Pentastomids and the tetrapod lung

J. RILEY[1] *and* R. J. HENDERSON[2]

[1] *Department of Biological Sciences, The University of Dundee, DD1 4HN*
[2] *Institute of Aquaculture, The University of Stirling, Stirling FK9 4LA*

SUMMARY

Pentastomids comprise a highly specialized taxon of arthropod-like parasites that probably became adapted to the lungs of amphibians and reptiles early in their long evolutionary history. Few other macroparasites exploit this particular niche. Pentastomids are often large, long-lived and yet they cause little observable pathology in lungs, despite being haematophagous. The lungs of all tetrapods are lined with pulmonary surfactant, a remarkable biological material consisting of a complex mixture of phospholipids, neutral lipids and proteins that has the unique ability to disperse over the air-liquid lining of the lung. In the lower tetrapods it acts as an anti-glue preventing adhesion of respiratory surfaces when lungs collapse during swallowing prey or upon expiration. In mammals, pulmonary surfactant also plays a critical role regulating the activity of alveolar macrophages, the predominant phagocytes of the lower airways and alveoli. This review outlines the evidence suggesting that lung-dwelling pentastomids, and also nymphs encysted in the tissues of mammalian intermediate hosts, evade immune surveillance and reduce inflammation by coating the chitinous cuticle with a their own stage-specific surfactant. The lipid composition of surfactant derived from lung instars of the pentastomid *Porocephalus crotali* cultured *in vitro* is very similar to that recovered from the lung of its snake host. Pentastomid surfactant, visualised as lamellate droplets within sub-parietal cells, is delivered to the cuticle via chitin-lined efferent ducts that erupt at a surface density of $< 400\,mm^{-2}$. The fidelity of the system, which ensures that every part of the cuticle surface is membrane-coated, testifies to its strategic importance. Two other extensive glands discharge membrane-associated (hydrophobic?) proteins onto the hooks and head; some have been purified and partly characterized but their role in minimising inflammatory responses is, as yet, undetermined.

Key words: Pentastomids, pulmonary surfactant, lipids, reptile lungs, immune evasion.

INTRODUCTION

The phylum Pentastomida comprises a highly specialized taxon of about 110 arthropod-like parasites whose adults infect the respiratory tracts of reptiles and other vertebrates (for general reviews see Riley, 1986, 1994: Table 1). The only pentastomids infecting non-reptile hosts include three species of *Raillietiella* in the lungs of amphibians (Krishnasamy *et al.* 1995), two species of *Reighardia* inhabiting the air sacs of birds (Dyck, 1975), and six species, all belonging to the genus *Linguatula*, from the nasal sinuses of mammals (Table 1). Clearly, the reptile respiratory tract is the favoured site of parasitism by pentastomids.

Reptiles have a simple respiratory system comprising a short uncomplicated nasal cavity, devoid of functional nasal sinuses, a long trachea or paired bronchi and a multicompartmented lung (Duncker, 1978: Fig. 1). Thus, in contrast to the bronchoalveolar lungs of mammals and the parabronchial (lung-airsac) system of birds, reptile lungs can be broadly classified as unicameral, paucicameral, or multicameral based on the degree of infolding of the lung wall (Perry, 1983). In most snakes and lizards, the anteriorly located respiratory surface of the simple bag-like unicameral lung (Fig. 1A, C, D, E) is followed by an extensive non-respiratory air sac (Daniels, Barr & Nicholas, 1989; Daniels, Smits & Orgeig, 1995). At the other extreme, the multicameral lungs of crocodilians possess numerous large chambers, each of which is subdivided, and these lungs have a spongy appearance due to pockets of trapped air (Fig. 1B).

An examination of the sites occupied by pentastomids reveals that a total of 95 species are recorded as growing to maturity in the lumen of the lungs of reptiles, living in intimate association with the respiratory surface. Exceptions to this general pattern are two genera, *Leiperia* and *Subtriquetra*, which inhabit the trachea and nasal sinuses respectively of their crocodilian hosts (Riley & Huchzermeyer, 1996), and at least one member of the genus *Elenia* which infests the throat of its varanid host (Bosch & Frank, 1986). In four other lung-dwelling genera, *Waddycephalus*, *Parasambonia*, *Kiricephalus* and *Cubirea*, the swollen and club-shaped head of females is permanently anchored deep in the lung wall, enclosed within a fibrous capsule of host origin, although in all cases males are freely mobile (Riley, 1992*a*).

Baer (1952) considered the distribution of extant pentastomids amongst their hosts and speculated about their evolutionary history. He concluded that reptiles were the ancestral hosts and that the association was probably very ancient, possibly originating in the Mesozoic. Recently discovered fossil evidence, however, indicates the association

 © 1999 Cambridge University Press

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

Table 1. An outline classification of the phylum Pentastomida (from Riley, 1992*a*)

Order	Family	Genus	No. of species	Definitive host	Intermediate host
Cephalobaenida	Cephalobaenidae	*Cephalobaena*	1	Snakes	?
		Raillietiella	> 35	Snakes, lizards, amphisbaenians, amphibians	Direct (?), insects, amphibians, lizards
	Reighardiidae	*Reighardia*	2	Marine birds	Direct (1 sp.)
Porocephalida	Sebekidae	*Sebekia*	12	Crocodilians (Chelonians)	Fish (snakes, lizards?)
		Alofia	5	Crocodilians	Fish
		Leiperia	2	Crocodilians	Fish
		Diesingia	1	Chelonians	?
		Selfia	1	Crocodilians	Fish
		Agema	1	Crocodilians	Fish
	Subtriquetridae	*Subtriquetra*	3 (?)	Crocodilians	Fish
	Sambonidae	*Sambonia*	4	Monitor lizards	Direct (1 sp.)
		Elenia	2	Monitor lizards	Amphibians, mammals
		Waddycephalus	10	Snakes	Amphibians, reptiles, mammals
		Parasambonia	2	Snakes	Amphibians, reptiles, mammals
	Porocephalidae	*Porocephalus*	8	Snakes	Snakes and/or mammals
		Kiricephalus	5	Snakes	Amphibians or lizards or mammals, and snakes
	Armilliferidae	*Armillifer*	7	Snakes	Mammals
		Cubirea	2	Snakes	?
		Gigliolella	1	Snakes	Mammals
	Linguatulidae	*Linguatula*	6	Mammals	Direct (1 sp.), mammals

may be considerably older even than that (Walossek & Müller, 1994; Walossek, Repetski & Müller, 1994). Baer (1952) concluded "It is therefore clear that conditions of so non-specialized a nature as occur in the lungs of reptiles have saved pentastomids from extinction and, consequently, this archaic group owes its present day existence to having adapted itself to parasitism". While the last part of Baer's statement is undoubtedly true, the intricacies of lung physiology were largely unknown in 1952 and Baer was unaware of the unique suite of adaptations that enable pentastomids to chronically infect the lung lumen (Riley, 1992*b*). Consequently, it is almost the converse of his statement that actually obtains; probably the main reason for pentastomid survival lies in the complex adaptations they show to the highly specialized conditions within the reptile lung. Throughout life pentastomids produce membrane-dominated excretory/secretory (E/S) products which coat the cuticle. We will argue that these probably evolved very early in the evolutionary history of pentastomids, as a direct response to the selective pressures of life in the membrane-delimited environment of the reptile lung. Subsequently, these mechanisms were further selected for life in the tissues of intermediate hosts, constituting one of the more unusual examples of immune evasion by a parasite.

Lung-dwelling pentastomids are typically large, some reaching 5–10 cm or more in length and < 1 cm in diameter (Fig. 1C, E). They are long-lived (many months to many years) and all imbibe blood pumped from capillaries in the lung epithelium (Thomas & Böckeler, 1992*a*, *b*). They crawl using the body wall in a manner analogous to that of dipteran maggots (von Haffner, 1972), through a microhabitat – the air/water interface of the lung lining – which is one of the most carefully regulated environments in the vertebrate body. Self & Kuntz (1967), Self (1972) and Self & Cosgrove (1972) commented upon the remarkable degree of compatibility between tissue-dwelling pentastomids and their mammalian and reptilian hosts, and concluded that the adaptation of the host to the parasite is so complete as to result in little observable pathological effect. The deployment of surfactant by pentastomids, a key adaptation resulting from a long association with the lungs of lower tetrapods, lies at the heart of this apparent stability. The elaborate structural and biochemical adaptations that underpin the prolonged existence of pentastomids in this extreme and delicate environment are the subjects of this paper.

Macroparasites and lungs

The majority of the vertebrate helminth endoparasites are enteric and, although the respiratory tract has been successfully invaded by a number of quite disparate taxa (Rose, 1976), relatively few species actually reside in or on the respiratory epithelium. The exceedingly small size of alveoli, of the order of tens of micrometers, is clearly an

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

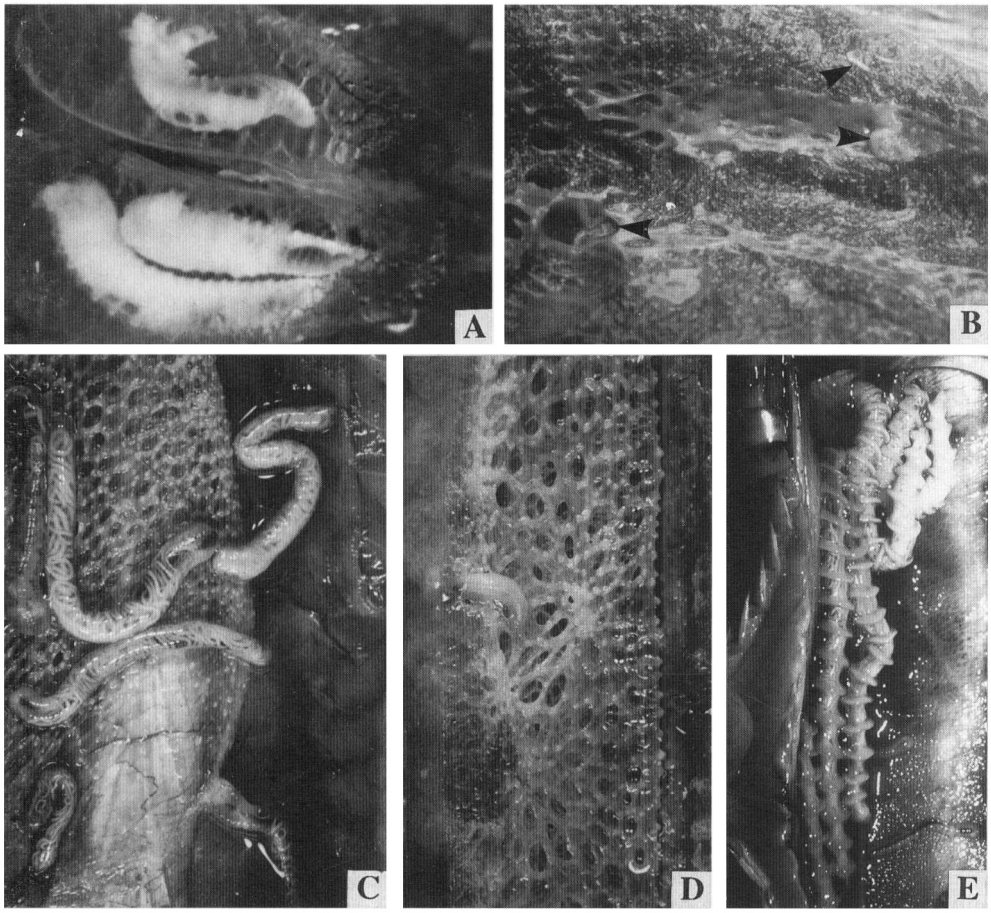


Fig. 1. Living pentastomids in the lungs of freshly dissected reptilian hosts. (A) The lungs of a gecko, *Hemidactylus brookii*, containing three large females and one small male *Raillietiella* sp. The heads of the females are orientated towards the fundus of the lung (left) and their tails extend towards the trachea (right). The individual gas exchange units (faveoli) carry substantial blood vessels and are clustered mainly around the top of the lung (right). ×56. (B) Part of the lung of an African dwarf crocodile (*Osteolaemus tetraspis*), which has been sliced open to reveal pentastomids (*Sebekia* and *Alofia*, arrows) in the bronchioles and air sacs of these paucicameral lungs. ×2·1. (C & D) *Porocephalus crotali* within the lung of a rattlesnake (*Crotalus atrox*); on the left are several females showing coils of the uterus through the thin chitinous cuticle and these mostly obscure the black, haematin-filled intestine. The right panel shows a single male that has penetrated into a single faveolus. Many faveoli are plainly visible, as are the smooth muscle bands (trabeculae) which encircle the neck of each of these respiratory units. ×0·9 and ×1·2 respectively. (E) *Armiller armillatus* within the lung of a gaboon viper (*Bitis gabonica*); the elevated annuli of the four females are plainly visible and two much smaller males are attached to the faveolar lining of the lung towards the top of the photograph. ×0·5.

important limiting factor in the case of mammals. Thus, the slender trichurid, metastrongylid, dictyocaulid and protostrongylid nematode lungworms of mammals, which measure 2–10 cm in length (Mehlhorn, 1988), actually lodge within the fine branches of the bronchi, as do certain syngamid nematodes in birds and mammals (Smyth, 1976). The ontogenetic migrations of many species of larval nematodes, ultimately destined for adulthood in the gut, may involve penetration of the lungs but passage to the bronchioles is usually transient (Rose, 1976).

Adult flukes of the genus *Paragonimus*, found deep within the lungs of a number of mammals species, are invariably located within a conspicuous thick-walled cyst composed of fibrous granulation tissue of host origin (Miyazaki, 1991). Granulation tissue also invests lung-infecting hydatid cysts of the dog tapeworm *Echinococcus granulosus*, and these are further protected by a thick striated cuticular layer enclosing the germinal epithelium (Miyazaki, 1991). Several digeneans, including *Haplometra*, *Haematoloechus*, *Pneumonocoeces* and *Pneumobites* infect the lungs of frogs and are truly intraluminal (reviewed by Tinsley & Earle, 1983), as are three digenean species infesting the lungs of turtles (Crandall, 1960) and snakes (Krull, 1930, 1931). However, these few helminth examples appear to represent only isolated successes in the colonisation of the lung environment.

Two monogeneans, species of *Pseudodiplorchis* and *Neodiplorchis*, infecting the spadefoot toad *Scaphiopus* also have an obligatory lung stage

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

associated with ontogenetic migration. However, this is strictly short term (3–4 weeks) and occurs when developing worms migrate from the nares and mouth of the toad to the definitive site for maturation, the urinary bladder (Tinsley & Earle, 1983).

Several groups of arthropods (the taxon most closely related to the pentastomids (Walossek & Müller, 1994)) also inhabit the vertebrate respiratory tract. These include the larvae of dipteran head bots (Oestridae and Cuterebridae) in the nasal cavities of mammals, and various acarines (Rhinonyssidae and Halarachnidae) in the nasal passages of birds and bronchi and bronchioles of mammals (Chandler & Read, 1981). However, only one family of mites, the Entonyssidae, are specialist parasites of the snake lung (Turk, 1947).

In order to determine the exact *in situ* location of pentastomids in lungs, hosts must be examined very soon after death because worms undertake lengthy *post-mortem* migrations (Riley, 1986). Unsurprisingly, such information is limited. Female raillietiellids in gecko lungs are nearly always orientated with their heads towards the fundus of the lung and their caudal extremities towards the trachea (Fig. 1A), which often contains long slicks of haematin-contaminated faecal material. One group of raillietiellids which infect small lizards possess large blunt-tipped posterior hooks (Ali, Riley & Self, 1985), and the head and projecting hooks are precisely contoured to fit snugly inside individual gas exchange units termed faveoli (Duncker, 1978: Fig. 1A). This arrangement holds the worm securely in place and the blunt tips of the hooks presumably minimise damage to the delicate respiratory epithelium. *Porocephalus crotali* and *Armillifer armillatus* mostly inhabit the faveolar region of the lung of their snake hosts (Fig. 1C, E) and males are small enough to occupy partly individual faveoli (Fig. 1D). *Sebekia*, *Agema* and *Alofia* species in dwarf crocodile lungs can be seen occasionally through the pleura, but deep incisions into the lung reveal that most worms live within the air spaces and fine bronchioles often very close to the pleura (Riley & Huchzermeyer, 1995: Fig. 1B).

The above inventory, although not exhaustive, emphasises that the phylum Pentastomida constitutes the highest-ranking taxon that has become irrevocably associated with the tetrapod lung. An important corollary is that for most pentastomids the parasite tegument is likely to interface intimately with the host respiratory surface.

The lung and pulmonary surfactant in endothermic tetrapods

The tetrapod respiratory system is equipped with protective mechanisms essential for the maintenance of lung homeostasis. A better understanding of the problems faced by reptilian systems can be obtained by comparing them with the protective mechanisms of mammals (Fowler, 1980). In the first instance, inhaled air must be warmed or cooled and cleansed. In mammals this begins as air is inhaled when particles may be trapped either by nasal hairs or on the sticky surface of the nasal mucosa and turbinates. Any particles penetrating the trachea, bronchi and bronchioles are caught by mucus and swept out of the tract by cilia at a rate of about 1 cm min^{-1}, a process which may be greatly assisted by coughing. Particles less than 10 μm in diameter can enter alveoli where they are removed by phagocytosis. The honeycomb arrangement of faveoli in reptile lungs (Fig. 1A, C, D, E) inhibits fluid removal and reptiles are also unable to sneeze or cough. These attributes alone may be sufficient to explain why bulky lung-dwelling pentastomids are mostly restricted to reptiles and amphibians (Table 1).

Virtually all of our knowledge of the interrelationships between the chemical constituents of pulmonary surfactant, the lung-lining material of tetrapods, is derived from mammalian studies (Farrell, 1982). Therefore, mammalian examples will be used to introduce the biology of the delicate interface to which pentastomids have become adapted. Note, however, that surfactant composition (Table 2) and function is broadly similar across all tetrapods (Daniels *et al.* 1998*a*, *b*).

The respiratory surface of tetrapod lungs is richly supplied by alveolar type-II cells, which synthesise and secrete pulmonary surfactant, a remarkable biological material that has physical properties critical for normal lung function. The only other alveolar epithelial cell, the type-I cell, covers 95% of the alveolar surface area but has no known role in surfactant metabolism (Hawgood & Poulain, 1995). Pulmonary surfactant consists of a complex mixture of phospholipids, neutral lipids and proteins and has the unique ability to disperse as a monolayer over the liquid inner lining of the lung (Harwood, 1987). In mammals and birds its primary function is to lower the surface tension of the thin fluid layer between the tissue and air, thereby reducing the work associated with inspiration and preventing alveolar collapse at low lung volumes (Farrell, 1982).

Thus, surfactant is present in two major pools; in the extracellular compartment it spreads as a film over the liquid lining of the lung whereas intracellularly it is sequestered within distinctive lamellar bodies that characterise alveolar type II cells (Smith, Smith & Ryan, 1972). Surfactant isolated from either source contains about 85–90% lipid by weight, of which 90–95% is composed of phospholipids, with cholesterol as the main neutral lipid (Harwood, 1987). Phosphatidylcholine (PC) accounts for 70–80% of total lipid, and about 60% of that is disaturated, both of the constituent fatty acids being saturated. The resulting molecule, dipalmitoylphosphatidylcholine (DPPC) is the key component that is

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

Table 2. The phospholipid composition of isolated lung surfactants (% wt/wt). For the phospholipid notations see text and Table 3

Class	Species	PC	PE	PG	PI+PS	Sph	Others	Source
Mammal	Rat	76	6	6	4	3	4	Harwood (1987)
	Rabbit	83	4	4	5	1	3	
	Man	69	4	7	5	5	7	
Bird	Turkey	86	6	—	7	tr.	1	
Reptile								
Snakes	Rattlesnake	68·5	—	—	11·5	11·5	17·7 (LPC)*	Daniels, Smits & Orgeig (1995)
	Garter snake	64	—	4·0	16·9	1·0	14·2 (LPC)*	
Lizard	*Ctenophorus nuchalis*	72·5	—	—	24·4	3·1	—	Daniels, Barr & Nicholas (1989)
Amphibian								
Salamander	*Amphiuma tridactylum*	77·5	1·0	—	17·5	4·0	—	Daniels, Orgeig, Wilson & Nicholas (1994)
Toad	*Bufo marinus*	62·5	9·0	13·0	8·5	6·5	—	

* LPC, lysophosphatidylcholine. tr. = trace.

responsible for the low surface tension and stability of the monolayer (Kuroki & Voelker, 1994). The phosphocholine in DPPC is polar and hydrophilic, while the two palmitic acid residues are non-polar and hydrophobic. The choline part of the molecule associates with the liquid phase in the alveoli and the palmitic acid orientates towards the air. DPPC molecules become closely packed together when the surface area is reduced, which confers great stability on the surface film (Wright & Clements, 1987). Phosphoglycerides comprised 90% of the total phospholipids and in all tetrapods the PC fraction dominates, but there is some variation between species in the composition of the various lipid classes (Harwood, 1987: Table 2). In ranking order of importance, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) constitute the bulk of the minor phospholipid component in mammals, and sphingomyelin (Sph) is the only significant sphingolipid (Table 2). Disaturated phospholipids (DSP) account for most of the phospholipid fraction but significant amounts of polyunsaturated fatty acids may be present in some mammals and these probably function as liquifiers (Bangham, 1987)

Although lipids constitute $< 90\%$ of surfactant mass in mammals, surfactant proteins (SP) make up the remainder and these are critical to every aspect of surfactant function. SP-A and SP-D are members of the collectin family of proteins that both regulate surfactant phospholipid secretion and uptake, as well as playing an important role in host defence within the lung. In particular, SP-A binds with broad specificity to a variety of microorganisms stimulating phagocytosis and chemotaxis in alveolar macrophages (Kuroki & Voelker, 1994; Wright, 1997). Two small surfactant proteins, SP-B and SP-C, are also synthesised and secreted by alveolar type II pneumocytes. Both are strongly hydrophobic and remain lipid-associated, and both are essential in modulating the processes of phospholipid adsorption and spreading (Perez-Gil, Casals & Marsh, 1995).

Surfactant has a complicated life cycle, first through synthesis, then to a regulated secretion to the alveolus, followed by monolayer formation and subsequent clearance by catabolic and recycling pathways (Wright & Clements, 1987). The change in the alveolar form of surfactant appears to depend on surface exposure of the surfactant and the presence of a serine protease (Gross, 1995), although the exact mechanisms remain poorly understood. A number of lines of evidence suggest that surfactant PC is retrieved from the alveolar pool and recycled by alveolar type II cells (Ikegami & Jobe, 1993). It is even possible for labelled PC, administered via the trachea (of rabbits), to be recycled via these cells (Hallman, Epstein & Gluck, 1981). The continuous recycling of surfactant, which in rabbits has an efficiency of between 25% and 90% (Rider, Ikegami & Jobe, 1990), has important implications for the E/S products of lung-dwelling pentastomids.

Lung surfactant in ectothermic tetrapods

Snakes and most lizards commonly possess simple bag-like lungs with air-sacs that are up to a 1000-fold larger and up to a 100-fold more compliant than those of comparably sized mammals. They are divided structurally and functionally into two regions; a proximal faveolar (respiratory) area, and a much larger non-respiratory saccular region (Daniels *et al.* 1989, 1995: Fig. 1). Upon inspiration, air passes over a honeycomb of bellows-shaped faveoli, where actual gas exchange occurs, into a saccate region lined with a simple squamous epithelium which stores air that can be made available for gas exchange during apnoea. Both regions contain large amounts of surfactant (Daniels *et al.* 1995).

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

The two main functions of surfactant in mammals are to provide alveolar stability and to increase compliance of the relatively stiff bronchoalveolar lung. Since the respiratory units of most non-mammalian vertebrates are > 1000 times larger, surfactant is not required for these functions (Daniels *et al.* 1998*a*). In non-mammalian vertebrates, surfactant appears to act as an anti-glue preventing adhesion of respiratory surfaces that may occur when lungs collapse during swallowing prey or upon expiration. Surfactant also controls lung fluid balance and this may represent the primitive function of surface active material in vertebrate lungs (Orgeig *et al.* 1997; Daniels *et al.* 1998*a*).

Lung surfactant has been investigated in a number of ectotherms (McGregor, Daniels & Nicholas, 1993; Daniels, Eskandari-Marandi & Nicholas, 1993; Daniels *et al.* 1994: Table 2). In toads and salamanders, DSP lower surface tension at the air-water interface in the lung, while cholesterol and unsaturated phospholipids assist spreading. Lizards possess significantly more surfactant per unit area of respiratory surface than do similar sized mammals, and increases in the cholesterol content of surfactant at low temperatures may promote fluidity and adsorption (Daniels *et al.* 1989). Snake faveolar surfactant contains very little cholesterol (3–8 %), but that of rattlesnakes (*Crotalus atrox*) differs from most other vertebrates in that it contains unusually high levels of lysophosphatidylcholine (LPC) and large amounts of Sph, whereas PG and PI are virtually absent (Table 2). However, LPC has detergent properties and can be toxic and consequently it does not normally accumulate in biological systems. Hence, the elevated levels recorded by Daniels *et al.* (1989) probably reflect some form of degradation of the sample prior to analysis.

Generally the saccular lungs and other gas-holding structures in non-mammals have 7–70 % more surfactant/cm^{-2} than occurs in the lungs of mammals. The lipid composition of surfactant is highly conserved within the vertebrates, except that in lungfish it is dominated by cholesterol whereas in higher tetrapods DSP is the dominant fraction (Daniels *et al.* 1998*b*). Finally, homology within a surfactant protein (SP-A) across a whole range of the vertebrate gas-holding structures including the swim-bladder of goldfish, points to a single evolutionary origin for the surfactant system (Sullivan *et al.* 1998).

The lung and regulation of immunity

Apart from the various biophysical properties of pulmonary surfactant outlined above, it is also crucially involved in the protection of the lungs from injuries and infections caused by inhaled particles and microorganisms (Griese, 1999): again our knowledge of these non-physiological functions stems largely from studies of mammalian systems. Alveolar macrophages are the predominant phagocytes of the lower airways and alveoli and it is now well established that pulmonary surfactant regulates their activity, thereby protecting alveoli from potentially damaging immune reactions (Wilsher, Hughes & Haslam, 1988; Geertsma *et al.* 1993). Although surfactant lipids can suppress a variety of immune cell functions, most notably lymphocyte proliferation, conversely SP-A and SP-D enhance phagocytosis by alveolar macrophages (Mariencheck *et al.* 1999; Restrepo *et al.* 1999). Thus surfactant lipids and proteins may be counter-regulatory, and changes in the lipid-to-protein ratio may be important in regulating the immune status of the lung. That these ratios change in certain disease states is clear, but it is not yet established whether this constitutes a cause or an effect (Wright, 1997).

THE ADAPTATIONS OF PENTASTOMIDS FOR LIFE IN THE TETRAPOD LUNG

The pentastomid cuticle and its relation to glands which secrete E/S products

In common with the larvae of many holometabolous insects, pentastomids crawl using their tegument (von Haffner, 1972; Riley, 1986), which remains soft and flexible throughout life. The cuticle comprises three main layers, the endo-, exo- and epicuticles (Mehlhorn, 1988). A number of lipid analogues intercalate with the outermost leaflet of the epicuticle, identified as cuticulin by analogy with the insect cuticle (Riley & Banaja, 1975). The technique of fluorescence recovery after photobleaching reveals that the lateral fluidity of this cuticulin layer is very much like that of a conventional plasma membrane (Ambrose & Riley, 1988*c*).

A long recognised characteristic of pentastomids is the abundance of large glandular cells suspended in the haemocoel (Doucet, 1965). Three of these glands, the sub-parietal cell (SPC) system, frontal glands (FG) and hook glands (HG), discharge E/S products onto the cuticle via minute chitin-lined ducts (Riley, 1986, 1992*b*: Fig. 2). FG and HG, restricted to the cephalic region in primitive cephalobaenid pentastomids (Table 1), are diffuse and masses of individual secretory lobules and associated efferent ducts empty secretion onto the anterior margin of the head, the buccal cavity and the hook pits (Riley, 1973; Riley, James & Banaja, 1979). The equivalent gland cells in the advanced Porocephalida are arranged into compact organs within which individual chitin-lined efferent ducts, each serving a secretory lobule, converge into common collecting ducts. Again these breach the cuticle at the hook pits or the anterior margin of the head at the frontal papillae (Riley, 1986; Ambrose & Riley, 1988*b*: Fig. 2).

By contrast, SPC are distributed similarly in both

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

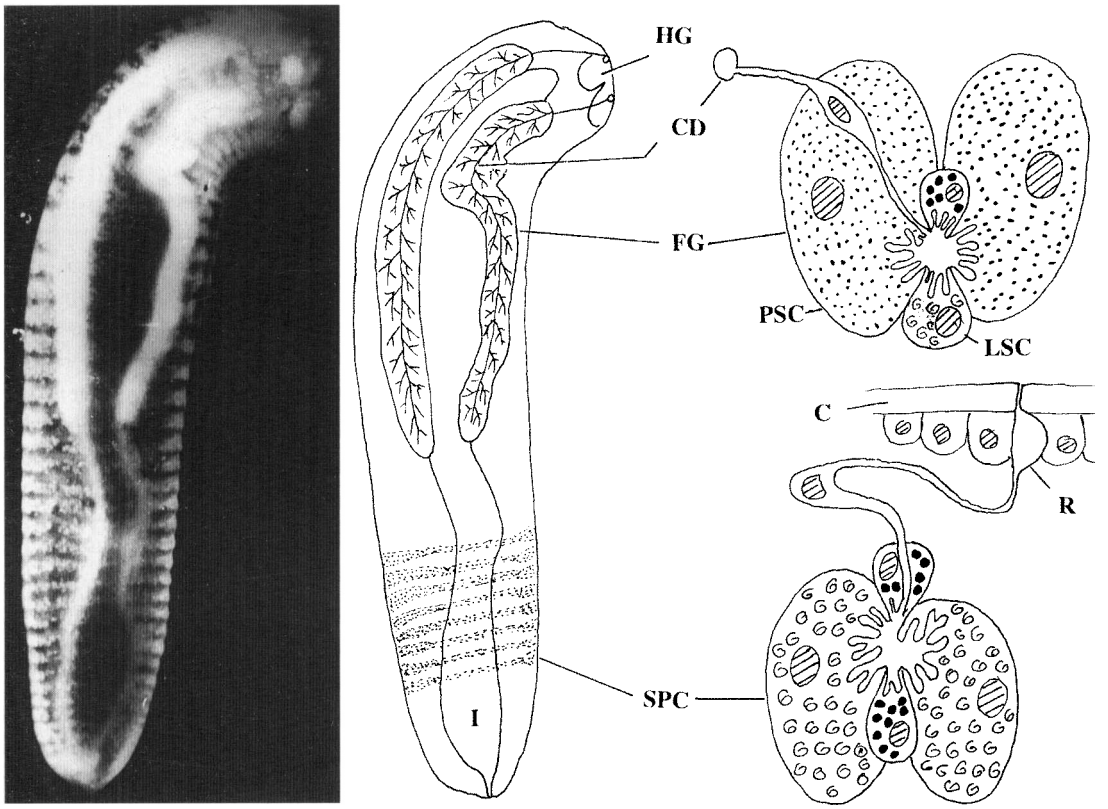


Fig. 2. An 11 mm long, living infective nymph of *Porocephalus crotali* (left), mounted on a slide in saline and flattened slightly by coverslip pressure, to show the disposition of the three major gland systems which produce surface-active material. The glands are drawn on a tracing of the nymph in the middle panel and the arrangement of efferent ducts, which eventually converge onto the collecting duct (CD) before discharging at the anterior margin of the head, have been added to frontal glands (FG). The right-hand panel shows an individual secretory lobule within FG, comprised of two large protein-secreting cells (PSC), a lipid secreting cell (LSC) and another small cell type, apparently also synthesising protein, but of a different type. The efferent duct is shown connecting to the collecting duct. Subparietal cells (SPC) are composed of four cells, all linked to the cuticle (C) by a sinuous efferent duct which expands to form a reservoir just below the level of the cuticle. The two large cells in each lobule secrete lamellate droplets and their ultrastructure closely resembles that of LSC in FG. The other two cells are filled with protein (?) droplets. Hook glands (HG) are drawn in outline only. (I = intestine). Diagrammatic and not to scale.

orders and appear as diffuse annular clusters of subtegumental secretory lobules, each connected to a single efferent duct (Fig. 2). The latter erupt over the entire surface of the cuticle, but are more densely concentrated in broad bands over the raised portion of each abdominal annulus (Doucet, 1965: Riley *et al.* 1979; Ambrose & Riley, 1988*b*). The potential of the SPC system to coat simultaneously the entire cuticle with secretion is striking. For example, microscopical examination of the cuticles of living pentastomids under short wavelength blue light generates bright autofluorescence, particularly in the chitin-lined efferent ducts of gland cells. Those serving SPC, though tiny (< 1 μm in diameter), can be easily counted and their surface density has been estimated at about 400 mm^{-2} (Ambrose & Riley, 1988*b*). Most emerge onto the cuticle near to the periphery of chloride cell pore caps (Doucet, 1965; Banaja, James & Riley, 1977), although it should be emphasised that every part of the cuticle receives SPC ducts. The subtlety of the system is quite remarkable and testifies to the strategic importance of the SPC system (Ambrose & Riley, 1989).

Precursor gland cells filled with secretory droplets and corresponding to HG, FG and SPC have been detected by light microscopy in primary larvae of *P. crotali* within the egg (Esslinger, 1962*b*). By the time nymphs have developed in rodent intermediate hosts to become infective to rattlesnake definitive hosts (Esslinger, 1962*a*), FG, for example, are macroscopic measuring 0.3×5 mm (Ambrose & Riley, 1988*a*: Fig. 2). Adult instars in snake lung (Fig. 1C, D) possess enormous FG, attaining lengths of 2 and 4 cm (in ♂♂ and ♀♀ respectively: Jones *et al.* 1991). The other two systems also increase in size with each moult and the available evidence suggests that, in both pentastomid orders, all instars are coated with E/S material throughout life. Inevitably, E/S products from each of the three glands must interact with the host's immune system; in the case of pre-adult and adult instars, this occurs necessarily within the environment of a lung.

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

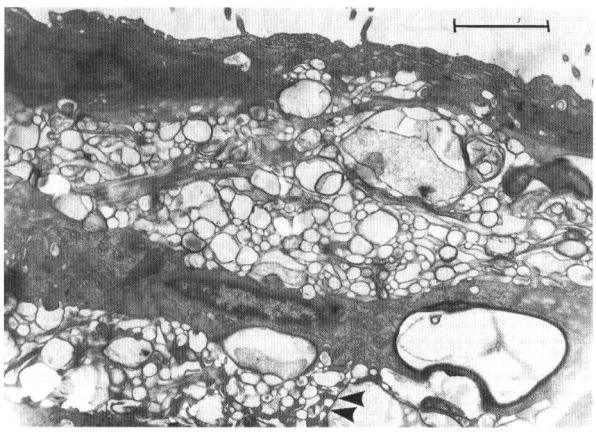


Fig. 3. An electron micrograph of host cells and SPC-derived vesicles on the ensheathing VIth instar cuticle surrounding an encysted VIIth instar of *Porocephalus crotali* (120 d post-infection) in the abdominal fat body of a rat. A fragment of cuticle is visible (bottom left) and the mat of epicuticular hairs (arrowed) holding a foamy layer of vesicles over the cuticle surface can be seen above it. Parts of two macrophages can be visualised and, although both are immersed in vesicles of parasite origin, they merely deform around them and remain totally unreactive. Collagen fibres, which form a very significant component of the final granuloma, are beginning to be deposited (middle left). Scale bar is 1 μm.

Pentastomid SPC-derived surfactant and the host immune response

Sub-parietal cell (SPC) secretion is predominantly membranous, which has the enormous advantage that evidence of SPC activity at the host/parasite interface can be visualised by conventional transmission electron microscopy (Ambrose and Riley, 1988*b*, *c*; 1989; Riley, 1992*b*). In *P. crotali* infections in rodent and rattlesnake hosts, individual SPC secretory lobules consist of four secretory cells, two large and two very small, which share a common efferent duct (Ambrose & Riley, 1988*b*: Fig. 2). The larger cells synthesise and secrete elaborate lamellate droplets. In terms of their ultrastructure and biosynthesis via Golgi bodies, these droplets are indistinguishable from pulmonary surfactant within alveolar type II cells, even to the extent of forming tubular myelin (Smith *et al.* 1972; Williams, 1977; Beckman & Dierichs, 1984; and see Fig. 17 in Ambrose & Riley, 1988*b*). Efferent ducts are equipped with bulbous reservoirs measuring approximately $3 \times 4{\cdot}5$ μm which lie entirely within ductule-secreting cells deep in the tegumental epithelium (Fig. 2). Reservoirs store membranous droplets prior to their discharge onto the cuticle surface through exceedingly narrow pores, 60–80 nm in diameter, enclosed within chitinous collars. In rodent hosts the end product of SPC secretory activity blankets the surface of the cuticle of growing nymphs of *P. crotali* in a foam of polymorphic vesicles and stacked membrane systems up to 12 μm thick (Ambrose & Riley, 1988*b*, *c*; 1989: Fig. 3). The epicuticle of instars II–VI is elevated to form a dense mat of hair-like processes that effectively anchors this foam layer to the parasite surface. Macrophages, eosinophils and other inflammatory cells freely penetrate the foam, and many become completely submerged within it, but significantly none reacts to it in any obvious way (Fig. 3). Equally, smooth-surfaced infective nymphs, recovered from the body cavity of rattlesnakes *en route* to the lung, are also membrane-clad, but now in sheets up to twenty deep. Membranous profiles can also be recovered by the centrifugation of culture medium in which either infective nymphs or subsequent lung-stages have been grown, and thus it seems that all instars are permanently enveloped in membranes (Riley, 1992*b*; Buckle, 1998).

Actively moulting larvae of *P. crotali* encysted in tissues of rodent intermediate hosts become the focus of intense granulomatous responses, characterized in the early stages by a pronounced eosinophilia (Ambrose & Riley, 1988*a*), and later by the recruitment of mast cells (McHardy, Riley & Huntley, 1993). Cytotoxic responses, as evidenced by degranulation and giant cell formation, are very significant particularly following ecdysis but this activity is localised and directed largely, if not exclusively, against the inner surface of cast cuticles (Fig. 4). The continuously exposed hairy membrane-clad outside surface of the cuticle remains immunologically inert, irrespective of whether it is 'living' or 'dead' (i.e. an integral part of a living nymph or cast/ensheathing) (Ambrose & Riley, 1989). Host cells have direct access to the parasite surface of the first six instars – these nymphs actually feed on eosinophils (Fig. 4) – but the smooth-surfaced seventh stage is quite different. Uniquely, it retains the hairy cast cuticle of the previous instar as a protective sheath which affords an unbroken barrier, totally blocking the access of host cells to the living worm within (Ambrose & Riley, 1988*a*, *b*). Clearly, membranes attached to the ensheathing cuticle, and indeed those associated with every cast cuticle, cannot be replenished and yet continue to down-regulate effector cells (Fig. 4). An important corollary is that, at least in intermediate hosts, membranes do not need to be continuously secreted in order to exert their protective function.

The VIIth instar is infective to rattlesnake definitive hosts (Esslinger, 1962*a*) and can be readily dissected free of its ensheathing cuticle (Buckle, Riley & Hill, 1997). When such 'naked' larvae are transplanted parenterally into naive or previously infected rodents, the cuticle bears the brunt of a sustained attack by macrophages and eosinophils and worms are eventually killed (Fig. 5). Marked differences in the onset and intensity of the inflammatory responses in the two categories of hosts point

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

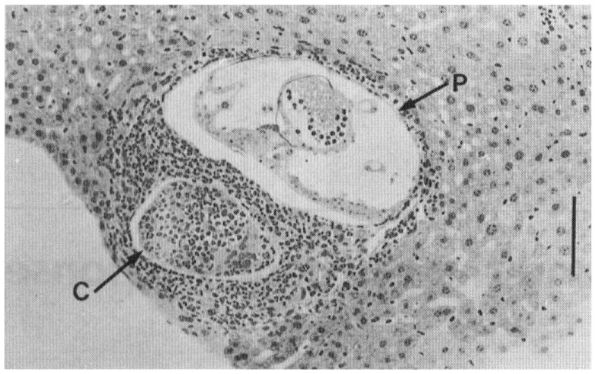


Fig. 4. A light micrograph of a sagittal section through a IIIrd instar of *P. crotali* in mouse liver, showing the onset of granuloma formation. A discrete layer of effector cells, mostly composed of macrophages and eosinophils, surrounds the parasite (P), and large populations of these cells invest a recently cast cuticle (C). The latter, filled with degranulating cells and transformed giant cells, will eventually constitute one of the many independent 'cuticle'granulomas' within the main lesion. By contrast, cells at the parasite surface appear inactive when observed by electron microscopy. The small round gut of the developing nymph is filled with eosinophils, to the virtual exclusion of other cell types. Reproduced with permission from Ambrose & Riley (1988*a*). Scale bar = 100 μm.

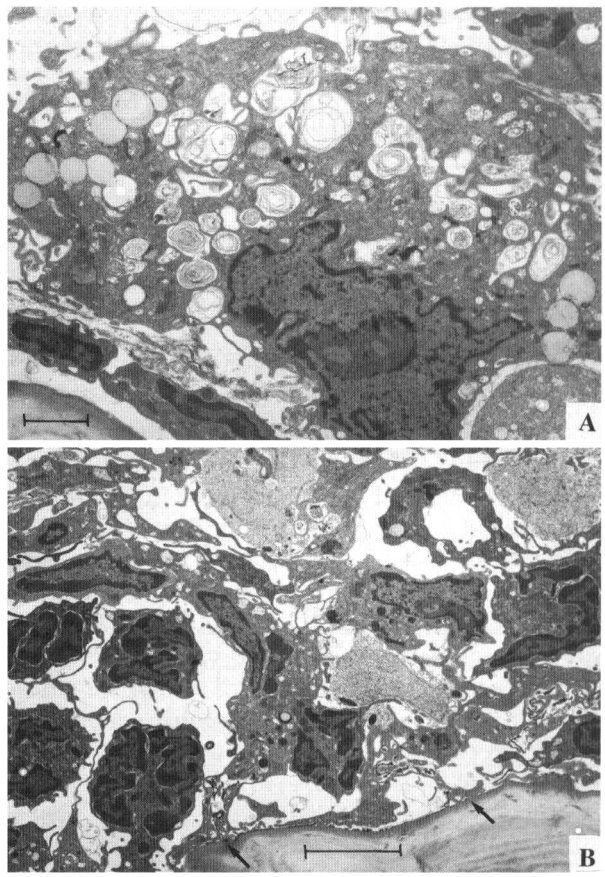


Fig. 5. Electron micrographs of cytotoxic activity around implanted 'naked' nymphs of *Porocephalus crotali* after 3 days in the body cavity of an infected mouse. A. Detail of a macrophage at the surface of the cuticle (bottom left) including numerous endocytotic vesicles, many containing concentric membranes that are highly reminiscent of SPC membranous E/S products. Normally, this material would be destined for deployment at the surface following successful transmission to a snake. However, under these circumstances of experimental manipulation, it appears to be incompatible with the rodent immune system with the result that it is endocytosed. B. The pseudopods of activated macrophages (in the centre of the micrograph) direct lysosomal contents (arrows) against the parasite cuticle (bottom right) amid a welter of granule discharge from eosinophils. The spent remains of the latter are visible as the lighter-staining, flocculent masses of cytoplasm. Reprinted, with permission, from Ambrose & Riley (1989). Scale bars are 2 μm.

to some form of specific recognition (Ambrose & Riley, 1989). Since both the ensheathing hairy VIth stage cuticle and the underlying smooth cuticle of the VIIth instar are membrane-clad (Ambrose & Riley, 1988*c*), stage-specific differences in the surfactants of instars I–VI and VII are thought to underpin these responses.

Ambrose & Riley (1989) postulated that the cuticles of instars I–VI were protected against the rodent phagocytes by so-called 'rodent-compatible' SPC-derived surfactant. Effectively so are VIIth instars in natural rodent infections but only because of protection afforded by the ensheathing VIth stage cuticle, complete with its covering layer of 'rodent-compatible' surfactant. Ambrose & Riley (1989) argued that the encysted VIIth instar in rodents was actually pre-adapted for life in a rattlesnake lung. This hypothesis is based on the observation that larvae remain viable in rodent tissues for several years and, following ingestion by a rattlesnake, penetrate through the stomach wall and into the body cavity within 24 h. Consequently, if VIIth instars are divested of their protective sheath by dissection, and immediately transplanted back into a rodent, only 'snake-compatible' surfactant is available for surface deployment. This is immediately recognised as 'incompatible' and endocytosed by macrophages (Fig. 5A). Subsequently, the worm surface attracts large populations of granulocytes, in various states of activation, which target the now unprotected and vulnerable cuticle (Fig. 5B) (Ambrose & Riley, 1989). By contrast, migrating infective nymphs, recovered from the body cavity after several days in snakes, are invariably 'clean' (totally free of investing cells (Ambrose & Riley, 1988*b*)). Following penetration of the lung after about 16 days post infection (Riley, 1981), it is possible that surfactant composition might change once again (Tables 3 and 4; Fig. 7)

Indirect fluorescent antibody tests on sectioned V–VIIth instar nymphs of *P. crotali* probed with infected rat serum (50–150 d.p.i.), or rabbit antiserum raised against purified SPC lamellate droplets

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

Table 3. The lipid class composition of E/S lipids secreted into MEM by cohorts of cultured *P. crotali* (PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CHOL, cholesterol; FFA, free fatty acids; SE, steryl esters)

Instar	Culture age	Worm no.	Extracted lipid μg worm^{-1} d^{-1}	PC	PS	PI	PG	PE	CHOL	FFA	SE
VII	15 h	186	37	33·4	tr	tr	8·4	8	14	20·9	13·5
VII	2 d	125	10·9	34·8	5·3	6·6	0·3	5·1	36·2	5·7	5·9
VIII	21 d	125	97·9	5·6**	6·6	—	—	1·6	22·4	17	46·7
IX	36 d	95	163·2	11·3**	0·7	1·6	2·3	2·1	25	11·4	45·7
X	114 d	90	16·9	29·1	2·6	4·5		7·6	36·6	16·2	3·4

** These samples contain relatively low PC and high SE compared to the other instars. tr = trace.

Table 4. Fatty acid composition (wt%) of lipids extracted from MEM in which 122 *P. crotali* infective nymphs had been cultured at the times indicated (blood medium fatty acids are included as a control). Chloroform-methanol extracts were dried down and methylated prior to analysis by gas chromatography. The identifications were confirmed by GC-mass spectrometry

Age of culture	Blood medium	0–22 h	6 d	10 d	20 d	37 d	
						0–80 min ⟶	22 h
Amount of lipid (mg)	0·988	0·404	0·036	0·227	0·048	0·063	0·046
Fatty acid							
16:0	19·6	13·7	21·4	19·0	15·7	21·4	18·0
16:1 n-7	0·6	1·3		1·4			1·3
18:0	19·3	6·8	20·4	18·4	16·0	21·0	10·6
18:1 n-9	14·8	4·3	11·5	6·5	11·6	11·2	10·4
18:2 n-6	22·2	2·3	6·3	3·3	7·7	10·4	8·6
18:3 n-3	3·9					2·0	1·6
20:0	0·3	7·1	7·2	4·7	9·5	2·9	8·5
20:1 n-9	0·2	10·4	13·0	12·0	8·4	1·3	11·5
20:3 n-6	2·1	0·3				1·5	
20:4 n-6	7·2	0·9		1·2	4·4	6·1	5·5
22:0	0·9	6·8	2·4	4·0	5·1	4·2	3·0
22:1 n-9	0·6	11·2	3·2	15·8	6·2	2·2	4·8
22:5 n-6	2·3					1·7	1·1
22:6 n-3	1·9					3·2	3·3
24:0	1·9	13·6	4·9	6·7	5·5	5·9	5·0
24:1 n-9	2·3	21·3	9·8	7·0	10·1	5·0	6·9

from VIIth instar nymphs, shed further light on the hypothesis of the stage-specificity of SPC surfactant (Jones, Henderson & Riley, 1992). Significantly, rabbit antiserum uniformly labelled SPC lamellate droplets and also morphologically identical cells in FG and HG but only in VIIth instars. Pooled serum from infected rats consistently failed to identify similar cells in any instars, although protein-secreting cells in FG and gut enzyme-secretory cells and gut contents labelled consistently (Jones & Riley, 1991; Jones *et al.* 1991). Ambrose & Riley (1988*b*, *c*, 1989) and Riley (1992*b*) concluded that although the parasite cuticle was potentially vulnerable to immune attack, this rarely occurred because of protective host compatible surfactant.

Jones *et al.* (1992) partially characterized the lipid and protein composition of SPC-derived lamellate droplets recovered by density gradient centrifugation of pooled homogenised cuticles dissected from the infective nymphs of *P. crotali*. Very small amounts of lipid were recovered and their purity was checked only by the electron microscopical examination of centrifuged pellets. Unsurprisingly there was some inconsistency between the three samples. Nonetheless, neutral lipids comprising cholesterol, cholesterol esters (= steryl esters) and triacylglycerol were dominant (65% of the total lipid), with PC being the most important phospholipid, together with significant amounts of Sph (5%) and PS/PI (5·6%). Palmitic acid (16:0) was the commonest fatty acid but overall there was nothing unusual about the lipid class composition. Anti-lamellate droplet antiserum raised in rabbits labelled both SPC and lipid-secreting cells in FG with equal

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

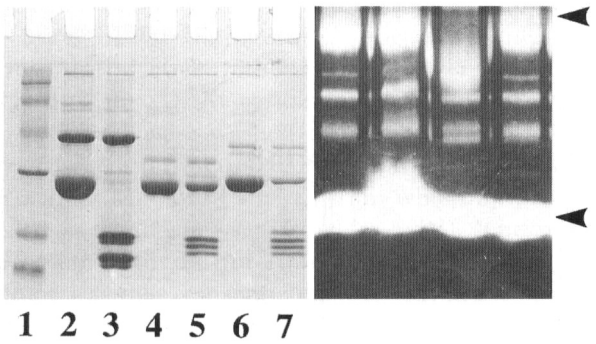


Fig. 6. The left panel is an SDS-PAGE analysis of *P. crotali* (lanes 2 and 3) and *Porocephalus clavatus* (lanes 4–7) frontal gland proteins recovered from pooled, dissected frontal glands. The low molecular weight marker lane proteins (lane 1) ascend through molecular masses of 21, 31, 45, 66, 97, 116 and 200 kDa. Note that in the unboiled lanes (3, 5 and 7), three of four bands of polypeptides are present in the 14–22 kDa range which aggregate to reinforce a band at approximately 30 kDa when boiled (lanes 2, 4 and 6). The metalloproteinase enzyme is at 48 kDa (in *P. crotali*) and 39 kDa (in *P. clavatus*). Lanes 2–5 non-reduced, Lanes 6 and 7 reduced. The Coomassie-stained gelatin substrate gel (right hand panel) shows enzyme activity in *P. crotali* associated with the metalloproteinase at 48 kDa (lower arrow) and a variety of other less active proteins of up to 200 kDa molecular mass (upper arrow).

intensity (Jones *et al.* 1992). Triton-X phase separation of the proteins associated with lamellate droplets sorted them into an aqueous phase (with a major band at 60 kDa), a detergent phase (with two hydrophobic proteins at 16 and 24 kDa) and an insoluble pellet.

Non-lipid E/S products of HG and FG

Morphologically identical cells to SPC are located in every secretory lobule within HG and FG (Fig. 2), and therefore membranes are likely to be implicated also in the function of the non-lipid E/S products discharged from these glands (Ambrose & Riley, 1988*a*). Nothing is known about the activity of the other secretory cell types present in HG, but since efferent ducts serving this gland discharge over the hooks – effectively the point of most intimate contact between host and parasite – this gland is likely to have important, but as yet undetermined, functions.

SDS-PAGE analysis of whole nymphal homogenate of *P. crotali* followed by Western-blotting and probing with immune rat serum revealed that only two bands labelled consistently. Both are metallo-proteinases, with molecular masses of 48 and 150 kDa, but only the former labelled strongly (Jones & Riley, 1991: Fig. 6). Subsequent double antibody labelling of sectioned nymphs demonstrated that the 48 kDa molecule originated as 2 μm diameter secretory droplets in the dominant cell type in FG (Jones *et al.* 1991: Fig. 2). 70% of cytoplasmic volume is occupied by this secretion (Ambrose & Riley, 1988*b*). Antibody responses to this enzyme, detected in the serum of infected rats from as early as 12 d.p.i., peaked at about 100–120 d.p.i., coincident with the termination of moulting. Characterization of proteins purified from pooled FG dissected from VIIth instars shows the 48 kDa species to be an elastase-like metallo-proteinase with an alkaline pH dependency. In addition to this proteinase, four low molecular weight peptides (16–22 kDa) were resolved as two doublets which, following boiling in SDS, with or without dithiothreitol, aggregated to form a single band at 31 kDa (Fig. 6). Aggregation may be explained in terms of a conformational change or, more likely, some form of heat-induced cross-linking. In either event, it is a very unusual phenomenon. Antibodies to these small peptides could not be demonstrated in the serum of *P. crotali*-infected rats. However, all of these proteins, together with the 150 kDa species, can be harvested from saline in which living infective nymphs, freed of their ensheathing cuticles by dissection, have been temporarily cultured (Jones *et al.* 1991; Buckle, 1998). The presence of proteases (Fig. 6) is consistent with a tissue-penetrating post-mortem role in transmission. Snakes swallow their prey whole (Riley, 1992*b*) and < 24 h at 28 °C are required for nymphs to escape from a cyst, migrate through tissues and then penetrate out through the skin of a rodent. However, it is difficult to reconcile this alleged *post-mortem* role with evidence of sustained secretion in rodent tissues and, because glands continue to grow and remain active in lung-stages (see below), with ongoing secretory activity in lungs.

Interestingly, Mariencheck *et al.* (1999) reported that SP-A opsonises a mucoid strain of *Pseudomonas aeruginosa*, thereby enhancing phagocytosis by alveolar macrophage. Moreover, it seems that the bacterium has the capacity to counter this activity by secreting a metalloproteinase that degrades SP-A (Mariencheck & Wright, 1999).

IN VITRO LIPID PRODUCTION BY *P. CROTALI* AND A COMPARISON WITH RATTLESNAKE LUNG SURFACTANT

A number of experiments have been conducted to study the formation and release of surfactant by *P. crotali* and all take advantage of the fact that these parasites can be grown *in vitro* from the VIIth instar to the adult stage (Buckle *et al.* 1997). The basic protocol was modified so that E/S lipids from putative 'lung worms' could be recovered from the culture medium. Two quite different methods were used to determine the nature of the lipids released *in vitro*, and each had its problems (Buckle, 1998). In the first case, [1–^{14}C] palmitic acid was added to

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

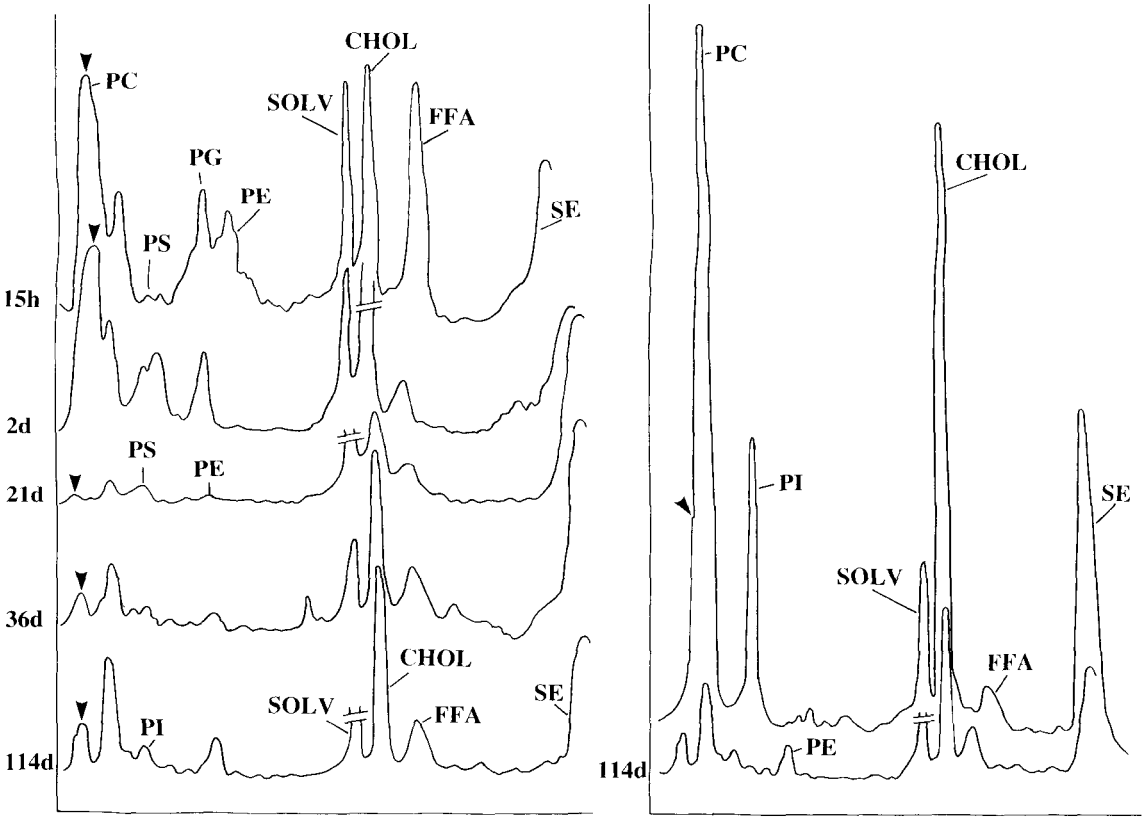


Fig. 7. The left hand panel comprises HPTLC scanning densitometer profiles showing the lipid class composition of E/S lipids, secreted into MEM by cohorts of cultured *P. crotali*. Each was traced from the original scan and then aligned in vertical register (these data are quantified in Table 3). The PC peaks are left, and a gradual shift in relative abundance from the unsaturated form (small arrow) to the saturated species immediately to its right, is apparent with increasing age (from 15 h to 114 d in culture). The abbreviations are explained in Table 3. (SOLV = solvent front). The right hand panel is the identical 114 d profile (left), but this time aligned below a densitometer trace of rattlesnake lung lavage; the latter was far more abundant and hence the big difference in the height of the peaks. The unsaturated PC peak (small arrow) appears as a tiny shoulder on the side of the saturated PC peak in the rattlesnake.

20 ml of blood culture medium – this was known to be the main fatty acid in both lung – and *P. crotali* surfactant (Buckle, 1998). Two batches of worms (80 newly-excysted VIIth instars and 100, 78 d old 'lung worms') were allowed to imbibe blood and thereby acquire label over 48 h. Worms were then washed thoroughly and transferred to normal blood medium. At two day intervals thereafter, 5–10 worms were removed and dissected in order to determine the tissue-localisation of label. All remaining worms were cultured normally, the medium changed every two days and lipids bulk-extracted. The radio-labelled lipid fraction was analysed by a combination of high performance thin-layer chromatography (HPTLC) and scintillation counting. After two days in cold medium, 74–80% of label was located in the head and cuticle of both groups, 13% resided in the gut, whereas the remainder was distributed though FG, body fluids and gut contents (Buckle, 1998). Significant loss of label occurred over the experimental period in both groups, but this was mostly limited to the head/cuticle. Approximately 35% of label was lost from this site after 14 d and 66% after 36 d. It was assumed that, at first, SPC in the head/cuticle acquired palmitate preferentially but that this was subsequently metabolised and exported across the cuticle to the bathing medium. Elsewhere in the worm, label was mostly locked into somatic tissues as part of the normal lipid pool

Extracting ^{14}C-labelled lipid metabolites from whole blood culture media proved difficult. Quenching affected subsequent scintillation counting and this precluded quantification of lipid fluxes. Adequate amounts of labelled lipid metabolites were recovered for analysis, but only from the cohort of 80 infective instars. Among polar lipids, 64% of label initially resided in PC, 18% in PI, 8% in PS, 6·5% in PE and 2·5% in PG. However, after 12 d, PC was the only secreted phospholipid to retain significant label – down to 9% of the original level (Buckle, 1998). At first, neutral lipid profiles were dominated by the release of free fatty acids, almost certainly composed of unabsorbed palmitic acid voided from the worm intestine. The remaining label was located in metabolites such as triacylglycerols (50%), sterols/partial acylglycerols (30%) and cholesterol (= steryl) esters (19%), in all of which label was lost rapidly over 2–6 d.

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

The other study of *in vitro* lipid release followed cohorts of 90–186 cultured worms continuously from the infective to the Xth instar over a period of 114 d (Table 3). At various intervals worms sieved from blood were washed thoroughly in saline and transferred for periods of 5–18 h into Minimum Essential Medium (MEM), from which lipids were extracted and analysed (Buckle, 1998). The major problem with the interpretation of MEM-derived lipids was that transcuticular E/S surfactant tended to be overwhelmed by faecal blood contaminants. Blood faecal material is indicated by the high proportion of sterol/cholesterol esters (the 21 and 36 d samples in Table 3). Cholesterol and cholesterol esters are known to be major components of the lipids of erythrocytes and plasma lipoproteins in vertebrates and the absorption of dietary cholesterol is slower and less complete than that of other lipids (Gurr & Harwood, 1991). This source of contamination is probably insignificant in the 15 h sample because infective instars do not have access to rodent cells and consequently their intestines would contain few digestible residues (Table 3). However, despite this problem, when the HPTLC scanning densitometer profiles of these various lipids are aligned in vertical register, a significant switch in relative abundance of two forms of PC is strongly associated with increasing worm age (Fig. 7, Table 3). The PC profile is composed of two peaks, the left being the predominantly unsaturated form and the right the disaturated species (Fig. 7). The former dominated the PC profile in the 15 h and 2 d worms, but declined relative to the saturated species thereafter. From 21 d, around the time worms enter the rattlesnake lung during natural infection (Riley, 1981), DSP is the dominant form and by 114 d it is 2·5 times more abundant than the other fraction. This apparent shift in the relative abundance of the two forms with increasing worm age is unlikely to have been influenced by faecal contamination (see below).

Finally, we lavaged a rattlesnake lung (following the protocol of Daniels *et al.* 1995), and analysed it by HPTLC and scanning densitometry. When its densitometer profile is aligned with that of the 114 d lung worms (mentioned above), a close correspondence in lipid peaks is evident (Buckle, 1998: Fig. 7). This observation lends credence to the hypothesis that, in lungs, pentastomids may reduce the antigenicity of the cuticle by coating it with a surfactant which is immunologically compatible with pulmonary surfactant (Riley, 1992*b*).

FUNCTIONAL CONSIDERATIONS

Rattlesnake faveolar lung contains more visible pulmonary surfactant than almost any other vertebrate although its exact function remains unclear (Daniels *et al.* 1995). Moreover, in common with surfactant in other tetrapods it will contain surfactant proteins that enable it to adsorb as a monolayer over the air/water interface of the lung, and much of it will be retrieved and recycled, as happens in mammals (Wright & Clements, 1987; Sullivan *et al.* 1998). Pentastomid surfactant will, presumably, contribute indirectly to the lung surfactant pool but there is no good reason to suppose that it is has to be presented to the host as a monolayer. Lipids in mammalian lung surfactants undergo remote assembly into an "active" form following secretion from type II pneumocytes, a process dependent upon a combination of surfactant proteins SP-A and SP-B (Hawgood & Poulain, 1995). *Porocephalus crotali* surface membranes apparently contain proteins (Jones *et al.* 1992; Buckle, 1998) but it remains to be discovered whether any of these are structurally or functionally analogous with the ubiquitous SP-A present in the pulmonary surfactants of tetrapods (Sullivan *et al.* 1998).

Certainly in the case of *P. crotali* in rodents, pentastomid surfactant is semi-permanent – it remains active even on cast cuticles, physically bound to the tegument by a combination of epicuticular hairs, host cells and connective tissue (Ambrose & Riley, 1988*a*: Fig. 3). Electron microscopy reveals that this surfactant exists as fluid-filled vesicles delimited by one or more bilayers that are somehow extruded onto the cuticle through exceedingly narrow pores (Ambrose & Riley, 1988*c*). Therefore, once pentastomid 'rodent-compatible' surfactant has been secreted, it does not behave like lung surfactant which passes through an unusual intermediate geometry comprising interwoven membranes known as tubular myelin in the liquid layer of the alveolar epithelium (Williams, 1977). We do not know whether surfactant produced by lung-dwelling pentastomids behaves similarly within the lung. Nonetheless, membranes enveloping these instars (Ambrose & Riley, 1988*c*) may require constant renewal as evidenced by the recovery of E/S lipids and membranes from *in vitro* culture medium up to 114 d post-excystation (Table 3).

We have repeatedly analysed fatty acids assumed to be associated with E/S lipids produced *in vitro* by cultured *P. crotali*, but no clear pattern has emerged (Jones *et al.* 1992; Buckle, 1998). This may have been the result of a combination of small sample size and contamination by faecal material. In a recent attempt to resolve this problem, a cohort of 122 *P. crotali* was cultured, but worms were very carefully sieved and gently washed in warm saline to minimise any trauma engendered during transfer to MEM. The 0–22 h and 6 d samples contained very little faecal contamination (easily visualised as black granular haematin) and although copious faecal material was liberated by the later stages, many worms appeared to retain their intestinal contents

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

for extended periods (< 18 h). The fatty acid composition of E/S lipids and that of fresh blood medium which was lipid-extracted as a control were compared. The blood extract was typical of mammalian somatic cell membranes in general in that it contained a high proportion of polyunsaturated fatty acids, especially the essential fatty acids for animal species (18:2 n-6, 18:3 n-3) and the products derived from them (20:4 n-6, 22:5 n-6, 22:6 n-3) (Table 4). In contrast, the 0–22 h VIIth instars had a high proportion of saturated and monoenoic fatty acids, with up to 24 carbons, which presumably must have been synthesised by the worms (Table 4). It is highly unusual to find these in abundance in any type of tissue - certain lipids in nervous tissues are the exception. Possibly, the comparatively long acyl tails on these fatty acids form bridges between opposed hydrophobic domains within the lipid bilayers constituting pentastomid surfactant (Fig. 3), conferring stability and durability.

The prime function of pentastomid surfactant may be simply to lower the antigenic profile of the cuticle but this is unlikely to be the sole function. For example, rodent phagocytes and granulocytes freely penetrate the vesicle layer around encysted *P. crotali* (Fig. 3) and yet, despite their being completely enveloped in material of parasite origin, they remain totally unreactive to it (Ambrose & Riley, 1989). The contrast between the behaviour of eosinophils and macrophages on either side of a cast cuticle is remarkable; on the vesicle-coated side essentially normal cells are found whereas on the underside a veritable orgy of activation and degranulation is evident. During moulting the hairy epicuticle appears to hold inflammatory cells at bay until a new protective vesicle layer is secreted (Ambrose & Riley, 1989).

These observations suggest that it is physical contact between surfactant and granulocytes that renders the latter inactive and thereby minimises inflammation. The PC and PG components of mammalian pulmonary surfactant phospholipids are immunosuppressive (Ansfield & Benson, 1980) and have a key role in down-regulating the activity of alveolar macrophages (Wilsher *et al.* 1988; Geertsma *et al.* 1993). It is tempting to speculate that pentastomid SPC system evolved to exploit this property but the evidence is, at best, circumstantial. There remains a great deal to be learnt about pentastomid surfactant. In concert with the membrane-associated (?) proteins emanating from HG and FG, it has a key role in immune-evasion and reducing inflammation, both of which are critical to the long-term survival of these bizarre and ancient inhabitants of the delicate environment that is the tetrapod lung.

Accepting the risk of appearing anthropomorphic, we leave you with this thought. The next time that you inadvertently bypass the first protective barrier of your respiratory tract, by inhaling something solid that should have been swallowed; when the paroxysms of coughing subside, consider the following (from Self & Kuntz, 1967). "We have one collection of over 100 adult *Porocephalus crotali* taken from the lung of a healthy diamond-back rattlesnake ... This supports the contention of host-parasite compatibility as a result of a long evolutionary association between them" (Fig. 1C).

REFERENCES

ALI, J. H., RILEY, J. & SELF, J. T. (1985). A review of the taxonomy and systematics of the pentastomid genus *Raillietiella* Sambon, 1910 with a description of a new species. *Systematic Parasitology* **7**, 111–123.

AMBROSE, N. C. & RILEY, J. (1988*a*). Light microscope observations of granulomatous reactions against developing *Porocephalus crotali* (Pentastomida: Porocephalida) in mouse and rat. *Parasitology* **97**, 1–16.

AMBROSE, N. C. & RILEY, J. (1988*b*). Fine structural aspects of secretory processes in a pentastomid arthropod parasite in its mouse and rattlesnake hosts. *Tissue and Cell* **20**, 381–404.

AMBROSE, N. C. & RILEY, J. (1988*c*). Studies on the host/parasite interface during the development of *Porocephalus crotali* (Pentastomida: Porocephalida) in rodent intermediate hosts with observations of protective surface membranes. *Tissue and Cell* **20**, 721–744.

AMBROSE, N. C. & RILEY, J. (1989). Further evidence for the protective role of sub-parietal cell membranous secretory product on the cuticle of a pentastomid arthropod parasite developing in its rodent intermediate host. *Tissue and Cell* **21**, 699–722.

ANSFIELD, M. J. & BENSON, B. J. (1980). Identification of the immunosuppressive components of canine pulmonary surface active material. *Journal of Immunology* **125**, 1093–1098.

BAER, J. G. (1952). *Ecology of Animal Parasites*. Urbana, The University of Illinois Press.

BANAJA, A. A., JAMES, J. L. & RILEY, J. (1977). Observations on the osmoregulatory system of pentastomids: the tegumental chloride cells. *International Journal for Parasitology* **7**, 27–40.

BANGHAM, A. D. (1987). Lung surfactant: how it does and does not work. *Lung* **165**, 17–25.

BECKMAN, H. J. & DIERICHS, R. (1984). Extramembranous particles and structural variations of tubular myelin figures in rat lung surfactant. *Journal of Ultrastructural Research* **86**, 57–66.

BOSCH, H. & FRANK, W. (1986). The Australian pentastomid genus *Elenia*, Heymons, 1932 – development in the intermediate and definitive host. *Proceedings of the Sixth International Congress of Parasitology*, p. 97.

BUCKLE, A. C. (1998). A study of the *in vitro*-released products (IVRP) of the pentastomid *Porocephalus crotali*. PhD Thesis, University of Dundee

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

BUCKLE, A. C., RILEY, J. & HILL, G. F. (1997). The *in vitro* development of the pentastomid *Porocephalus crotali* from the infective instar to the adult stage. *Parasitology* **115**, 503–512.

CHANDLER, A. C. & READ, C. P. (1981). *Introduction to Parasitology*. 10th Edition. New York and London, John Wiley & Sons.

CRANDALL, R. B. (1960). The life history and affinities of the turtle lung fluke, *Heronimus chelydrae* McCallum, 1902. *Journal of Parasitology* **46**, 63–64.

DANIELS, C. B., BARR, H. A. & NICHOLAS, T. E. (1989). A comparison of the surfactant associated lipids derived from reptilian and mammalian lungs. *Respiratory Physiology* **75**, 335–348.

DANIELS, C. B., ESKANDRI-MARANDI, B. D. & NICHOLAS, T. E. (1993). The role of surfactant in the static lung mechanics of the lizard *Ctenophorus nuchalis*. *Respiration Physiology* **94**, 11–23.

DANIELS, C. B., LOPATKO, O. V. & ORGEIG, S. (1998*a*). Evolution of surface activity related functions of vertebrate pulmonary surfactant. *Clinical and Experimental Pharmacology and Physiology* **25**, 716–721.

DANIELS, C. B., ORGEIG, S., WILSON, J. & NICHOLAS, T. E. (1994). Pulmonary-type surfactants in the lungs of terrestrial and aquatic amphibians. *Respiration Physiology* **95**, 249–258.

DANIELS, C. B., ORGEIG, S., WOOD, P. G., SULLIVAN, L. C., LOPATKO, O. V. & SMITS, A. W. (1998*b*). The changing state of surfactant lipids: new insights from ancient animals. *American Zoologist* **38**, 305–320.

DANIELS, C. B., SMITS, A. W. & ORGEIG, S. (1995). Pulmonary surfactant lipids in the faveolar and saccular lung regions of snakes. *Physiological Zoology* **68**, 812–830.

DOUCET, J. (1965). Contribution à l'étude anatomique, histologique et histochemique des pentastomes (Pentastomida). *Mémoires de l'Office de la Recherche Scientifique et Technique Outre-Mer, Paris* **14**, 1–50.

DUNCKER, H. R. (1978). Funktionsmorphologie des Atemapparates und Coelomgliederung bei Reptilien, Vögeln und Säugern. *Verhandlungen der Deutschen Zoologischen Gesellschaft*, 99–132.

DYCK, J. (1975). *Reighardia sternae* sp. nov., a new pentastomid from the guillemot. *Norwegian Journal of Zoology* **23**, 97–109.

ESSLINGER, J. H. (1962*a*). Development of *Porocephalus crotali* (Humboldt, 1808) (Pentastomida) in experimental intermediate hosts. *Journal of Parasitology* **48**, 452–456.

ESSLINGER, J. H. (1962*b*). Morphology of the egg and larva of *Porocephalus crotali* (Pentastomida). *Journal of Parasitology* **48**, 457–462.

FARRELL, P. M. (1982). *Lung Development: Biological and Clinical Perspectives*. London, Academic Press.

FOWLER, M. E. (1980). Differential diagnosis of pneumonia in reptiles. In *Reproductive Biology and Diseases of Captive Reptiles* (ed. Murphy, J. B. & Collins, J. T.), pp. 227–223. Society for the Study of Amphibians and Reptiles.

GEERTSMA, M. F., BROOS, H. R., VAN DEN BARSELAAR, P. H. & VAN FURTH, R. (1993). Lung surfactant suppresses oxygen-dependent bactericidal functions of human blood monocytes by inhibiting the assembly of the NADPH oxidase. *The Journal of Immunology* **150**, 2391–2400.

GRIESE, M. (1999). Pulmonary surfactant in health and human lung disease: state of the art. *European Respiratory Journal* **13**, 1455–1476.

GROSS, N. J. (1995). Extracellular metabolism of pulmonary surfactant: the role of a new serine protease. *Annual Review of Physiology* **57**, 135–150.

GURR, M. I. & HARWOOD, J. L. (1991). *Lipid Biochemistry: An Introduction*. 4th Edition (ed. Gurr, M. I. & Harwood, J. L.). London, Chapman and Hall.

HALLMAN, M., EPSTEIN, B. L. & GLUCK, L. (1981). Analysis of labelling and clearing of lung surfactant phospholipids in rabbit. Evidence of bidirectional surfactant flux between lamellar bodies and alveolar lavage. *Journal of Clinical Investigation* **68**, 742–751.

HARWOOD, J. L. (1987). Lung surfactant. *Progress in Lipid Research* **26**, 211–256.

HAWGOOD, S. & POULAIN, F. R. (1995). Functions of the surfactant proteins: a perspective. *Pediatric Pulmonology* **19**, 99–104.

IKEGAMI, M. & JOBE, A. H. (1993). Surfactant metabolism. *Seminars in Perinatology* **17**, 233–240.

JONES, D. A. C., HENDERSON, J. R. & RILEY, J. (1992). Preliminary characterization of the lipid and protein components of the protective surface membranes of a pentastomid *Porocephalus crotali*. *Parasitology* **104**, 469–478.

JONES, D. A. C. & RILEY, J. (1991). An ELISA for the detection of pentastomid infections in the rat. *Parasitology* **103**, 331–337.

JONES, D. A. C., RILEY, J., KERBY, N. W. & KNOX, D. P. (1991). Isolation and preliminary characterization of a 48-kilodalton metalloproteinase of the excretory/secretory components of the frontal glands of *Porocephalus* pentastomids. *Molecular and Biochemical Parasitology* **46**, 61–72.

KRISHNASAMY, M., JEFFERY, J., INDER SINGH, K. & OOTHUMAN, P. (1995). *Raillietiella rileyi*, a new species of pentastomid from the lung of toad, *Bufo melanostictus* from Malaysia. *Tropical Biomedicine* **12**, 31–38.

KRULL, W. H. (1930). The life history of two North American frog lung flukes. *Journal of Parasitology* **16**, 207–212.

KRULL, W. H. (1931). Life history studies on two frog lung flukes, *Pneumonoeces medioplexus* and *Pneumobites parviplexus*. *Transactions of the American Microscopical Society* **50**, 215–277.

KUROKI, Y. & VOELKER, D. R. (1994). Pulmonary surfactant proteins. *Journal of Biological Chemistry* **269**, 25943–25946.

MARIENCHECK, W. I., SAVOR, J., DONG, Q., TINO, M. J. & WRIGHT, J. R. (1999). Surfactant protein A enhances alveolar macrophage phagocytosis of a live, mucoid strain of *Pseudomonas aeruginosa*. *American Journal of Physiology* **277**, 777–786.

MARIENCHECK, W. I. & WRIGHT, J. R. (1999). A metalloproteinase secreted by *Pseudomonas aeruginosa* degrades pulmonary surfactant protein A (SP-A). *American Journal of Respiratory and Clinical Care Medicine* **159**, No. 355, p. A506.

MCGREGOR, L. K., DANIELS, C. B. & NICHOLAS, T. E. (1993).

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

Lung ultrastructure and the surfactant-like system of the central netted dragon, *Ctenophorus nuchalis*. *Copeia* **2**, 326–333.

MCHARDY, P., RILEY, J. & HUNTLY, J. F. (1993). The recruitment of mast cells, exclusively of the mucosal phenotype, into granulomatous lesions caused by the pentastomid parasite *Porocephalus crotali*: recruitment is irrespective of site. *Parasitology* **106**, 47–54.

MEHLHORN, H. (1988). *Parasitology in Focus*. Berlin, Heidelberg, New York, London, Paris, Tokyo, Springer Verlag.

MIYAZAKI, I. (1991). *Helminthic Zoonoses*. South Eastern Medical Information Center, Publication No. 62. Tokyo, International Medical Foundation of Japan.

ORGEIG, S., SMITS, A. W., DANIELS, C. B. & HERMAN, J. K. (1997). Surfactant regulates pulmonary fluid balance in reptiles. *American Journal of Physiology – Regulatory Integrative and Comparative Physiology* **42**, 2103–2021.

PEREZ-GIL, J., CASALS, C. & MARSH, D. (1995). Interactions of hydrophobic lung surfactant proteins SP-B and SP-C with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol bilayers studied by electron spin resonance spectroscopy. *American Chemical Society* **34**, 3964–3971.

PERRY, S. F. (1983). Reptilian lungs: functional anatomy and evolution. *Journal of Advances in Anatomy, Embryology and Cell Biology* **79**, 1–80.

RESTREPO, C. I., DONG, Q., SAVOV, J., MARIENCHECK, W. I. & WRIGHT, J. R. (1999). Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. *American Journal of Respiratory Cell and Molecular Biology* **21**, 576–585.

RIDER, E. D., IKEGAMI, M. & JOBE, A. H. (1990). Intrapulmonary catabolism of surfactant-saturated phosphatidylcholine in rabbits. *Journal of Applied Physiology* **69**, 1856–1862.

RILEY, J. (1973). A redescription of *Reighardia sternae* Diesing 1864 (Pentastomida: Cephalobaenida) with some observations on the glandular systems of pentastomids. *Zeitschrift für Morphologie der Tiere* **76**, 243–259.

RILEY, J. (1981). Some observations on the development of *Porocephalus crotali* (Pentastomida: Porocephalida) the Western Diamondback Rattlesnake (*Crotalus atrox*). *International Journal for Parasitology* **11**, 127–131.

RILEY, J. (1986). The biology of pentastomids. In *Advances in Parasitology* **25**, 46–128 (ed. Baker, J. R. & Muller, R.). London, Academic Press.

RILEY, J. (1992*a*). Sexual differentiation and behaviour of pentastomids. In *Reproductive Biology of Invertebrates* **Vol. V.** (ed. Adiyodi, K. G. and R. G.), pp. 401–411. Oxford and IBH Publishing Co.

RILEY, J. (1992*b*). Pentastomids and the immune response. *Parasitology Today* **8**, 133–137.

RILEY, J. (1994). Reproductive strategies of pentastomids. In *Reproductive Biology of Invertebrates* **Vol. VI.** (ed. Adiyodi, K. G. and R. G.), pp. 293–307. Oxford and IBH Publishing Co.

RILEY, J. & BANAJA, A. A. (1975). Some ultrastructural observations on the integument of a pentastomid. *Tissue and Cell* **7**, 33–50.

RILEY, J. & HUCHZERMEYER, F. W. (1995). Pentastomid parasites of the family Sebekidae Fain, 1961 in West African dwarf crocodiles *Osteolaemus tetraspis* Cope, 1851 from the Congo, with a description of *Alofia parva* n. sp. *Onderstepoort Journal of Veterinary Research* **62**, 157–162.

RILEY, J. & HUCHZERMEYER, F. W. (1996). A reassessment of the pentastomid genus *Leiperia* Sambon, 1922, with a description of a new species from both the Indopacific crocodile *Crocodylus porosus* and Johnston's crocodile *C. johnsoni* in Australia. *Systematic Parasitology* **34**, 53–66.

RILEY, J., JAMES, J. L. & BANAJA, A. A. (1979). The possible role of the frontal and sub-parietal gland systems of the pentastomid *Reighardia sternae* (Diesing, 1864) in the evasion of the host immune response. *Parasitology* **78**, 53–66.

ROSE, J. H. (1976). Lungs. In *Ecological Aspects of Parasitology* (ed. Kennedy, C. R.), pp. 227–242. Amsterdam, North-Holland Publishing Company.

SELF, J. T. (1972). Pentastomiasis: host responses to larval and nymphal infections. *Transactions of the American Microscopical Society* **91**, 2–8.

SELF, J. T. & COSGROVE, G. E. (1972). Pentastomida. In *Pathology of Simian Primates* (ed. Fiennes, R. N. T-W.), pp. 194–204. Basel, Karger.

SELF, J. T. & KUNTZ, R. E. (1967). Host-parasite relationships in some Pentastomida. *Journal of Parasitology* **53**, 202–206.

SMITH, D. S., SMITH, U. & RYAN, J. W. (1972). Freeze-fractured lamella body membranes of the rat lung great alveolar cell. *Tissue and Cell* **4**, 457–468.

SMYTH, J. D. (1976). *Introduction to Animal Parasitology*. 2nd Edition. London, Hodder and Stoughton.

SULLIVAN, L. C., DANIELS, C. B., PHILLIPS, I. D., ORGEIG, S. & WHITSETT, J. A. (1998). Conservation of surfactant protein A: evidence for a single origin for vertebrate pulmonary surfactant. *Journal of Molecular Evolution* **46**, 131–138.

THOMAS, G. & BÖCKELER, W. (1992*a*). Light and electron microscopical investigations on the feeding mechanism of *Reighardia sternae* (Pentastomida: Cephalobaenida). *Zoologischer Jahrbücher Anatomie* **122**, 1–12.

THOMAS, G. & BÖCKELER, W. (1992*b*). Light and electron microscopical investigations of the midgut epithelium of different Cephalobaenida (Pentastomida) during digestion. *Parasitological Research* **78**, 587–593.

TINSLEY, R. C. & EARLE, C. M. (1983). Invasion of vertebrate lungs by the polystomatid monogeneans *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis*. *Parasitology* **86**, 501–517.

TURK, F. A. (1947). Studies of Acari IV. A review of the lung mites of snakes. *Parasitology* **38**, 17–26.

VON HAFFNER, K. (1972). Die Muskultur und Ortsbewegung der Stachellarven (= Wandernlarven, Nymphen) von *Neolinguatula nuttali* (Pentastomida). *Zoologischer Anzeiger* **189**, 235–244.

WALOSSEK, D. & MÜLLER, K. J. (1994). Pentastomid parasites from the Lower Palaeozoic of Sweden. *Transactions of the Royal Society of Edinburgh, Earth Sciences* **85**, 1–37.

WALOSSEK, D., REPETSKI, J. E. & MÜLLER, K. J. (1994). An exceptionally preserved parasitic arthropod,

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press

Heymonsicambria taylori n. sp. (Arthropoda incertae sedis: Pentastomida), from Cambrian-Ordovician boundary beds of Newfoundland, Canada. *Canadian Journal of Earth Sciences* **31**, 1664–1671.

WILLIAMS, M. C. (1977). Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. *Journal of Cell Biology* **72**, 260–277.

WILSHER, M. L., HUGHES, D. A. & HASLAM, P. L. (1988). Immunoregulatory properties of pulmonary surfactant: effect of lung lining fluid on proliferation of human blood lymphocytes. *Thorax* **43**, 354–359.

WRIGHT, J. R. (1997). Immunoregulatory functions of surfactant. *Physiological Reviews* **77**, 931–962.

WRIGHT, J. R. & CLEMENTS, J. A. (1987). Metabolism and turnover of lung surfactant. *American Review of Respiratory Diseases* **135**, 426–444.

https://doi.org/10.1017/S0031182000084651 Published online by Cambridge University Press