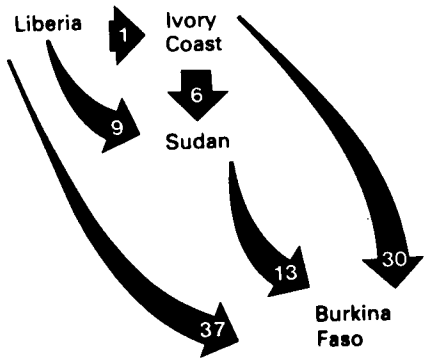

FIG. 1. Pictorial image depicting the relative degree of relatedness between two forest (Liberia and Ivory Coast) and two savannah (Burkina Faso and Sudan) populations of *Onchocerca volvulus* determined by enzyme electrophoresis. The figures refer to standard genetic distances (Nei's D × 1000) and show that the divergence between populations is small.

6. Dirofilaria immitis

A comparative study, using quite large sample sizes, of *D. immitis* from different regions of Australia and from Japan failed to show any genetic diversity between the populations. Of 11 enzymes, ten were identical and completely monomorphic and HK showed a marked sexual difference (Flockhart, unpublished observations).

IV. METABOLIC PATHWAYS IN HELMINTHS

This part of the review is concerned with the major variations in pathways of energy metabolism in parasitic helminths. This is not to say that other metabolic pathways may not vary, but simply reflects the greatest amount of effort that has been put into studies of helminth metabolism in the last 30 years. Thus, although the pathway of proline synthesis has been intensively studied in liver fluke by Isseroff and his colleagues (Isseroff, 1980), it is not nearly so well known in other helminths. Similarly, Jaffe and coworkers have performed some elegant research on the metabolism of purines, pyrimidines and folic acid in helminths (e.g. Jaffe and Chrin, 1981) but the necessary detailed comparative studies do not exist that would enable assessment of variability within species or even between species.

The patterns of energy metabolism encountered among parasitic helminths fall into three broad types, the first two of which are illustrated in Fig.

2. Type 1 contains the so-called homolactate fermenters (Saz, 1981). In these organisms, carbohydrate is degraded by the Embden-Meyerhof pathway to lactic acid. Lactic acid is not accumulated for reoxidation as it is in, say, vertebrates, but excreted. Examples of type 1 fermenters are the filarial worms and the schistosomes. However, recent work has highlighted some puzzling features about their metabolism. Thus, *Dirofilaria immitis* possesses high activities of malic dehydrogenase for which no role can be postulated if simple glycolysis is the sole energy-generating pathway. Further, Mendis and Townson (1985) have demonstrated that filariae possess electron transport systems similar to those of other parasitic nematodes, and cannot now be considered to be homolactate fermenters. Finally, recent evidence (Van Oordt *et al.*, in press) suggests that *Schistosoma mansoni* depends to a great extent on aerobic metabolism. It is therefore extremely doubtful if homolactate fermentation actually occurs in helminths.

Type 2 fermentation is characterized by a carbon dioxide fixation step (Bryant, 1975). Respiratory substrate, such as carbohydrate, is degraded to the level of phosphoenolpyruvate. There are then two possibilities for further metabolism. In the presence of pyruvate kinase, pyruvate and ATP may be formed, with the subsequent conversion of pyruvate to ethanol or lactate. These are then excreted, the latter as lactic acid. Alternatively, phosphoenolpyruvate carboxykinase catalyses the fixation of a molecule of carbon dioxide into phosphoenolpyruvate, with the formation of oxaloacetate and a nucleotide triphosphate. Malic dehydrogenase then converts oxaloacetate to malate. These two branches are exactly equivalent in terms of energy yield and redox balance. In each case a single molecule of nucleotide triphosphate is formed, and the conversion to lactate or malate is a reduction step involving a single molecule of NADH. At the levels of either lactate or malate, therefore, there is a net yield of two or three molecules of ATP (depending whether the starting point was glucose or a hexose unit from glycogen), and the reducing equivalents (NADH) generated in the early part of the pathway are exactly utilized. Lactic acid, as mentioned above, is an end-product, but malate enters the mitochondrion where it acts as the mitochondrial substrate.

Malate stands at a second branch-point of metabolism, and undergoes a dismutation within the mitochondrion. In the reducing sequence, it is dehydrated to fumarate. Fumarate acts as the substrate for an important enzyme complex, the fumarate reductase system. Fumarate reductase accepts reducing equivalents, generated in the oxidizing arm of the malate dismutation, and transfers the electrons to fumarate which is thus reduced to succinate. Succinate may be excreted as succinic acid, or converted by a second enzyme complex, the succinate decarboxylase system, to propionate. At each of these steps a single molecule of ATP is generated.

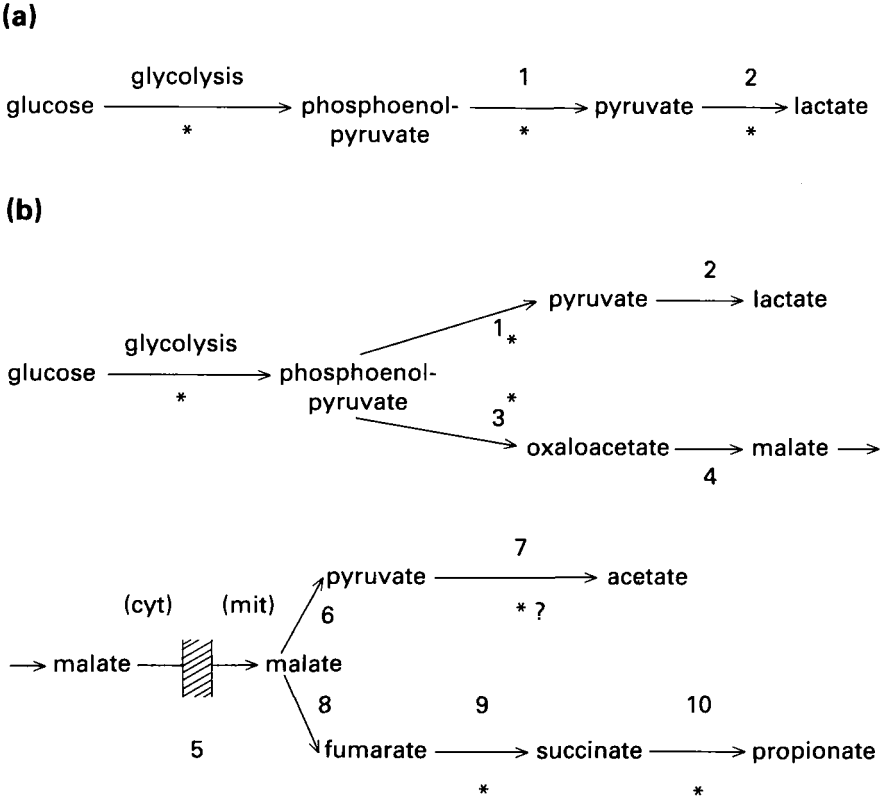

FIG. 2. Two types of energy metabolism in parasitic helminths. (a) Homolactate fermentation. (b) Malate dismutation.

Reaction 3 involves a carboxylation step; decarboxylation occurs at 6, 7 and 10. Reducing equivalents are generated at reactions 6 and 7; one reducing equivalent is used at reaction 9. Thus, when the mitochondrial compartment is in redox balance and malate is the sole substrate, twice as much propionate as acetate is produced.

Key 1, pyruvate kinase; 2, lactate dehydrogenase; 3, phosphoenolpyruvate carboxykinase; 4, malate dehydrogenase; 5, mitochondrial membrane; 6, malic enzyme; 7, pyruvate dehydrogenase complex; 8, fumarase; 9, fumarate reductase; 10, succinate decarboxylase complex.

*indicates reactions at which ATP is synthesized from ADP. cyt, cytosol; mit, mitochondrion.

Propionic acid is excreted. In the oxidizing sequence, malate is oxidatively decarboxylated to pyruvate, which is further oxidized to acetate by the pyruvate dehydrogenase complex. Each of these reactions yields a single reducing equivalent which is utilized at the fumarate reductase step. Thus, in perfect redox balance, two molecules of succinate or propionate (or one of each) will be generated for each molecule of acetate formed. Acetate is excreted as acetic acid.

However, it is rare in practice to observe such perfect stoichiometry, and there are many possible explanations for this. The first is that measurements of acid production can only be made *in vitro*, and the parasite may be maintained in far from ideal circumstances. Another is that the pathways may not be exactly as they have been described above. For example, evidence is accumulating that pyruvate can enter the mitochondrion, and may act as the mitochondrial substrate. If that were the case, it could be reduced to malate, which would then be converted to fumarate, which in turn would be reduced to succinate. Under these circumstances, for one succinate molecule to be produced, two molecules of acetate must be formed. Malate and pyruvate utilization by mitochondria need not necessarily be mutually exclusive, either, and it is possible that the simultaneous metabolism of one molecule of malate and one of pyruvate may occur (Tielens *et al.*, 1981). When this happens, there is no need for the interconversion of malate and pyruvate, and the formation of one molecule of acetate will provide the reducing equivalent for the reduction of one malate molecule. A final explanation for the different stoichiometries frequently observed in the formation of end-products by parasitic helminths may be found in the fact that the pyruvate may also leave the mitochondrion. If this were the case, then pyruvate generated in the malate dismutation may not go on to the pyruvate dehydrogenase oxidation step, but leave the mitochondrion for the cytosol where it would be converted to lactate. Thus the redox balance of both cellular compartments would be affected. This simple analysis makes it clear that a given parasite may have a number of options before it for balancing its energy and redox budgets, and it is often instructive to carry out the simple calculation, as for *Echinococcus granulosus* below, based on experimental evidence of end-product analysis.

Type 3 fermentation is best exemplified by *Ascaris*, and a number of other nematodes inhabiting the vertebrate intestine. It is characterized by a series of reactions between mitochondrial end-products that yield branched-chain fatty acids (Suarez de Mata *et al.*, 1977; Komuniecki *et al.*, 1981a,b; Rioux and Komuniecki, 1984). For example, two propionate molecules condense, with the utilization of two reducing equivalents, to yield 2-methylvalerate. Acetate and propionate similarly give rise to 2-methylbutyrate. Alternatively, they may condense to 2-methylacetoacetate, undergo reductive

rearrangement to form 3-hydroxy-2-methylbutyrate and 2-methylcrotonate (tiglate), which in turn is reduced to 2-methylbutyrate. Yet another pathway, also involving two reductive steps, involves the condensation of two molecules of propionate to 3-keto-2-methylpentanoate, reduction to 3-hydroxy-2-methylpentanoate, dehydration to 2-methyl-pentanoate and final reduction to 2-methylvalerate. The precise location of the enzymes concerned is uncertain but there is good evidence that they are mitochondrial. The source of the reducing equivalents to drive these pathways is also uncertain. They are mentioned here for the sake of completeness. Too little is known about them in general other than *Ascaris* to permit conclusions on variation to be drawn.

Although these types of fermentation pathways predominate, in many parasitic helminths some or all of the enzymes of the tricarboxylic acid cycle may be present, perhaps in the larval stage if not in the adult. The extent to which they contribute to energy metabolism is not clear, even in species, such as *Nippostrongylus brasiliensis*, that are said to be aerobic (Alphey, 1972). The parasitic helminths are clearly not a metabolically homogeneous group, in the sense that they all adhere to one pattern of metabolism.

V. Strain Variations of Metabolism

In this section, we list some specific examples of strain variation at the functional metabolic level. Many of them are now well substantiated, but others are perhaps little more than hearsay. However, the list of examples shows clearly that the phenomenon of strain variation of metabolism pervades all classes of helminth parasites.

A. *Echinococcus granulosus*

The biochemistry and physiology of *Echinococcus* has recently been reviewed by McManus and Bryant (in press). Very little is known about the metabolism of the adult parasite, which probably conforms most nearly to type 2 metabolism. On the other hand, the ease of access to material has permitted a considerable amount of study of energy metabolism in protoscoleces. The protoscoleces also possess a type 2 metabolism, and the most important respiratory end-products are lactic and succinic acids, but acetic acid is also produced, together with some ethanol. Pyruvic acid may also be generated, in small quantities.

Echinococcus granulosus provides some of the most remarkable examples of strain variation at the biological, physiological and biochemical levels.

There are marked compositional differences between horse and sheep strains (McManus and Smyth, 1978) but of much greater interest are the differences in energy metabolism. Aerobically and anaerobically, the major end-products produced by protoscoleces from the sheep strain, when incubated *in vitro*, were acetic and succinic acids. Those produced by the horse strain were mainly lactic and some succinic acid.

The full extent of variation in this parasite is unknown. McManus (1981) uncovered a most interesting complex of strains in Kenya. He found a whole spectrum of metabolic patterns in protoscoleces from a variety of hosts, with proportions of end-products varying from 5% lactic acid produced by goat material to 45% by human parasites. Acetic acid production varied from 13% (camel) to 55% (sheep), and succinic acid from 15% (sheep) to 55% (cattle). Australian strains of *Echinococcus* also vary. Protoscoleces isolated from sheep in NSW produce relatively more succinic acid and relatively less lactic acid than those isolated from sheep in Tasmania. A wallaby strain produced relatively more succinic acid and relatively less lactic acid than those isolated from sheep in Tasmania. A wallaby strain produced almost no succinic acid, 61% lactic acid and 35% acetic acid (C. A. Behm, C. Bryant and R. C. A. Thompson, unpublished data).

This information clearly shows that the different isolates of *E. granulosus* have adopted different strategies in different hosts. At present, we are bedevilled by a lack of precise information about the metabolic pathways concerned. However, as mentioned earlier, it is possible to make calculations about the theoretical energy yield of respiratory metabolic pathways in redox balance, on the basis of the proportion of end-products formed. The maximum yield of ATP from phosphoenolpyruvate in mitochondria in redox balance, where malate is the sole mitochondrial substrate, and the proportion of end-products is 33.3% acetic and 66.6% succinic acid, is 100 moles for each 50 moles of end-products (McManus and Bryant, in press). When energy yields for the Kenyan complex are calculated, a range of values per 50 moles of end-products is obtained, from 78 in the human material to 90 in the sheep. These differences are significant, and lower values can be accounted for by the shift towards lactic acid production. It would thus appear that there is a trade-off, that the lower figure exhibited by the human material is a compromise made necessary by some other, unknown, imperative.

One can speculate on what this imperative is. Perhaps it is some quirk of the physiology of the host, a characteristic of the immune system, or some peculiarity of the environment in which the parasite has to develop. Perhaps it is simply an accident that that particular host picked up a parasite with those particular characteristics. Alternatively, the appropriate type of parasite may be chosen, as it were, by the host. In order even to begin to answer

these questions, much more information than we now possess is needed. For example, we do not know if all human-derived material has the characteristics that McManus (1981) describes. Probably not, because there are many different types of humans—more even than types of sheep in Australia and we have already noted differences between sheep protoscoleces of different geographical origin. But some light, however, can be shed on the effect of the host on the establishment of a parasite type by the next example.

B. *Hymenolepis diminuta*

Hymenolepis diminuta is generally considered to conform to the type 2 metabolic pattern described above. Characteristically, it is thought to excrete only three respiratory end-products (apart from carbon dioxide), lactic, succinic and acetic acids. Lactic acid is cytosolic in origin and is produced by the action of the glycolytic pathway; succinic and acetic acids are mitochondrial end-products.

Ovington and Bryant (1981), in trying to test a hypothesis that the high pCO_2 in the small intestine of the rat, rather than the low pO_2, was the main determinant of anaerobic metabolic pathways (Podesta *et al.*, 1976), measured end-product formation by *H. diminuta* under a range of *in vitro* conditions. They observed that an Australian strain of parasite (ANU) had a pattern of end-product formation that was markedly different from the North American one. In short, the ANU strain of *H. diminuta* was found to excrete mainly lactic and acetic acids. Under the conditions of the experiments performed at that time, succinic acid was generally less than 5 to 10% of the total, a typical analysis being 5, 11 and 84% for succinic, acetic and lactic acids, respectively. This contrasts markedly with values found in the literature for the end-products of *H. diminuta* maintained under similar conditions. Read (1956) reported 80 to 90% lactic acid, with the remainder being succinic acid; Laurie (1957) generally agreed with this. Fairbairn *et al.* (1961) found that the predominating end-product was succinic acid (62%), with acetic acid (25%) and lactic acid (13%) being produced in much smaller amounts. Watts and Fairbairn (1974) achieved more or less the same results, but Coles and Simpkin (1977) found acetic acid (50%) to be the major end-product!

To conclude that only one of these groups of workers is correct is to do all the others an injustice. Even 30 years ago, the techniques required to measure the three acids were simple, and to suggest that they were not properly applied is almost libel. The only sensible conclusion is that the parasite–host system varied between the groups of research workers and that each group reliably reported what it measured.

The possible sources of variation are many and difficult to identify. The reports are not exact, and there are many factors that could be responsible: different definitive hosts (rats of various strains); different intermediate hosts (species or strains of beetle); incompletely described experimental conditions; ages of hosts; ages of parasites; differences in cultivation practices; and, last but not least, variation in the tapeworm itself.

The complexity of this problem is well illustrated in an important paper by Mettrick and Rahman (1984). These workers cultivated both the Canadian (Toronto) and the Australian (ANU) strains under identical conditions which were slightly different from those used by the Australian workers. They confirmed that there were consistent differences between the ANU and Toronto strains. They also noted that, although the Toronto strain consistently excreted more succinic acid than the ANU one, the production of succinic acid by the ANU strain was consistently higher than reported by the Australian group. This observation was confirmed by Kohlhagen *et al.* (in press) who were also able to compare the two strains. They found an excellent agreement between their results and those of the Canadian workers for proportions of end-products formed under aerobic conditions, although the Toronto strain apparently produced a greater total amount of end-products. One important discrepancy was that the succinic acid production was consistently higher (20–40%) in the ANU strain than earlier reports indicated (less than 10%). The most probable explanation for this, as Mettrick and Rahman (1984) pointed out, is that the earlier work was performed with *Tenebrio molitor* as the intermediate host, while their work and that of Kohlhagen *et al.* (in press) was done with *Tribolium confusum.*

Support for this view comes from some unpublished research of R. A. Cornish. She compared the metabolism of adult worms of the ANU and Toronto strains after they had each been passaged into similar rats through either *Tenebrio molitor* or *Tribolium confusum.* In each case, adult worms that had been passaged through *Tribolium confusum* gave significantly higher ATP/ADP ratios and adenylate energy charges (AEC) than those that had *Tenebrio molitor* as an intermediate host. In the Toronto strain, the increases were 1.12 to 1.47 and 0.69 to 0.73 for the ATP/ADP ratios and AEC's respectively. In the ANU strain, they were 1.40 to 1.71 and 0.71 to 0.76. Just as striking were the changes in lactic acid excretion in the adults on incubation *in vitro.* Adult worms that had been passaged through *Tribolium confusum* excreted about 35% more lactic acid. In Bryant (1983), the names of the intermediate hosts have been inadvertently exchanged (*Tribolium* is *confusum* indeed!).

We can only speculate about the reasons for these differences. Beetles are infected by first starving them and then permitting them to feed on a gravid proglottis of *H. diminuta.* Each beetle therefore consumes many eggs and it

is possible that only those cysticercoids survive that are in some way matched to the environmental conditions offered by the beetles. Each beetle, therefore, by putting limits on development, "selects" a different type of larva. Each type of larva possesses a suite of advantageous characters that enable its survival in the beetle but which also have consequences for the adults, in that they may even appear to be counteradaptive at this stage of the parasite's life cycle. The immediate advantage of larval survival is paid for by the adult as a small decrement in energy status.

The end-products of respiration merely mirror the metabolism of *H. diminuta*. In fact, when the activities of some of the important enzymes in their production are measured, considerable differences are apparent between the ANU and Toronto strains of *H. diminuta* (Kohlhagen *et al.* in press). The ANU strain has five times as much pyruvate kinase activity and lactic dehydrogenase activity as the Toronto strain, and only half to one-fifth of the malic enzyme activity. This alone might be sufficient to account for the overall differences in end-product formation, indicating as it does an emphasis on the cytosolic process of glycolysis and a de-emphasis of mitochondrial energy metabolism in the ANU strain. To underline the point, however, the Toronto strain has more than 15 times the activity of fumarase, and about four times the activity of fumarate reductase, both of which are important mitochondrial enzymes. Further, the kinetics for fumarase suggest that it is effective at much lower concentrations of its substrates than the enzyme from the ANU strain.

It is clear, then, that the ANU strain of *H. diminuta* tends towards a type 1 metabolism, while the Toronto strain tends towards type 2. The reasons why this should be so are far from clear. Recent studies using ^{13}C NMR (Behm, Bryant and Jones, unpublished observations) confirm their existence. A third strain "OSU", obtained from Professor Pappas of the Ohio State University, USA also shows some metabolic differences. Pappas (personal communication) can differentiate between four isolates on the basis of a number of morphological criteria, although the significance of the variations, especially at the physiological and biochemical levels, remains uncertain. These are the ARME "strain" (currently maintained at the University of Keele, UK), the "OSU" strain, the "ANU" strain and the Toronto "TOR" strain. If there are four strains, why should there not be more? It would be interesting to know if the strains are effete, surviving only because of the intensive cultivation that they receive, or whether they are viable in the wild. Certainly, bearing in mind the arguments that have been advanced for *Echinococcus granulosus*, one would expect those strains more dependent on glycolysis to be at a disadvantge, because they have to process more carbohydrate for the same energy return as strains more dependent in mitochondrial processes. By this reasoning, the ANU strain is most distant

from the wild type, but may be the most recent cultivar. It has been maintained in Australia for the last 20 years, whereas it seems likely that the North American strain was isolated perhaps 40 or more years ago. Considerations of this sort are no doubt misleading, though, as the event that establishes a cultivar with a unique metabolic profile may occur immediately on collection or after an unknown number of years in cultivation.

C. BENZIMIDAZOLE-RESISTANT NEMATODES

Where resistance to anthelmintics occurs in helminths it is likely that altered respiratory metabolic function will be detected, either directly in measurements of enzyme activities, or indirectly as changes in the pattern of metabolic end-products. Such changes do not necessarily confer resistance —in fact, unless the enzymes concerned are associated with detoxification pathways, it is likely that they will prove to be only correlates without causation. Presumably, they are symptomatic of a genome that in some other aspect confers on the organism the property of resistance. If the pathways concerned are less efficient, then that is the price the organism has to pay to complete its life-cycle in an inimical environment.

Sangster (1983) has carried out a detailed study on two strains of *Trichostrongylus colubriformis*, one susceptible and one resistant to thiabendazole. *T. colubriformis* conforms to a type 2 metabolism, with succinate and lactate as minor respiratory end-products, the major ones being ethanol, acetate, propionic and acetic acids when incubated *in vitro*. Ward and Huskisson (1980) have suggested that the pathways that produce the alcohols are reversible, and Sangster's (1983) results indicate that they might be in one of the strains he tested. He noted that, while the susceptible strain of *T. colubriformis* continued to accumulate all end-products in the medium, the resistant strain consistently excreted ethanol and propanol for the first two hours of incubation and resorbed them from the medium thereafter. Such differences between strains highlight the difficulties faced by comparative biochemists when trying to elucidate metabolic pathways, and the dangers of extrapolation from one species to another, let alone strains!

3-O-methyl glucose is used instead of glucose in order to measure the rate of glucose uptake across membranes. It enters the worms on the glucose carrier but is not metabolized further, so kinetic studies are not confused by the continuous depletion of the internal pool. The resistant strain of *T. colubriformis* took up methyl glucose more rapidly than the susceptible one. Further, its carbohydrate reserves were more readily depleted (Sangster, 1983). This implies the presence of a more active carbohydrate metabolism in the resistant strain. It contrasts with what was found for *Haemonchus*

contortus (Rew *et al.* 1982; Bennet and Bryant, 1984), in which the susceptible strain took up methyl glucose and utilized its carbohydrate reserves more rapidly. Once again, this supports the view that these differences are concerned with strain variation but not with resistance to benzimidazoles.

Haemonchus contortus is a classic example of strain variation, and one of particular interest because the various strains studied have appeared in response to the prolonged use of benzimidazole anthelmintics in the wild as well as in the laboratory. The first suggestion that metabolic strain variation was a feature of this parasite came from Prichard (1973). At that time, there was considerable interest in the possibility that fumarate reductase was a target for anthelmintic action. Prichard (1973) compared the effect of thiabendazole on one resistant and one susceptible strain. The former strain was isolated by Merck, Sharp and Dohme (Australia) from Theodore in Queensland and the latter by the Commonwealth Scientific and Industrial Research Organization's Division of Animal Health in Sydney, New South Wales. Although it was not central to his thesis, he demonstrated in four separate experiments that the susceptible strain possessed somewhere between two and ten times as much fumarate reductase activity as the resistant strain. In contrast to this, Malkin and Comacho (1972) detected no difference between their resistant "Ogdensburg" and susceptible "Branchburg" strains from New Jersey, and neither did Romanowski *et al.* (1975) in their Beltsville strains from Maryland.

Suspecting that the variation in fumarate reductase activity reported by Prichard (1973) might be symptomatic of anthelmintic resistance, Bryant and Bennet (1983) and Bennet and Bryant (1984) undertook detailed studies of energy metabolism in one susceptible, one mebendazole-resistant and one thiabendazole-resistant strain of *Haemonchus contortus*. Although their suspicions proved unfounded, they showed that the thiabendazole-resistant strain, but not the mebendazole-resistant one, possessed less than half the fumarate reductase activity of the susceptible strain.

Other differences between the strains were also noted. The susceptible worms used their reserves of glycogen to sustain energy metabolism during incubation *in vitro* to a much greater extent than did either of the resistant strains. *Haemonchus contortus* exhibits type 2 metabolism, and the respiratory end-products detected in all three strains were propionic and acetic acids and propanol, with smaller amounts of ethanol, lactic and malic acids. The total amounts of end-products generated during incubation did not differ between the strains; however, there were marked differences between their relative proportions. Briefly, in comparison with the susceptible strain, carbon flow was diverted away from propionate formation to ethanol in the mebendazole-resistant strain, and the thiabendazole-resistant strain produced considerably less ethanol. Finally, both resistant strains placed con-

siderably greater emphasis on aerobic metabolic pathways, as determined by cyanide-sensitive CO_2 generation from labelled glucose.

These various observations can be reconciled into different modes of metabolic adaptation. The greater aerobic capacity of the resistant strains is consistent with their apparently reduced utilization of carbohydrate reserves; presumably they derive more energy per hexose unit because of higher metabolic efficiency. Lacking this, the susceptible strain channels most of its resources through its anaerobic mitochondria whose end-products are propionate and acetate. The thiabendazole-resistant strain gets the best of both worlds, as its anaerobic capacity is the same as that of the susceptible strain. The mebendazole-resistant strain utilizes less anaerobic capacity.

Quite clearly, these metabolic strategies are effective, as each strain survives—providing that the ecosystem is constrained by the continued presence of benzimidazole challenge. The extent of the compromise with efficiency, compared with the wild type, cannot even be guessed at, even if it were profitable to do so, because the end justifies the means. Neither of the strategies adopted by the resistant strains can be said to confer resistance; rather, they are the ancillary characteristics of two among many *Haemonchus contortus* genomes that also have the property of benzimidazole resistance. At present it seems likely that resistance depends on the capacity of worm tubulin to bind the anthelmintic. Lacey (in press) has tested the tubulin from each of the strains and has indeed shown that the affinity of the resistant strains for the benzimidazole anthelmintics is about one-sixth to one-tenth that of the susceptible strain.

D. OTHER POSSIBLE EXAMPLES

Boczon (in press) has recently compared aspects of the carbohydrate metabolism of *Trichinella spiralis spiralis* and *Trichinella spiralis pseudospiralis*. They have been recognized as distinct entities because histopathological studies suggest that '*pseudospiralis*' induces greater changes in the muscles which it invades (Gabryel *et al.*, 1980). In particular, it is suggested that, as a capsule does not form around the parasite, it has greater opportunities for interaction with its host.

Trichinella appears to approach most nearly a type 3 metabolism, as its major respiratory end-products are n-valeric, acetic and propionic acids, and it has little dependence on tricarboxylic acid cycle activity (Boczon, 1976). Determination of the activities of a number of the enzymes of respiratory metabolism (lactate dehydrogenase, malate dehydrogenase and phosphoenolpyruvate carboxykinase) showed that they were greater in

"*spiralis*," but there were no significant kinetic differences (Boczon, in press). However, the properties of malic enzyme varied considerably. In both strains, the sigmoidicity of the saturation curves suggest that malic enzyme is regulatory, and a variety of regulators (oxaloacetate, ATP and ADP, and possibly fumarate) were identified. The two enzymes, however, showed different specificities for NAD and NADP. The "*pseudospiralis*" enzyme possesses an equal affinity for both cofactors, whereas that from "*spiralis*" displayed an affinity for NADP 2½ times greater than for NAD. In this, it is similar to the enzyme from *Ascaris* muscle.

However, one swallow does not make a spring. There is no suggestion that this difference in a single enzyme, nor the greater enzyme activities observed in "*spiralis*", are responsible for the changes in histomorphology mentioned earlier. However, they can be regarded as symptomatic, and undoubtedly a more detailed examination of the myriad Trichinellas would identify many other differences in metabolic properties.

Another helminth over which there has been some debate is *Nippostrongylus brasiliensis*. When Kelly, *et al.* (1975) published an account of the anthelmintic efficacy of mebendazole against this nematode, their results were greeted with some disbelief by researchers of Janssen Pharmacia in Belgium (Chevis, personal communication). One of us (CB) also caused surprise when, in a conference discussion, he commented that *N. brasiliensis* possessed a measurable fumarate reductase activity. It is therefore with considerable relief that we note the observations of Fry and Brazeley (1984), who report that the specific activity of fumarate reductase in *N. brasiliensis* is similar to that in *Ascaridia galli*, at 40 $nmol^{-1}\ min^{-1}\ mg^{-1}$ protein. In view of the experience with *Haemonchus contortus* (discussed above) these conflicting reports no longer surprise. Undoubtedly, each group of workers was reporting accurately results which were obtained with different cultivars. Each cultivar presumably differed in the emphasis placed on certain metabolic pathways and in susceptibility to mebendazole. This somewhat anecdotal account sounds a note of caution in the use of laboratory models in anthelmintic evaluation; each host–parasite system should be represented by several cultivars of different geographical origin.

VI. Conclusions

In this review we have been concerned with demonstrating that variation within species is the usual condition for living organisms. We concede that this conclusion is not new, and that anyone with a smattering of evolutionary biology will feel compelled to ask why we have seen fit to document a biological truism through so many pages of text. We reply that it is because

the problem of strain variation is particularly complex for parasitologists, whatever aspect of the discipline they pursue. Two features of the biology of parasites render them particularly susceptible to the evolutionary processes that induce variation. Firstly, they are opportunistic, which is evident in the fact that most parasites have a great capacity for amplifying their genomes, either by sexual or asexual means. Secondly, they are exposed to the extreme selection procedures of the host–parasite relationship. The coevolution of host and parasite is of increasing interest to evolutionary biologists, who begin to suspect that parasites are much more important in directing evolution than has hitherto been imagined (see Dawkins (1982) for an entertaining discussion of this point).

A knowledge of strain variation is important in studies of epidemiology and essential for control measures; it enables the rationalization of conflicting physiological and biochemical data. By charting strain variation in helminths thus far, we have tried to increase awareness of this phenomenon and alert others to its pitfalls. Strain variation in helminths is both commonplace and problematical, but there is now a substantial armoury of techniques derived from biochemistry that can be brought to its diagnosis. Variation is made manifest by studies of enzyme polymorphism and metabolism, and we hope that future work will illuminate the ways in which both phenomena contribute to parasite fitness. Meanwhile, the literature on strain variation will continue in confusion unless parasitologists specify, in much greater detail than is now the case, the host–parasite systems on which they work.

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