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Histochemical correlations between egg capsule laminae and the female gonoduct reveal the process of capsule formation in *Dicathais orbita* (Neogastropoda: Muricidae)

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Summary

Limited information is currently available on the process of encapsulation and degree of variation in the micromorphology of egg capsules deposited by muricid gastropods. In this study, a histochemical approach was employed to determine the origin of egg capsule laminae within the female gonoduct of *Dicathais orbita*. Comparisons of capsule micromorphology from this Australian Rapaninae, with species from the Muricinae and Ocenebrinae, suggest that whole capsule and lamina 1 (L1) thickness, L2 fiber orientation and intracapsular fluid biochemistry display interspecific variability. Furthermore, the method of capsule laminae deposition was found to be substantially different to previous descriptions of muricid capsule formation. Simultaneous examination of capsule and gonoduct biochemistry in *D. orbita* revealed that the intracapsular fluid and inner capsule wall (L3) contain secretions from the posterior lobe of the capsule gland; the thick middle lamina (L2) is derived from the lateral capsule gland lobes; and the outer layers (L1 & L0) include dorsal capsule lobe, albumen gland and pedal gland components. The insight gained into the processes involved in capsule formation imply that inter-specific differences in capsule micromorphology are dependent on the timing of egg entry during capsule formation, and the protein content of posterior lobe secretory products coupled with the presence or absence of albumen gland secretions. This investigation supplements existing evidence on the origin of capsule components and variability in capsule micromorphology in the Muricidae by providing a simple method for deciphering the complex process of encapsulation in neogastropods.

Introduction

Neogastropod embryos are maintained within protective capsules on nutritive sources during development into planktotrophic veligers, veliconcha or metamorphosed juveniles (Spight, 1977; Middelfart, 1994; Romero, 2004). Encapsulation of early ontogenetic stages is facilitated by the secretion of capsule laminae around embryos within the capsule gland, and by sculpturing, hardening and attachment of capsules by the ventral pedal gland (Hyman, 1968; Purchon, 1977; Fretter, 1941; Sullivan and Maugel, 1984; D'Asaro, 1988). The capsule macromorphology resulting from pedal molding is often species specific (D'Asaro, 1988, 1991). Similarly, discrete differences in capsule micromorphology have been observed with respect to the organization and composition of capsule laminae (Fretter, 1941; D'Asaro, 1988, 1991; Middelfart, 1992), which suggests possible differences in the process of encapsulation within Neogastropoda.

Of the neogastropods, members of the Muricidae construct some of the most structurally and chemically complex capsules. Muricid capsules are typically composed of 3-4 proteinaceous and carbohydrate-rich laminae (Table 1). The outermost fibrous laminae (L1-2) impart protection (Sullivan and Maugel, 1984; D'Asaro, 1988; Garrido & Gallardo, 1993; Rawlings, 1995; Ram et al., 2000; Lim et al., 2007) and structural support (D'Asaro, 1988) to the capsule. The third lamina (L3) is homogeneous in composition and often considered an extension of the apical plug, which is part of the hatching mechanism in many species (D'Asaro, 1988). The fourth lamina (L4) is largely composed of albumen (Bayne, 1968; Fretter, 1941; D'Asaro, 1988) and along with the third lamina, surrounds the intracapsular fluid and embryos. Despite the wealth of information available on capsule micromorphology, understanding of the formative process is hindered by the rarity of observing capsule during manufacture.

Table 1

Only on one occasion has a capsule been observed within the gonoduct of a muricid. Based on observations of a near complete capsule in *Nucella lapillus*, Fretter (1941) was able to propose the function of the various capsule gland lobes. In this species, the lateral, dorsal and anteroventral lobes were found to secrete the innermost proteinaceous layer of the capsule. These lobes then discharge an additional mucoid substance, which is mixed by epithelial cilia to form the fibrous outer capsule layers. Upon the release of eggs into the lumen, the posterior tips of the capsule gland secrete mucous which is propelled anteriorly with the eggs to line the interior. These tips continue to secrete mucous, which accumulates and becomes the operculum of the capsule escape aperture.

Evidence suggests that the encapsulation process of *N. lapillus* should not be generalized for the whole family. Descriptions of neogastropod capsules by D'Asaro (1988) and Sullivan and Maugel (1984), suggest that the process of laminae deposition may differ from that depicted by Fretter (1941). Muricoidean capsule micromorphology has also been shown to display within family variation (D'Asaro, 1988), although representative species from only two subfamilies have been examined to date (Table 1). Capsule micromorphology has not been previously examined in species from the large monophyletic subfamily Rapaninae, despite observations of capsule macromorphology by Kool (1993) suggesting that further data on Muricidae egg capsules may reveal interesting evolutionary trends. Furthermore, comparative analyses by Miloslavich (1996) indicate that the biochemical composition of laminae may depend on the nutritive

importance of the capsule to developing embryos and hence, the mode of intracapsular development. However, the current data on egg capsule composition is only available for species with direct development (Table 1). The Rapaninae, *Dicathais orbita*, has planktotrophic larval development (Phillips, 1969). Consequently, further studies on this species will broaden the taxonomic and ontogenetic developmental representation of investigations into the formation of Muricidae egg capsules.

Differences in capsule micromorphology ultimately reflect variations in the diversity and composition of maternal oviduct secretions and the processes involved in encapsulation. To gain a broader understanding of this complex procedure, an alternative method to the rare opportunity of observing capsule formation is required. Correlations between the biochemical properties of gonoduct sections and post deposition capsule laminae could be employed to determine the structures responsible for variations in capsule micromorphology. These relationships may also allow the processes involved in encapsulation to be deciphered. Furthermore, comparisons with hardened capsules may expand on formative descriptions of partially formed capsules (Fretter, 1941) by providing information on layers contributed by the ventral pedal gland. This investigation will examine the histochemical properties of capsules from *D. orbita*, in comparison with the staining reactions of gonoduct secretions in an effort to determine the process of laminae deposition. Ultimately, it is hoped that this investigation will provide a method for deciphering the process of encapsulation and increase our understanding of the origin of variations in capsule biochemistry and micromorphology.

Materials and Methods

Three *D. orbita* females were collected directly from egg masses along the rocky subtidal platforms along the metropolitan coastline of South Australia during November and December 2005. Fifteen egg capsules were also sampled from three separately spawned collective egg masses. Care was taken to maintain basal membrane integrity to prevent the expulsion of encapsulated juveniles. Digital images of egg capsules were taken under a stereo-dissecting microscope (Olympus, SZH) for descriptions of external capsule morphology. Morphometric measurements were obtained using an eye-piece micrometer (0.01mm). Three capsules from each egg mass were ruptured with a scalpel, and a wet-mount prepared of the encapsulated juveniles. Juveniles were examined under a compound light microscope (Olympus, BH-2), and the phase of intracapsular development determined in accordance with Roller and Stickle (1988) and Romero et al. (2004). Prior to fixation and embedding, each capsule was pierced with a scalpel to permit adequate fixative and paraffin to enter the intracapsular space. Egg capsules were prepared and stained for histochemical examination in an identical manner to female gonoducts.

The shell of each female specimen was removed by cracking with a vice at the junction of the primary body whorl and spire, and the soft body removed by severing the columnar muscle. The soft body was then transferred to a dissecting tray and submersed in filtered (0.22µm) seawater to reduce osmotic stress. The pallial gonoduct was then separated from the rest of the visceral mass by incisions along the lateral margins of the columnar muscle, between the ctenidium and hypobranchial gland and the ingesting and digestive glands. The pallial gonoduct of each specimen was then fixed in 10% neutral buffered formalin for 6hrs, dehydrated through an ethanol series, cleared in chloroform

and embedded in paraffin. Twenty four serial transverse sections (6µm) were acquired from the anterior and posterior capsule gland, and the albumen gland. A minimum of sixteen serial sections were obtained from each egg mass replicate.

Six transverse sections from each gonoduct region, and four from each egg mass, were stained for biochemical comparisons. Application of haematoxylin and eosin has previously allowed differentiation of gonoduct secretory products (Fretter, 1941; Jaramillo, 1991; Aungtonya, 1997), while Toluidine Blue has permitted the identification of homologous capsule components (Bayne, 1968; D'Asaro, 1988). Thus, Modified Harris Haematoxylin and Eosin Y with Phloxine B (Thompson, 1966) was applied for the differentiation of eosinophilic and basophilic structures, while Toluidine Blue (Kramer and Windrum, 1954) was applied for the identification of neutral (Wägele et al., 2006) and sulphated mucopolysaccharides (Kramer and Windrum, 1954). To provide further discriminative power and biochemical information, Periodic Acid Schiff (McManus, 1946) and p-dimethylaminobenzaldehyde-nitrite (Adams, 1957), counterstained with nuclear fast red (C.I. 60760), were also applied for the demonstration of glycoproteins and tryptophan, respectively. Sections were examined and photographed under a compound light microscope (Olympus, BH-2). Measurements of egg capsule laminae and yolk granules (0.01µm) were made in Adobe Photoshop®, version 5.5 (Adobe Systems Incorporated, 1999) in accordance with digital images of a stage micrometer.

Results

Encapsulated larvae and egg capsules

Encapsulated larvae displayed heterogeneous intracapsular development between capsules within and between replicate egg masses. Despite these intraspecific variations,

the majority of larvae within one replicate egg mass were characterized by a large vegetal pole containing vitelline material, and smaller animal or supranuclear pole. The presence of cilia on the animal pole coupled with the absence of a blastopore indicated that these larvae were at the stereoblastula stage. Larvae within the remaining two egg mass replicates were significantly more advanced, displaying features in accordance with early veligers and veligers. Early veligers possessed a posterior protoconch, evidence of an operculum, bilobulate ciliated velum and pigmented eye spots, while veligers displayed in addition, a ventral foot, digestive gland and protoconch ornamentation. Although greatly reduced in comparison to stereoblastulas, a considerable number of yolk granules were still present at the veliger stage. In transverse sections, eosinophilic yolk granules (width = 6.29 ± 1.07µm, length = 7.23 ± 2.94µm) stained weakly orthochromatic with Toluidine Blue and were Periodic Acid Schiff (PAS) and tryptophan positive.

The egg capsules of *D. orbita* (height = 6.41 ± 1.03mm, width = 5.61 ± 0.91mm), are composed of two convex walls joined by lateral sutures, which give the capsule an elliptical shape in cross section (Fig. 1). The walls taper towards the basal membrane, before broadening into the peduncle (Fig. 1a). The concave apical plate contains a centered oval operculum with a mean length of 0.6 ± 0.03mm and width of 0.5 ± 0.04mm (Fig. 1b). The apical plate is bisected by a suture, which is slightly to off canter and extends to a low apical ridge.

Fig. 1

In transverse sections, the capsule wall has a mean width of 36.59 ± 3.72µm and is composed of 4 laminae (L1-4) along with an additional non-structural outer lamina (L0) (Fig. 2). The innermost lamina (L4) was only visible when stained in haematoxylin and eosin (H&E) (Fig. 2b). This lamina lacks distinct structural detail and has a mean width of 0.59 ± 0.09µm. The third lamina (L3) also appears homogeneous in composition (Fig. 2) and has a mean width of 1.15 ± 0.11µm. The second lamina (L2) is much wider than the other laminae (28.89 ± 1.01µm) and is composed of three distinct regions (Fig. 2). The lateral regions contain densely packed circular fibers, while the fibers of medial region are arranged into a loose network of circular fibers, giving a vacuolated appearance. The mean width of the outer structural lamina (L1) was noticeably greater in capsules containing stereoblastulas (7.19 ± 0.61µm, Fig. 2a) than those with veligers (2.67 ± 0.73µm, Fig 2b). The loosely packed fibers of L1 collectively display perpendicular orientation to the capsule long axis, although many individual fibers were entangled and unorientated. The surface of this lamina appears to undulate in a semi-regulated fashion (Fig. 2). The outer non-structural lamina (L0) was only detected in stereoblastula capsules (Fig. 2a). This lamina is very thin (1.12 ± 0.2µm) and homogeneous in composition. The intracapsular fluid (IF) enclosed within capsules from all egg masses was consistently fibrous in composition.

Fig. 2

The biochemical compositions of capsule laminae and intracapsular fluid are given in Table 2. L1 stained strongly basophilic and metachromatic with Toluidine Blue, while L2

was strongly eosinophilic. The basophilic intracapsular fluid also produced purple metachromasia with Toluidine Blue and displayed strong staining reactions with PAS and n-DMAB-nitrite. L3 stained in an identical manner, but only weak reactions were observed. Although L4 was only detected using H &E, it was distinctly different to the adjacent L3, with eosinophilic rather than basophilic properties.

Table 2

Albumen, capsule and ventral pedal glands

From posterior to anterior, the gonoduct of *D. orbita* is composed of a dorsal ovary, a right lateral albumen gland, an ingesting gland and a capsule gland. The duct from the ovary opens into the ventral lumen of the albumen gland, which is formed between left and right lateral lobes (Fig 3a). Subepithelial acini of these lobes release two types of fibrous secretion, with distinctly different biochemical staining reactions (Table 2). These secretions are released into parallel ducts, which open into the dorsoventral lumen (Fig. 3a) through micro-pores in the ciliated columnar epithelium. The dorsal lumen of the albumen gland unites with the ventral channel, which progresses in an anterior direction to open into the ventral posterior capsule gland.

The capsule gland is composed of five glandular lobes, a posterior lobe (Fig. 3b), an anteroventral lobe, a dorsal lobe and left and right lateral lobes (Fig. 3c). Although secretory products of the posterior, anteroventral and lateral lobes are all packaged into spherules suspended in homogeneous secretion, the biochemistry of these lobes varies (Table 2). Spherules of the posterior (Fig. 3b) and anteroventral lobes both stain for

tryptophan; however posterior lobe spherules exhibit metachromasia with Toluidine Blue, while those of the anteroventral lobe stain orthochromatically (Fig. 3c). With the exception of the ventral portion of the right lateral lobe (Table 2), the lateral lobes fail to stain for this amino acid (Fig. 3b). Secretions from the dorsal lobe are morphologically distinct, appearing as a fibrous material, which stains metachromatically with Toluidine Blue (Fig. 3c).

Fig. 3

The posterior capsule gland is composed of a right lateral posterior lobe lined along the left lateral margin by dorsal and right lateral lobe gland cells (Fig. 3b). A remnant left lateral lobe is also present, proximal to the anterior ingesting gland (Fig. 3b). At this point, the dorsoventral lumen of the capsule gland is continuous with the ventral channel (Fig. 3b). Anteriorly, dorsal lobe cells become a discrete dorsal lobe, and the anterior ingesting gland and posterior lobe recede to be replaced by left and right lateral lobes, respectively (Fig. 3c). An anteroventral lobe, which develops from the ventral region of the right lateral lobe, divides the dorsoventral lumen into transverse lumina (Fig. 3c). The right transverse lumen is terminal, while the left unites with the ventral channel. Just posterior of the vaginal opening, the dorsal lobe divides bilaterally and extends in a ventral direction to replace the lateral lobes, while the anteroventral lobe reunites with the ventral portion of the right dorsal lobe.

An anterior and a posterior (Fig. 3a) ventral pedal gland are present in female *D. orbita.* The anterior pedal gland is as a small protrusion in the subepithelial musculature

located ventral and mid-way along the capsule gland, while the posterior is as a prominent longitudinally folded muscular papilla (Fig. 3a) extending the length of the ingesting gland. The secretory activity of these glands appears restricted to ciliated columnar epithelial cells, as both lack subepithelial gland cells. The diversity of secretory epithelial cells varies from anterior to posterior. The epithelium of both ventral pedal glands is composed of goblet cells interspaced with another secretory cell type, while the posterior pedal gland contains an additional third secretory cell type. The secretion of both cell types is packaged into spherules, which display distinct biochemical properties (Table 2). The histochemical staining reactions of secretions from albumen and capsule gland acini, and epithelial cells of the ventral pedal glands are detailed in Table 2.

Discussion

This study reveals that egg capsule biochemistry has remained relatively constant throughout Muricidae evolution. The histochemical properties of capsule laminae from the planktotrophic Rapanine, *D. orbita* (Table 2), were comparable to previous reports of those from direct developing species in the divergent subfamilies Ocenebrinae and Muricinae (Table 1). Nevertheless, inter-species variability was observed in capsule micromorphology with respect to total capsule and L1 thickness, L2 fiber orientation and intracapsular fluid composition. Furthermore, morphological changes in outer laminar laminae were observed over the course of larval development in *D. orbita*.. Correlations between the biochemistry of female gonoduct secretions and capsule constituents enabled the process of encapsulation to be deciphered in *D. orbita* and revealed that capsule formation is a complicated process incorporating distinct secretions from specific regions of the gonoduct. Comparison to the process described in other muricids indicates that the

observed inter-species variability in capsule micromorphology is primarily related to oviduct functional anatomy and the biochemical origin of intracapsular fluid. Increased replication is required to encompass a range of larval stages in representative species from all Muricidae subfamilies to establish the evolutionary and developmental significance of these variations in capsule micromorphology

Four structural laminae (L1-4) and an additional non-structural lamina (L0) comprised the capsules of *D. orbita* (Fig. 2a). A thin layer (L0) of proteinaceous material (Table 2) was observed on surface of fresh egg capsules. Previous research into the ultrastructure of *D. orbita* egg capsules (Lim et al., 2007), revealed a "crust" on the surface of ≤1wk old capsules. The age-dependent correlation between changes observed in SEM micrographs by Lim et al., (2007) and those observed in this histochemical study, strongly suggest that the previously described "crust" is the outer lamina layer L0 detected in this investigation (Fig. 2a). Although this lamina has only been previously described in buccinid capsules (Sullivan and Maugel, 1984), the rapid degradation and slight thickness (approx. 1µm) of this lamina could explain the lack of previous reports from histochemical studies of Muricidae capsules.

The outermost structural lamina (L1) was characterized by transverse fibers embedded in carbohydrate. The detection of sulphated mucopolysaccharides (Table 2) appears typical of this lamina in both the Muricinae and Ocenebrinae (Table 1). Undulations in the surface of L1 (Fig. 2a) correlate with ridges observed on the exterior of *D. orbita* capsules by SEM (Lim et al., 2007). Surface corrugations have also been detected in Muricinae capsules and are thought to result from pedal molding (D'Asaro, 1988), which may explain the mixture of orientated and unorientated fibers observed in

L1 of *D. orbita* capsules. The morphology of this lamina also varied with capsule maturity, decreasing in thickness by over 60% between stereoblastula (Fig. 2a) and veliger stage (Fig. 2b) capsules. Degradation and finally delamination of this outer fibrous layer has also been observed in SEM analyses of veliger stage *D. orbita* capsules (Lim et al., 2007). Shedding of this outer fibrous lamina was thought to occur in response to excessive fouling (Lim et al., 2007). However, as L1 degradation also correlates with the maturity of encapsulated larvae, delamination may be more directly related to the exchange of gases (Booth, 1995) and accumulated toxic waste through the capsule wall. Thus, delamination of heavily fouled outer layers may ensure adequate diffusion of respiratory gases as the metabolic demands of developing larvae increase.

Of the data currently available, direct developing species (Table 1) have greater L1 and/or whole capsule widths in comparison to the planktotrophic species, *D. orbita*. As direct developing muricids have an extended duration of encapsulation (Romero et al., 2004), these species may have evolved thicker capsules to facilitate multiple shedding events. Furthermore, the doubling of capsule wall thickness between *Urosalpinx cinerae* and *N. lapillus* correlates well with encapsulated development periods of 60 days and 120 days, respectively (Romero et al., 2004). Intraspecific variation in capsule wall thickness in the direct developer *Nucella emarginata* has also been attributed to the vulnerability of capsules to benthic predators (Rawlings, 1990, 1994). Therefore, the increased thickness of capsule walls could be a particularly important form of defense in species that invest significant energetic resources in nutritive nurse eggs to support only a few well developed hatching juveniles with extended periods of benthic exposure. Increased egg capsule wall strength and greater relative energetic investment in capsule laminae has

been previously documented for *Conus* species with longer periods of encapsulated development (Perron, 1981). Although a broader representation of Muricidae species with planktotrophic development is required, outer laminae thickness appears to be influenced by developmental mode and environmental pressures influencing the survivorship of encapsulated embryos and juveniles.

The second lamina (L2) constitutes approximately 75% of the capsule wall and is best described as a protein-carbohydrate matrix, containing dense lateral and loose medial circular proteinaceous fibers embedded in neutral mucopolysaccharides (Table 2). The layered structure (Bayne, 1968; Fretter, 1941; D'Asaro, 1988; Hawkins & Hutchinson, 1988; Ram et al., 2000) and large contribution of this lamina to the total capsule width is typical of all Muricidae capsules (Table 1). However, the arrangement of fibers in *D. orbita* differs from that described in previously examined species (Table 1). L2 fibers of the Muricinae and Ocenebrinae are of longitudinal and perpendicular or circular orientation, respectively, while those of *D. orbita* are only of circular direction. Similarly, the presence of neutral mucopolysaccharides in L2 also appears to be restricted to certain muricid species (Table 1). Consequently, the biochemical composition of L2 appears to be a plastic feature in Muricidae egg capsules that can not be explained simply by subfamilial affiliation.

The third lamina (L3) of *D. orbita* is homogeneous in composition and composed of proteins and carbohydrates (Table 2). The staining reactions of this lamina indicate the presence of glycoprotein and the amino acid tryptophan, which is consistent with previous reports from *Ocenebra erinacea* and *N. lapillus* (Table 1). The presence of mucopolysaccharides in L3 also appears common to all Muricinae and Ocenebrinae

capsules examined with Toluidine Blue (Table 1). The innermost homogeneous lamina (L4) of *D. orbita* was only detectable when stained with eosin (Table 2). Although this laminae is often indistinct from the third under light microscopy (Rawlings, 1995), it has been previously detected in many Muricidae, and shown to contain mucopolysaccharides (Table 1).

The biochemical composition of intracapsular fluid was also of interest, as L4 is thought to be largely composed of albumen (Bayne, 1968; Fretter, 1941; D'Asaro, 1988). However in *D. orbita*, L4 was shown to be eosinophilic, while the intracapsular fluid displayed basophilic properties (Table 2). Interestingly, the intracapsular fluid stained in an identical manner to L3 (Table 2). Detection of tryptophan was unexpected as application of the p-DMAB-nitrite method to the intracapsular fluid of *O. erinacea* and *N. lapillus* capsules failed to produce a positive reaction (Table 1). Furthermore, the histochemical method applied can react with serotonin (Adams, 1957), which is involved in the control of morphogenesis (Cote et al., 2007). Tryptophan can also oxidize to produce the coenzymes NAD and NADP (Bender and Olufunwa, 1988), which play an important role in cellular metabolism. Consequently, this amino acid may provide an essential resource for developing larvae within the intracapsular fluid.

A major advance in our understanding of the process of encapsulation in the Muricidae was gained by correlating the histochemical properties of secretions from capsule and albumen gland lobes, and ventral pedal gland secretory cells, with capsule constituents (Table 2). Identical reactions from the five histochemical stains applied indicate that L0 is composed of secretions from the ventral portion of the right lateral and anteroventral lobe of the capsule gland, along with secretory cell type one of the ventral

pedal gland. The outermost structural lamina (L1) appears to originate from dorsal capsule gland lobe and albumen gland secretion 2, while the staining properties of L2 correlate with those of the lateral capsule gland lobes. The inner lamina (L3) contains identical biochemical secretions to the posterior capsule gland lobe, as does the intracapsular fluid (Table 2). Due to the limited biochemical data obtained for L4, this lamina could be composed of either lateral or anteroventral capsule gland lobe secretions or secretion 1 from the albumen gland. Overall these correlations suggest that L1-3 originate exclusively from the capsule gland, while L1 and L0 appear to contain in addition, albumen and ventral pedal gland components, respectively. This is the first time that histochemical staining has been used to successfully trace the origin of egg capsule components to specific regions of the female gonoduct.

The order of laminae deposition greatly depends on when fertilized eggs enter the capsule gland. If they enter prior to secretion of capsule laminae as suggested by Sullivan and Maugel (1984) and D'Asaro (1988), then capsule laminae originating from the capsule gland would theoretically be secreted in an L4 or L3 to L1 sequence. However, if the outer structural laminae are secreted first, and the eggs forced into the center of the capsule (Fretter, 1941), then L3 or L2 to L1 would be secreted first, followed by the influx of albumen and formation of the albumen retaining lamina. Although the exact sequence of events occurring during capsule formation is difficult to describe in the absence of direct observations, information gained through coupling capsule micromorphology and biochemical origin with oviduct morphology, provides a good indication of processes involved.

The exclusive presence of circular fibers in *D. orbita* capsule laminae strongly suggests that the eggs are already present within the capsule gland prior to secretion of the capsule wall. In contrast, longitudinal fiber orientation in *N. lapillus* and *O. erinacea* capsules occurs during the forcible anterior movement of albumen and eggs into the already secreted L2 and L1 (Fretter, 1941), while circular fibers arise from the ciliary currents of capsule gland epithelial cells (Fretter, 1941). Correlations between intracapsular fluid and posterior lobe secretions in *D. orbita* (Table 2) further suggest that these are propelled anteriorly with the mass of eggs upon entry to the posterior capsule gland. However, in some Muricidae (Table 1) intracapsular fluid is structurally stratified, which may reflect the inclusion of both albumen and posterior capsule gland lobe secretions (Fretter, 1941; D'Asaro, 1988). Despite being commonly termed "albumen", no contribution from the albumen gland was detectable in the intracapsular fluid of *D. orbita* capsules.

Correlations in biochemistry suggest that L3 is also secreted by the posterior lobe (Table 2). Although this lobe is restricted to the posterior half of the capsule gland, secretions may also be received by the ventral channel and distributed anteriorly by epithelial cilia. Previous investigations indicate that the apical capsule region is constructed within the posterior capsule gland (Fretter, 1941) and the inner apical operculum is formed during L3 deposition (Fretter, 1941; D'Asaro, 1988). Thus formation of the operculum during this stage of capsule formation would be facilitated by the posterior positioning of this lobe. The left of centre positioning of the apical plate suture in *D. orbita* (Fig. 1) is reflected in the presence of one dominant right posterior lobe and a remnant left lateral lobe (Fig. 3), which is in contrast to the centered suture

formed by symmetrical posterior lobes in *N. lapillus* (Fretter, 1941). The lateral lobes occupy the entire length of the capsule gland and function in the deposition of L2. The fibrous content of this lamina most likely originates from proteins packaged within secretory spherules (Flower, 1973), while the material comprising interfibrillar spaces would arise from the homogeneous secretion in which spherules are embedded.

Finally, the outermost structural lamina (L1) is secreted by the dorsal lobe, which extends ventrally in the anterior and posterior capsule gland. This lamina may also be supplemented by albumen secretions received from the ventral channel. Once complete, the capsule is transferred to the ventral pedal glands where longitudinal folds in the epithelium produce ridges in the capsule surface. During exit from the capsule gland, L1 comes into contact with the ventral right lateral and the anteroventral lobe, which begin to secrete L0. Secretory cell type 1 continues to secrete this lamina, as suggested for capsules of the buccinid *Ilyanassa obsoleta* (Sullivan and Maugel, 1984). The occurrence of an additional secretory cell type in the posterior ventral pedal gland epithelium (Table 2) suggests that this may function in either capsule hardening or secretion of an adhesive for attachment to the substrate (Sullivan and Maugel, 1984).

Observed interspecies differences in egg capsule micromorphology therefore appear to depend on both the timing of egg entry during capsule formation (i.e. L2 fiber orientation) and origin of laminae and intracapsular fluid secretions within the female gonoduct. For example, capsule gland lobes contributing to structural laminae in *N. lapillus* contain essentially identical double secretions (Fretter, 1941), while those of *D. orbita* possess single secretions, which are biochemically distinct (Table 2). The chemical diversity and discrete structure of laminae in *N. lapillus* arises from the selective release

of single or combined secretions from all lobes in synchrony (Fretter, 1941), while secretions are released sequentially from different lobes in *D. orbita,* depending on the laminae being secreted. Thus, combining information on capsule laminae origin and oviduct morphology provides insight into the process of encapsulation that can aid in the interpretation of observed interspecific differences in capsule micromorphology.

In summary, the capsules of *D. orbita*, a planktotrophic developer from the subfamily Rapaninae, are composed of four structural laminae (L1-4) and an outer non-structural lamina (L0). Although capsules were found to be of comparable micromorphology to direct developing Muricinae and Ocenebrinae species, interspecific differences in L1 and total capsule thickness, L2 fiber orientation and intracapsular fluid biochemistry were detected. The method of laminae deposition also varied considerably between *D. orbita* and previous accounts of encapsulation in *N. lapillus* (Fretter, 1941). Information gained on the process of encapsulation by correlating capsule and gonoduct biochemistry and morphology indicate that variations in capsule micromorphology depend on capsule constituent origin and oviduct functional anatomy. Application of the method presented in this investigation to Muricidae species across all subfamilies and modes of development may reveal further evolutionary trends, which have been overlooked in during examination of capsules alone. Overall, this investigation has provided a simple method for understanding the complex process of encapsulation and deciphering the origin of micromorphological capsule differences.

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Figure Legends

Figure 1. Egg capsules of *D. orbita* containing encapsulated juveniles, showing details of; A) the lateral, and B) apical morphology. Ap, apical plate; Ar, apical ridge; As, apical suture; Bm, basal membrane; Ls, lateral suture; Op, operculum; P, peduncle. Scale bars = 1mm.

Figure 2. Transverse sections of a *D. orbita* capsule wall stained in H&E demonstrating; A) the non-structural lamina (L0) and thick outer fibrous lamina (L1) characteristic of stereoblastula stage egg capsules, along with the innermost laminae (L3 & L4, insert); and B) the reduced thickness of L1 in veliger stage capsules. Scale bars = 10µm.

Figure 3. Morphology and histochemistry of the female *D. orbita* gonoduct showing; A) albumen; and B) posterior capsule gland morphology stained in p-DMAB-n for tryptophan (blue); as well as C) the anterior capsule gland stained in Toluidine Blue for mucopolysaccharides. Ags1, albumen gland secretion 1; Ags2, albumen gland secretion 2; AVCg, anteroventral capsule gland lobe; DC, dorsal lobe cells; DCg, dorsal capsule gland lobe;; Ig, ingesting gland; LCg, left lateral capsule gland lobe, Lu, lumen; Pg, pedal gland; R, rectum; RCg, right lateral capsule gland lobe; RCgv, ventral portion of right capsule gland lobe; Vc, ventral channel. Scale bars = 1mm.

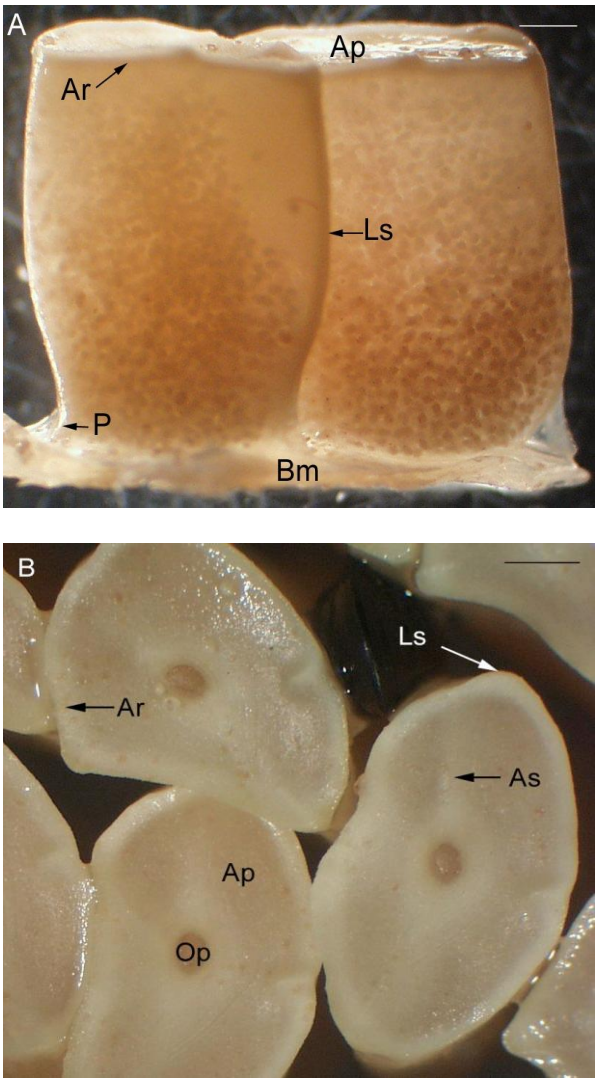


Figure 1

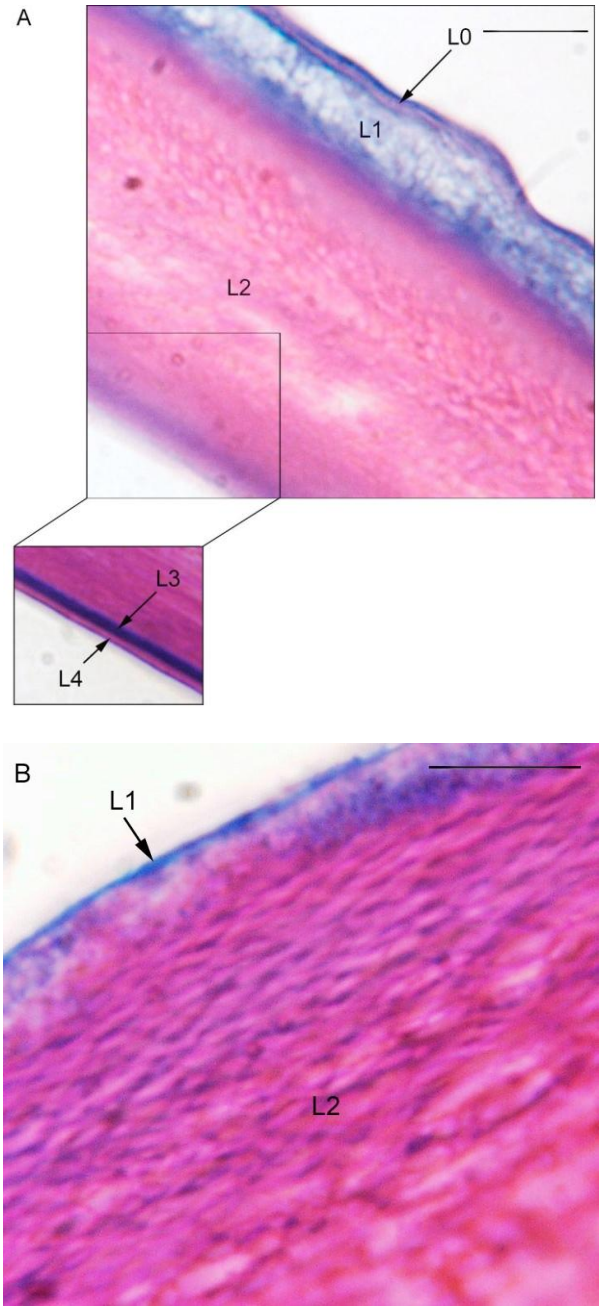
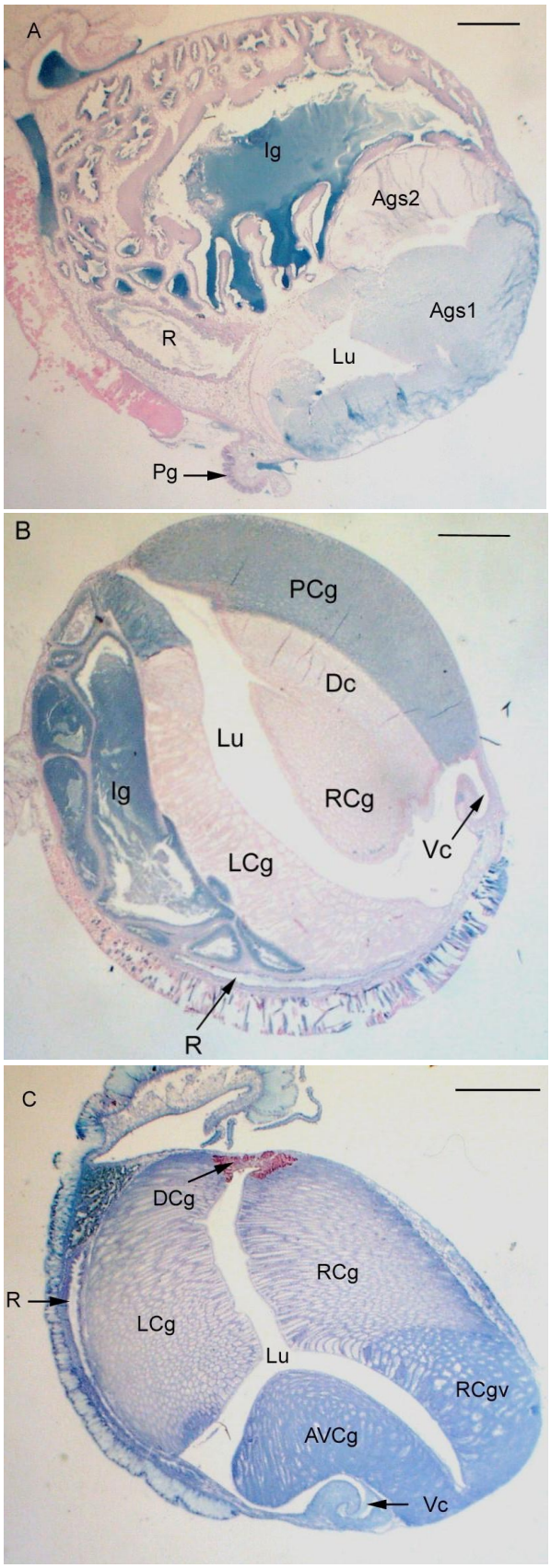


Figure 2.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DCg
RCg
R
LCg
Lu
RCgv
AVCg
Vc

Figure 3.

Table 1. Mode of development, micromorphological and biochemical staining features of egg capsules from the Muricidae. MPS, Mucopolysaccharide staining with Toluidine Blue; Meta, metachromatic; Ortho, orthochromatic; NO, negative reaction; NT, not tested. Fiber orientation in L2 is classified as Circ, circular; Long, longitudinal and; Perp, perpendicular. NR, not reported.

Subfamily	Species	Development Mode	L1	L2	L3	L4	Albumen	Capsule width	Reference
Muricinae	*Chicoreus* (*Murex*) *florifer dilectus*	Direct	MPS (Meta) 4µm	MPS (Meta) 54µm Long & Perp	MPS (Meta) 2µm	MPS (Meta) 4µm	MPS (Meta) 2 Types	64µm	D'Asaro, 1970a; D'Asaro, 1988
Muricinae	*Phyllonotus* (*Murex*) *pomum*	Direct	MPS (Meta) 1-2µm	MPS (Ortho) 47µm Long & Perp	MPS (Meta) 1-2µm	MPS (Meta) 2µm	MPS (Meta) 2 Types	51µm	D'Asaro, 1970a; D'Asaro, 1988
Ocenebrinae	*Eupleura caudata*	Direct	MPS (Meta)	NA waiting for ref Gruber	NA waiting for ref Gruber	MPS (Meta)	MPS (Meta) 2 Types	NR	Spight, 1976; Guber, 1982
Ocenebrinae	*Urosalpinx cinerea*	Direct	MPS (Ortho) 30µm	MPS (Ortho) 75µm Long & Circ	MPS NR 2µm	MPS (Ortho) 2µm	NR	109µm	Hancock, 1956; Tamarin & Carriker, 1967; Costello *et al.*, 1957; Spight, 1976
Ocenebrinae	*Ocenebra erinacea*	Direct	MPS NT Amino acids Glycoprotein	MPS NT Amino acids Glycoprotein Long & Circ	MPS NT Tryptophan Amino acids Glycoprotein	MPS NT Amino acids Glycoprotein	MPS NT Amino acids Glycoprotein 1 Type	200µm	Hawkins & Hutchinson, 1988 Fretter, 1941; Spight, 1976
Ocenebrinae	*Nucella lapillus*	Direct	MPS (Meta) Amino acids Tyrosine	NO MPS Carbohydrate Amino acids Tyrosine Long & Circ	MPS (Meta) Amino acids Tryptophan	NR	MPS (Meta) 1 Type	NR	Fretter, 1941; Costello *et al.*, 1957; Bayne, 1968; Spight, 1976; Pechenik *et al.*, 1983

Table 2. The biochemical composition and comparison of gonoduct secretions and egg capsule constituents in *D. orbita*. Interfibrillar spaces in capsule laminae L1 and L2 and the protoplasm of capsule gland acini stained identically to fibrous laminae components and acini spherules, respectively. Av, anteroventral lobe; D, dorsal lobe; Gc, goblet cell; L, lamina; La, lateral lobes; NA, not available; Rv, right ventral lobe; S, secretion; Sc, secretory cell; ++, strong staining reaction; +, weak staining reaction; -, negative staining reaction.

Stain	Substance	Albumen Gland		Capsule Gland					Pedal Glands		Capsule Laminae						IF
		S1	S2	D	La	Av	Rv	P	Sc1	Sc2	Gc	L0	L1	L2	L3	L4	
Haematoxylin	Basophilic	-	+	+	-	-	-	++	-	+	-	-	++	-	+	-	++
Eosin	Eosinophilic	+	-	-	++	+	+	-	+	-	+	+	-	++	-	+	-
Toluidine Blue	Neutral MPS	+	-	-	+	++	++	-	+	-	+	+	-	+	-	NA	-
	Sulphated MPS	-	++	++	-	-	-	++	-	-	-	-	++	-	+	NA	++
PAS	Glycoprotein	++	+	+	+	+	+	++	+	-	++	+	+	+	+	NA	++
p-DMAB-Nitrite	Tryptophan	+	-	-	-	++	++	++	+	-	-	+	-	-	+	NA	++