Journal of Helminthology (2004) **78,** 77–85 DOI: 10.1079/JOH2003199

Pallisentis rexus from the Chiang Mai Basin, Thailand: ultrastructural studies on egg envelope development and the mechanism of egg expansion

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Abstract

Pallisentis rexus Wongkham & Whitfield, 1999 (Eoacanthocephala: Quadrigyridae) infects the freshwater snakehead fish, *Channa striata*, in the Chiang Mai Basin, Thailand. All stages of egg development within the body cavity of the female parasite were observed, using transmission electron microscopy. Changes in mature eggs after contact with water were also investigated. The mature egg has five egg envelopes separated from each other by four gaps. The fertilization membrane, which is formed first, is pushed centrifugally by other, subsequently formed, envelopes and gaps, which produces a final total shell thickness of 8–36 μm around the acanthor. The disappearance of the outermost layer and the unpleating of an adjacent inner layer causes the expansion of eggs on contact with water. The volume of an expanded egg is approximately 27 times that of an unexpanded one, but the density of eggs is reduced from a value greater than water to one almost equal to water. This is believed to aid the dispersion of eggs.

Introduction

Pallisentis rexus (Wongkham & Whitfield, 1999) infects the freshwater snakehead fish, *Channa striata*, in the Chiang Mai Basin, northern Thailand. The parasite completes its larval development (by reaching the cystacanth stage) within 13 days in *Cyclops* sp. and can employ several species of copepods as intermediate hosts (Wongkham, 1998).

The structure of the egg envelope has been studied in many species of acanthocephalans. Crompton (1985) concluded that the parasite eggshell is composed of three to four envelopes. The precise number is dependent on the species of acanthocephalan concerned. The ultrastructure of the acanthocephalan eggshell has been studied by Whitfield (1973) and Marchand (1984a,b) in several species of Acanthocephala. In all species it was reported that there are four solid envelopes separated by four fluid/granular gaps. Peter *et al.* (1991), Taraschewski & Peter (1992) and Taraschewski *et al.* (1992) have extended the findings of Marchand (1984a,b) in the Archiacanthocephala, Palaeacanthocephala and Eoacanthocephala.

The observations of the present study were aimed at providing a detailed description of the development and final configuration of the egg envelopes of the eoacanthocephalan, *P. rexus*. These studies have also facilitated the first ultrastuctural study into the mechanism by which mature eggs of acanthocephalans in the genus *Pallisentis* expand enormously when they

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contact fresh water (Rai, 1967; George & Nadakal, 1973; Wongkham, 1998).

Materials and methods

Living specimens of locally caught *Channa striata* were purchased from several markets in Chiang Mai town. Living gravid female worms of *P. rexus* parasites were obtained at autopsy from the fish intestine and washed in normal saline. They were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for at least 3–6 h at 8–10°C and washed in the same buffer. To aid fixative penetration, the bodies of worms were cut transversely into sections 2–3 mm in length. Central body sections containing eggs within the pseudocoelome were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at pH 7.2, dehydrated in ethanol and propylene oxide, and embedded in Spurr resin. Ultrathin sections were cut on a Reichert Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Jeol 100CX MKII transmission electron microscope (TEM). The sections were examined for the different stages of egg development.

Live gravid female *P. rexus* parasites from purchased fishes were placed in a culture dish containing spring water. Released mature eggs were immediately transferred to a new dish and stored in spring-water for 5–7 days until fully expanded. They were concentrated by centrifugation in water at 5000 rpm and then processed as above for electron microscopy. Sections were examined to investigate the expanded configuration of parts of the egg envelope series. Some eggs were kept in spring water for longer periods of time to investigate possible changes after expansion.

Results

Ultrastructure of the mature eggs within the female parasite body cavity

In the mature egg, a number of egg envelopes are concentrically arranged around the enclosed acanthor. There are four solid envelopes (E1–E4) corresponding to those which have been described in other acanthocephalan eggs (Peter *et al.*, 1991; Taraschewski & Peter, 1992; Taraschewski *et al.*, 1992). There is also, however, an additional external envelope (E0) in the case of *P. rexus* eggs, which seems to be derived from the oocyte fertilization membrane. These envelopes are separated by four gaps (G0–G3), which are filled with different, presumably liquid material (figs 1 and 2). There is also a gap (G4) between E4 and the acanthor. This gap is filled with highly osmiophilic material. E0, measuring 0.05–0.15 μm in thickness, is an electron-dense layer. E1 is an electron-dense layer with a thickness of approximately 0.05–0.15 μm. A characteristic of this layer is its regular folding or pleating with pleat amplitudes of 2–3 μm (fig. 2). The inner clefts of the pleating, which are

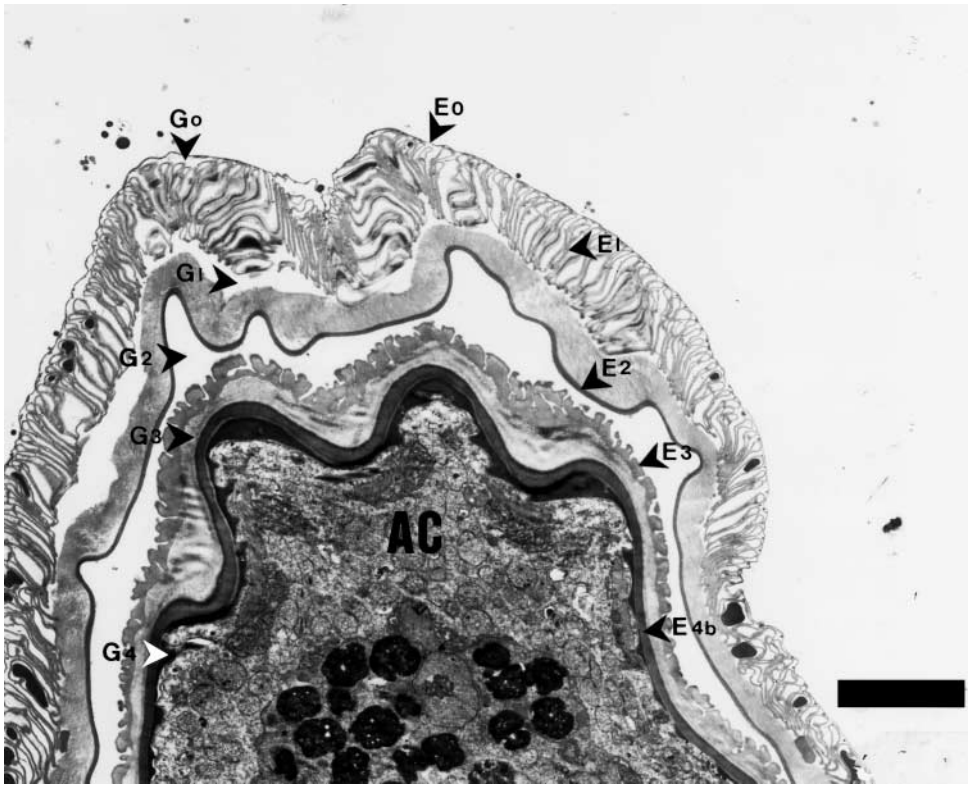

Fig. 1. Transmission electron micrograph of mature egg envelopes. The solid layers are labelled as E0–E4 and the gaps as G0–G4. The acanthor (AC) is clearly seen with a nuclear mass at its centre. Scale bar = 5 μm.

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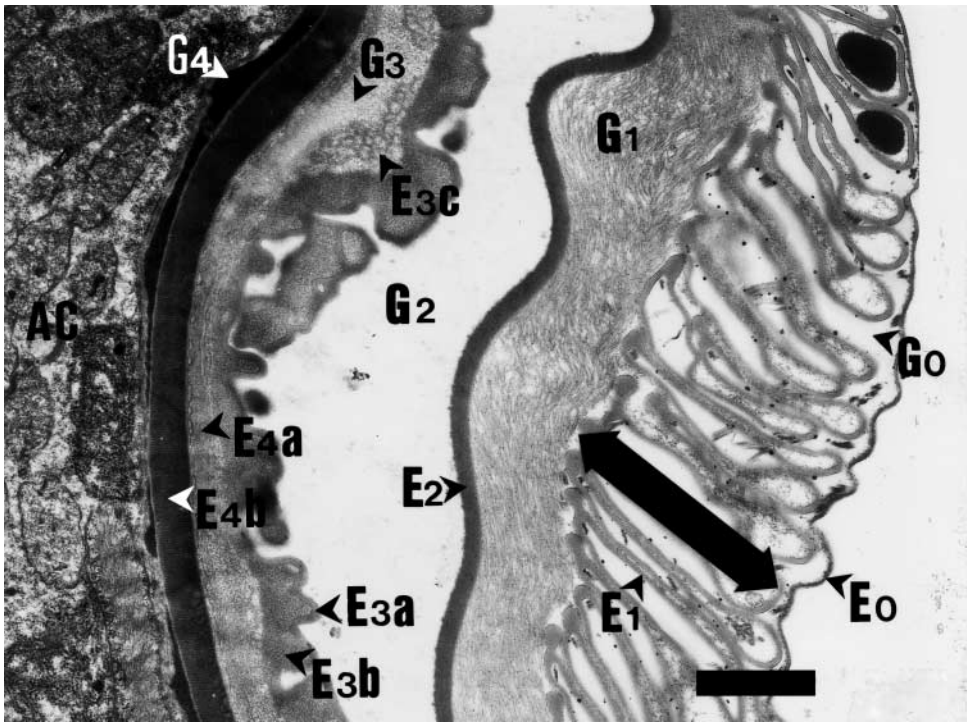

Fig. 2. Transmission electron micrograph of mature egg envelopes. Envelopes and gaps are labelled as in fig. 1. The pleat amplitude of E1 is indicated by a double-headed arrow. Scale bar = 1 μm.

confluent with gap G1, contain osmiophilic granulation. The outer clefts of E1 are part of the gap G0, which is filled with electron-lucent material enclosed by the outermost layer E0.

The gap G1, between the basal part of E1 and E2 is 0.7–1.6 μm wide, and, like the inner clefts of E1, contains dense osmiophilic filamentous material. E2 is a further electron-dense layer measuring of 0.1–0.3 μm in thickness. Gap G2, situated between E2 and E3 is filled with electron-lucent material and a few osmiophilic granules. This gap is seen by light microscopy as a clear zone between E2 and the acanthor. The width of this gap at each pole of the mature egg is approximately 15–27 μm and 5–10 μm at the equator.

E3 consists of three clearly demarcated layers (fig. 2). E3a is a thin corrugated electron-dense membrane-like layer measuring 0.04–0.1 μm in width. This layer is characterized by irregular clefts and curvatures inside which are two additional layers of electron-dense material (E3b and E3c). The fine dense osmiophilic granules of E3b are immediately beneath the inner clefts of E3a. The patchy clumps of coarse electron-dense granules of E3c (0.0–0.06 μm in width) are attached to the inner side of E3b (0.05–0.1 μm in width). These two layers (E3b and E3c) are separated from E4 by gap G3. This gap measures 0.0–0.7 μm in width with osmiophilic granular contents.

E4 can be distinguished into two layers, the outer E4a layer and the inner E4b layer (fig. 3). E4a measured 0.01–0.05 μm in width and is composed of dense osmiophilic granules in an irregular layer above E4b. E4b, the innermost solid layer of the envelope series is thick and dense and measures 0.2–0.5 μm in width. It has a very characteristic internal substructure which consists, in transverse section, of an alternating series of electron-dense and electron-lucid lines which are arranged perpendicular to the underlying acanthor surface (figs 2 and 3). The dense line to dense line spacing in this array is about 20 nm. The G4 gap between E4 and the outer surface of the acanthor is filled with extremely electron dense material.

Egg envelope development

The initial stage of envelope development is represented by a single electron-dense layer called here the fertilization membrane (Fm) (fig. 4A), with a thickness of 0.05–0.15 μm. This membrane is probably synonymous with E0 in later terminology. A translucent zone (Gz) measuring 0.05–0.5 μm between the fertilization membrane and the developing acanthor is also clearly observed. This translucent zone is filled with osmiophilic granules and all the other egg envelopes are formed within this zone.

The fertilization membrane, which is formed first, is gradually pushed centrifugally by the other envelopes and gaps which are formed internal to the fertilization membrane (figs 4 and 5). The process of envelope synthesis and secretion, from the surface of the developing acanthor, is associated with an accumulation of granular endoplasmic reticulum and secretory vesicles at the periphery of the outer layer of embryonic cytoplasm (fig. 4); these organelles may also be concerned with processes other than envelope synthesis. The images in

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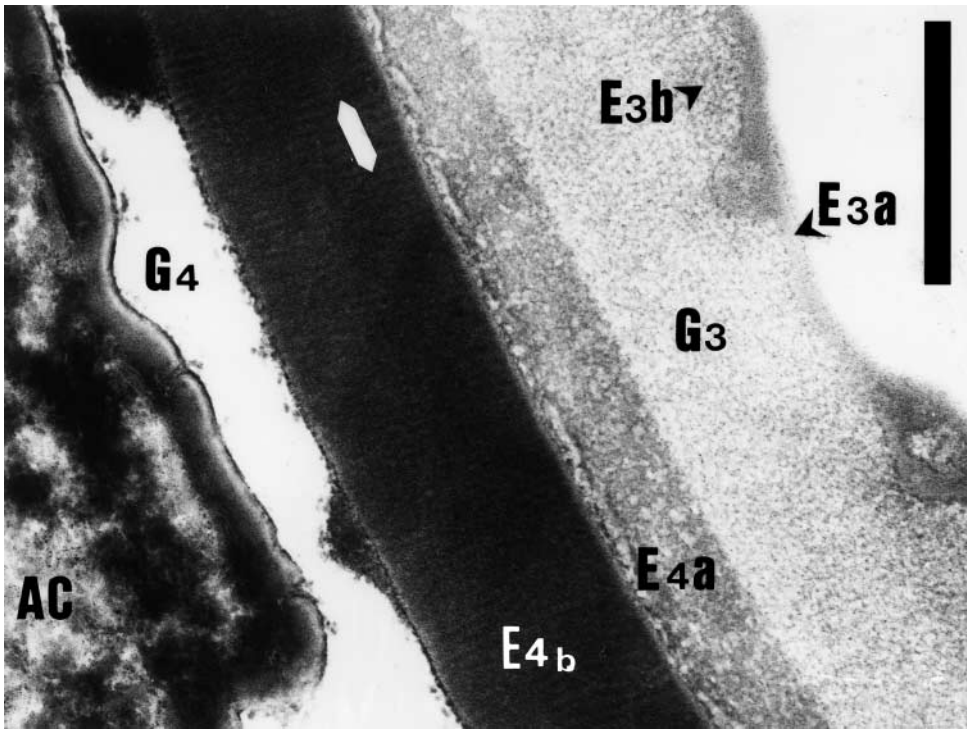

Fig. 3. Transmission electron micrograph of egg envelopes E3 and E4. Labels in this figure are as described in fig. 5H. The distance between seven adjacent dense lines in E4b is marked with a double-headed white arrow. Scale bar = 500 nm.

fig. 4 represent stages in the generation of the egg envelope series from a stage when only the fertilization membrane is present (fig. 4A) to a much later stage (fig. 4F) in which the elaboration of envelope E4b is beginning.

The full sequence of envelope development stages is illustrated in a series of scale diagrams in fig. 5. It shows the enormous expansion of the gap between the fertilization membrane (later E0) and the developing acanthor. This gap, within which the envelope series develops, is initially only 0.05–0.2 μm in width, but by the time that the mature egg envelope series is in place, the gap is 8–36 μm wide.

Although a large number of stages in the process of envelope secretion have been observed in different TEM images, it is not possible to accommodate all of them in a single unambiguous sequence. The sequence shown in figs 4 and 5, however, seems to describe the ordered formation of almost all envelopes and their sub-layers. The eight developmental stages illustrated diagrammatically in fig. 5 constitute three major phases of synthesis.

In the first phase (stages A–D, fig. 5) there is little expansion of the initial Gz gap but the foundations of envelopes E2 and E3 are laid down as simple dense layers. It is possible that the dense layer inside E2 and immediately above the embryonic surface is the foundation of E4. In the second phase (stages E–F, fig. 5), considerable expansion of the initial Gz gap begins to occur and E2 and E3 begin to become more elaborate. Simultaneously, the complex pleated layer of envelope E1 forms in the gap between the fertilization membrane and E2, within very dense material filling the gap between the fertilization membrane and E1. By stage F (fig. 5) there are more explicit signs that E4 is in place. In the final phase (stages G and H, fig. 5) even more Gz expansion occurs and major alterations happen to both gap G1 and envelope E4. G1 becomes filled and expanded with fibrous material and the ordered structure of E4b becomes apparent. By the end of stage H, the mature egg envelope series is in position.

Egg expansion in water

Ultrastructural analysis of expanded eggs (figs 6A & B) shows that EO disappears when eggs come into contact with spring water and that layer E1 beneath it unpleats and expands providing the physical basis for egg expansion. The initial unpleating of E1 always begins at the equator of the egg and symmetrically expands the boundary of the unpleating to both poles (fig. 7). This results in increasing width and length of the eggs and within 6–72 h in spring water, the mature eggs can reach their fully expanded length (0.42–1.40 mm, with a mean of 0.79 ± 0.24 SD). There is, however, a pleated part of E1 which remains at both poles of the fully expanded egg, which is referred to here as a 'folded cap' (fig. 7). The fully expanded E1 envelope is fusiform in shape and contains the unexpanded components of the egg, that is: E2, E3, E4, G2, G3, G4 and the acanthor. In the expanded egg these parts are presumably bathed in a mixture of the G1 contents and spring water which has permeated E1. The expanded egg is almost eight times the length and approximately twice the width of the unexpanded mature egg (table 1).

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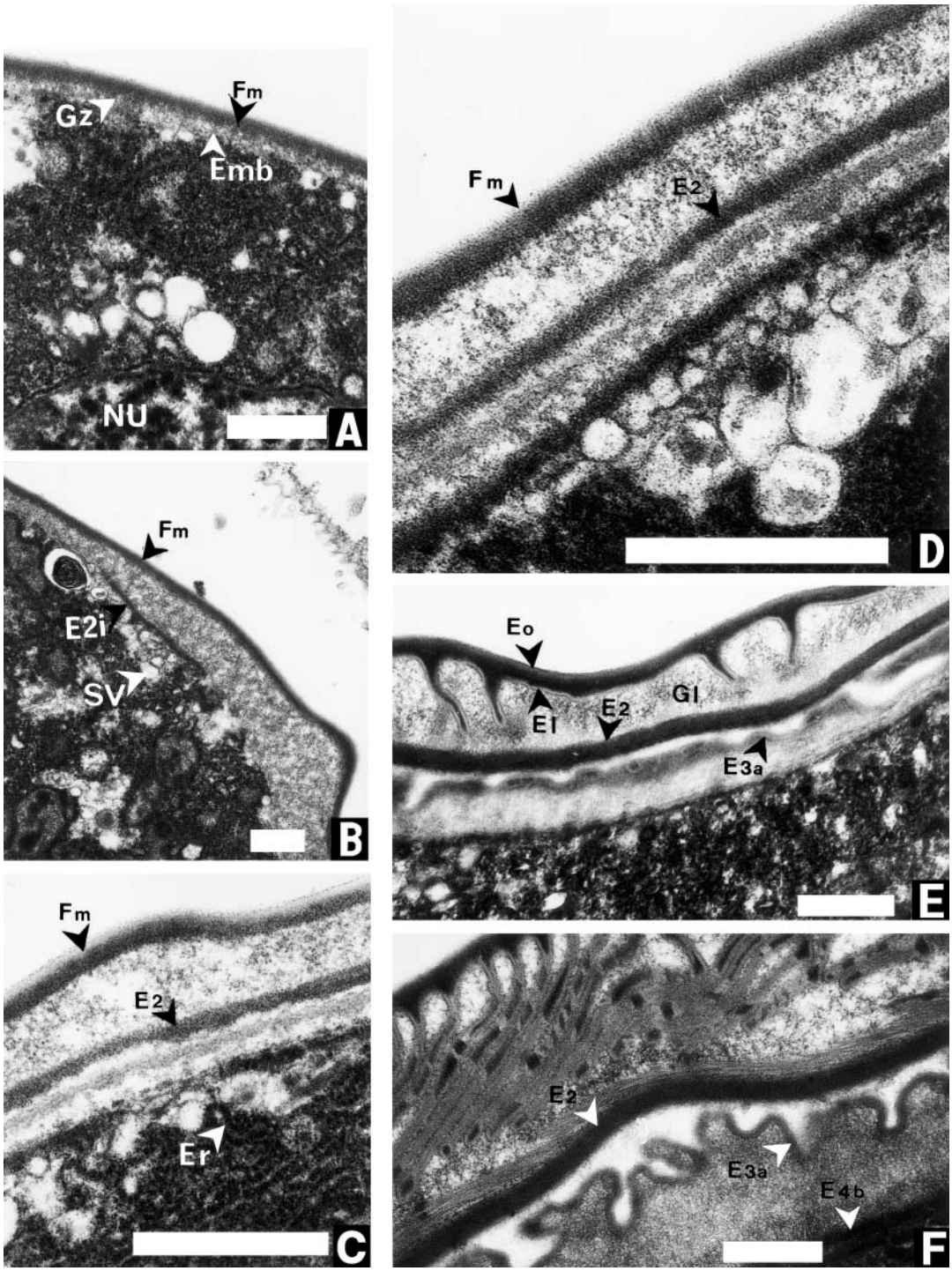

Fig. 4. Transmission electron micrographs of entire egg envelope sequence at six different stages of development (A–F). Each scale bar = 0.5 μm. A. There is an initial gap (Gz) between the fertilization membrane (Fm) and the embryonic membrane (Emb). A nucleus of an embryo (NU) can also be seen. B. E2i is the initial stage of E2 subtended by secretory vesicles (SV). C. E2 is pushed outwards while E3 and E4 form beneath. Note the active endoplasmic reticulum (Er) and secretory vesicles at the surface of the acanthor. D. Later stage of development with four solid layers being formed. E. The fertilization membrane can be separated into two parts E0 and E1. E2 and E3a are also distinguishable. F. E2, E3a and E4b are distinguishable and pleats in E1 are obvious.

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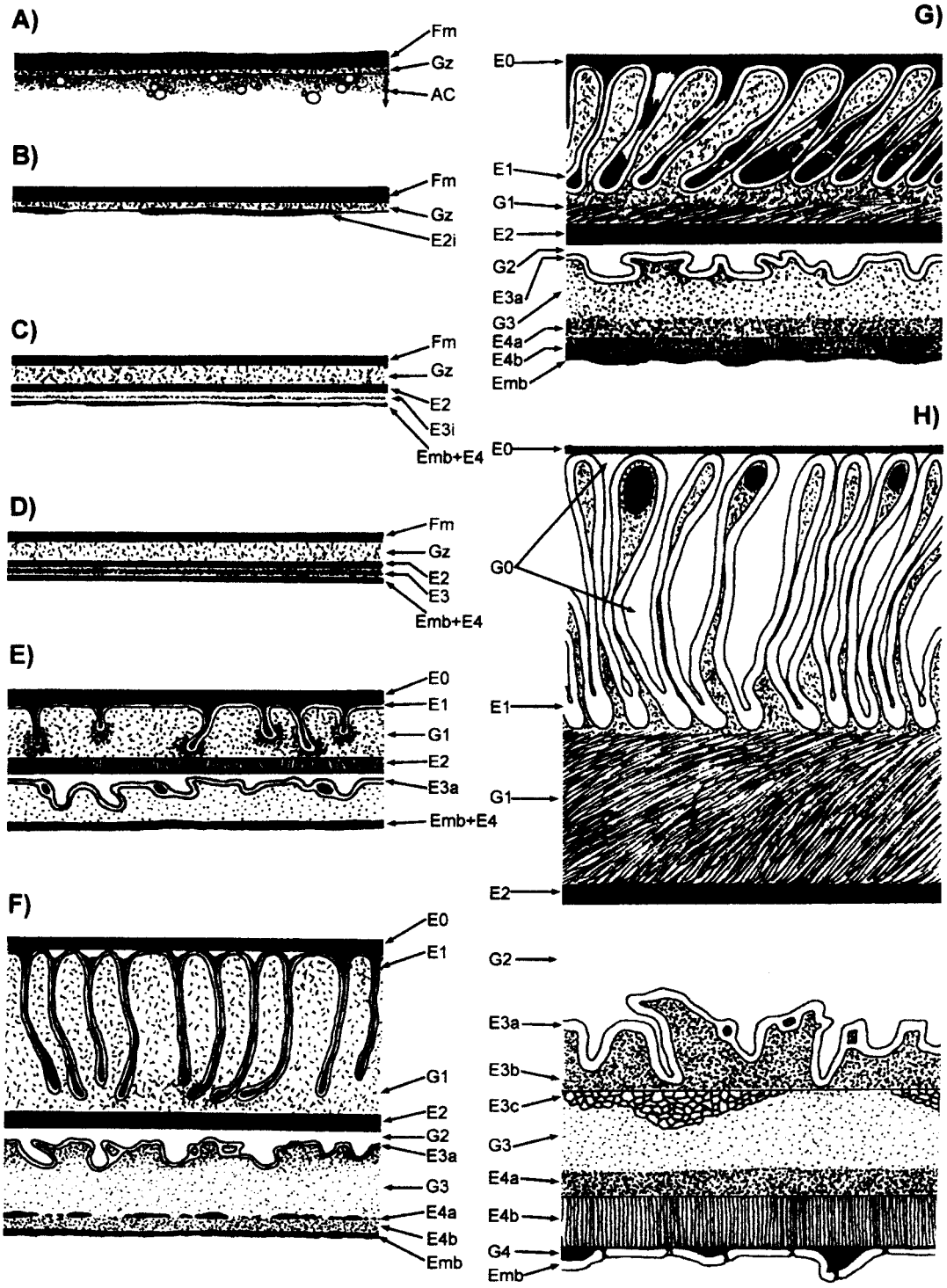

Fig. 5. Scale diagrams representing egg envelope formation in chronological sequence A–H. Each diagram represents a width of 3 μm of the developing envelope series.

Approximately 80% of expanded eggs stored in the spring water were found to be resting in the vertical position with an egg pole 'attached' to the glass surface. These adhesive expanded eggs were not easily removed or detached by stirring the surrounding water since they remained attached to the glass surface by means of the folded cap and swung with water movements. Eggs kept in spring water longer than 2–3 days lost their polar adhesive property. Bacteria often covered the outer surface of expanded eggs left in spring water for a few

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Table 1. *Pallisentis rexus*: the linear dimensions and volume of unexpanded and expanded eggs.

Egg	Size range (length × width in mm)	Mean length (mm)	Mean width (mm)	Estimated volume (mm^3)
Unexpanded	0.096–0.116 × 0.036–0.048	0.107	0.042	0.000099
Expanded	0.42–1.4 × 0.06–0.11	0.790	0.080	0.002646

Volume, V estimated from $V = 4/3\pi r^2 h$ where r = 1/2 mean width and h = 1/2 mean length.

weeks, but were never found in G1 or in the other gaps beyond (fig. 6).

Discussion

Structure of the egg envelopes

The structural arrangement of mature egg envelopes for *P. rexus* is similar to that described for *P. golvani* by Marchand (1984b). The size and thickness of each layer, however, differs between the two species (table 2).

The initial stage of formation of E0 is similar to that described for the fertilization membrane in *Acanthosentis acanthuri* (Eoacanthocephala) by Marchand (1984a), *Moniliformis dubius* (Archiacanthocephala) by Wright (1971) and *Polymorphus minutus* (Palaeacanthocephala) by Whitfield (1973). Most authors agree that the fertilization membrane remains around the developing acanthor as the outer envelope of a series. West (1964) and Wright (1971) have proposed that this membrane is the innermost envelope, but have failed to demonstrate explicit evidence to support their opinion. It is clear in the present study that this membrane is initially secreted by the fertilized egg, after which it is gradually pushed centrifugally by the development of the new envelopes and the gaps. The derivatives of the fertilization membrane therefore remain as the outermost envelope of the shell. The same phenomenon has been described by Stranack (1972) in *Pomphorhynchus laevis*, Whitfield (1973) in *Polymorphus minutus* and Marchand (1984a) in *A. acanthuri*.

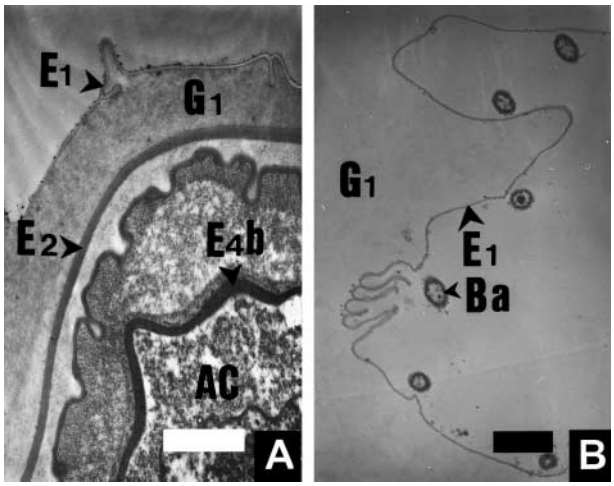

Fig. 6. Transmission electron micrographs of the unpleating of E1 in contact with spring water (E0 has disappeared). A. Early expansion stage with most pleats of E1 straightened out and some fibrous material still present in G1 (scale bar = 1 μm). B. Later stage of expansion of E1 with bacteria (Ba) on its outer surface and very little fibrous material visible inside (scale bar = 10 μm).

The homogeneous transparent nature of E1 seems to characterize all species of eoacanthocephalans (Marchand, 1984a,b). The pleated ultrastructural configuration of this layer was probably misunderstood by Marchand (1984b) who referred to it as 'irregular shaped tubules' in *Pallisentis golvani*. However, the density of pleats in this layer in *P. golvani* (approx. 30 pleats per 3 μm) illustrated in the photomicrograph provided by Marchand (1984b) is more than that in *P. rexus* (approx. 10–12 pleats per 3 μm). The pleated configuration of E1 clearly plays a crucial role in facilitating egg expansion.

E1, referred to as E1b by Marchand (1984a,b), has different appearances among other eoacanthocephalan species. In three genera this envelope has a corrugated character with a different overall configuration and a lesser degree of pleating than that found in *Pallisentis*. Examples include the two species described by Marchand (1984a), *Neoechinorhynchus agilis* and *Tenuisentis niloticus*, as well as *Neoechinorhynchus rutili* described by Taraschewski *et al.* (1992). Egg expansion has not been described in these species.

E1 has a filamentous structure in three species of the genus *Acanthosentis*, namely: *A. acanthuri* (Marchand, 1984a), and *A. tilapiae* and *A. papilio* (Marchand, 1984b). The shape, size and characteristics of the filaments are different in each species. In addition, there are interior connections between the E1 and E2 layer in *N. rutili* (Taraschewski *et al.*, 1992) which maintain the distance between the two layers. These structures have never been found in *P. rexus*.

E2, with its dense, concentrically arranged filaments, is apparently similar in the mature eggs of all eoacanthocephalan species (Marchand, 1984 a,b; Taraschewski *et al.*, 1992). In *P. rexus*, these fine filaments develop early during eggshell formation with an arrangement perpendicular to the surface of the acanthor. These filaments later become reinforced and appear as an electron-dense layer during the later stages of development. In all the investigated eoacanthocephalan species, this envelope contains keratin (Marchand, 1984 a,b; Taraschewski *et al.*, 1992). The chemical composition of the E2 layer supports the suggestion that its role is one of protection and impermeability to water.

The characteristics of E3 and E4 are very similar among all species of eoacanthocephalans that have been studied before (Marchand, 1984 a,b; Taraschewski *et al.*, 1992). E3 may be accompanied by one or two amorphous electron-dense materials, which differ between the species. The chemical composition of this layer is different in the three classes of Acanthocephala (Taraschewski *et al.*, 1992).

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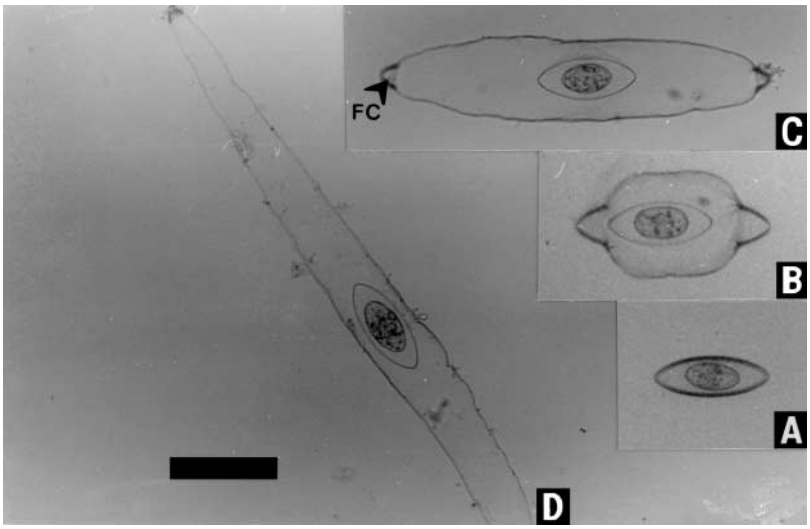

Fig. 7. Photomicrographs of sequential stages of mature egg expansion in spring water. Four eggs are illustrated at the same magnification (scale bar = 100 μm) and at successive stages of expansion, A–D: A, unexpanded egg; B, envelope E1 has begun to expand in its equatorial regions with the polar regions still unexpanded; C, most of E1 has now expanded but there remains at each polar region a small folded cap (FC); D, maximum expansion of E1.

The structure of E4 in *P. rexus* is similar to that described for other species of eoacanthocephalan (Marchand, 1984a,b; Taraschewski *et al.*, 1992). Peter *et al.* (1991) and Taraschewski *et al.* (1992), concluded that neither chitin nor keratin has been found in this layer in eoacanthocephalans.

Wongkham (1982) microscopically examined expanded eggs of *Pallisentis* sp. (almost certainly *P. rexus*, as in this study) during the process of experimental infection of the intermediate host (*Cyclops* sp.). The copepod consumed the acanthor and most of the egg envelopes including E2, but in most cases had not eaten the expanded E1 envelope. A tear was always visible in the rejected layer. We can probably conclude, therefore, that the envelope layers from E2 inwards may play an important role in the protection of the acanthor from potential mechanical damage caused by the copepod mouthparts.

Table 2. Thickness (μm) of the egg envelopes and gaps in *Pallisentis golvani* and *P. rexus*.

Egg envelopes	*P. golvani*	*P. rexus*
E0	0.02	0.05–0.15
E1 thickness	0.01*	0.05–0.15
E1 amplitude of pleats	1.0–1.4*	2–3
G1	0.1–0.5	0.7–1.6
E2	0.04–0.05	0.1–0.3
G2	0.1–1.0	5–27
E3a	0.01	0.04–0.1
E3b	0.035–0.7	0.05–0.1
E3c	–	0.0–0.06
G3	0.0–0.15	0.0–0.7
E4a	0.03	0.01–0.5
E4b	0.15–0.2	0.2–0.5
Total (approx)	1–4	8–36

The name and the width of each envelope of *P. golvani* is adapted from those described by Marchand (1984b). *Measured from the images in Marchand (1984b).

Egg expansion

Egg expansion in contact with water seems to be a common and distinctive feature of the genus *Pallisentis*. Two other members of this genus have been described as undergoing egg expansion, namely *P. nagpurensis* (George & Nadakal, 1973) and *P. panadei* (Rai, 1967). However, the nature of the expansion in these species was only described in these publications in a preliminary manner and the descriptions do not include information on the expansion mechanism. From the results of the present study on the structure of eggs, before and after expansion, three factors can be hypothesized to control the expansion process: (i) the elimination of E0 which acts as a 'shrink-wrap' around E1 in its pleated configuration; (ii) the subsequent increase in the permeability of E1 to water; and (iii) an osmotic pressure difference between the two sides of E1, causing an ingress of water through E1 resulting in the hydration and solution of the dense filamentous material (see fig. 2) in the space under E1. This ingress of water increases the volume within E1 and hydraulically expands E1 causing the straightening of its pleated configuration.

E0 is well preserved in the environmental medium of the female parasite body cavity. Only in certain environmental conditions after egg release is E0 eliminated. Wongkham (1998) has shown that E0 elimination and egg expansion occurs most rapidly and completely at pH values between 5.0 and 6.5 and in aqueous solutions containing low molarities of mineral salts, equivalent to around 0.02 M NaCl. Only after the disappearance of the E0, can E1 unpleat and expand. As the unpleating and expansion of E1 occurred, G1 increased in width. E2

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and the remaining egg envelope layers, together with all the gaps (G2–G4), have never been found to expand or increase in width and length. This characteristic is probably indicative of the rigid nature of E2 and its impermeability to water.

The unexpanded egg has a density significantly greater than that of water and sinks rapidly to the bottom of a water column. When the egg expands, its volume is approximately 27 times that of the unexpanded egg and the whole of this expansion seems to be caused by ingress of the water (with a density of $1\,g\,ml^{-1}$). If the density of the unexpanded egg is assumed to be, say, $1.2\,g\,ml^{-1}$, it follows that the density of the expanded egg will be $27.2 \div 27$ which is $1.0074\,g\,ml^{-1}$. In other words, the expansion has reduced the density of the egg to a value very close to that of water. This helps to explain the observation that expanded eggs were moved and dispersed with greater ease in water than the unexpanded eggs.

The phenomenon of egg expansion may facilitate copepod infection by two potential mechanisms given the previous finding by Wongkham (1998) that the acanthocephalan can successfully employ a range of copepod species as intermediate hosts. In the first few days after release from a female worm, the expanded egg retains a sticky property on both poles and will attach to the substratum in a 'vertical' orientation. In this 'benthic' phase the fixed position and extension of the egg about 1 mm above the substratum surface may increase the chances of encounters with bottom-browsing benthic copepod species. On losing their sticky property, the eggs (with water-like density) spend their longer planktonic period floating freely in the stirred region of the water column, which increases their infection chances with respect to planktonic copepod species.

The chemical composition of the gaps in the egg envelope series may also play an important role in increasing the chance of infection. According to Taraschewski *et al.* (1992) all the gaps of the egg shell of the eoacanthocephalans, *Neoechinorhynchus rutili* and *Paratenuisentis ambiguus* contain different kinds of polysaccharide compounds. It is assumed that these polysaccharides are also present in *Pallisentis rexus*. The intermediate host may be attracted by these polysaccharides in the G1 as nutrients which in turn improve the probability of infection.

The regular pleating of E1 appears to be a unique characteristic of the genus *Pallisentis* and is obviously associated with the layer's capacity to expand. The mechanism by which the pleated E1 between E0 and E2 is formed remains an area to be studied. Presumably the constituents of E1 are secreted into the space between E0 and E2 and here self-assemble into the E1 layer. Why this self-assembly results in an already pleated configuration is unclear.

Acknowledgements

We would like to express our special thanks to Dr R.G. Bailey (King's College London) and Associate Professor O.B. Parkobvitayakit (Chiang Mai University) for their help and advice. Thanks are also due to Mr J. Pacey, Dr T. Brain and Ms Jane Storey at the EM-Unit, Division of Life Sciences, King's College London, UK, for their assistance.

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(Accepted 22 May 2003)