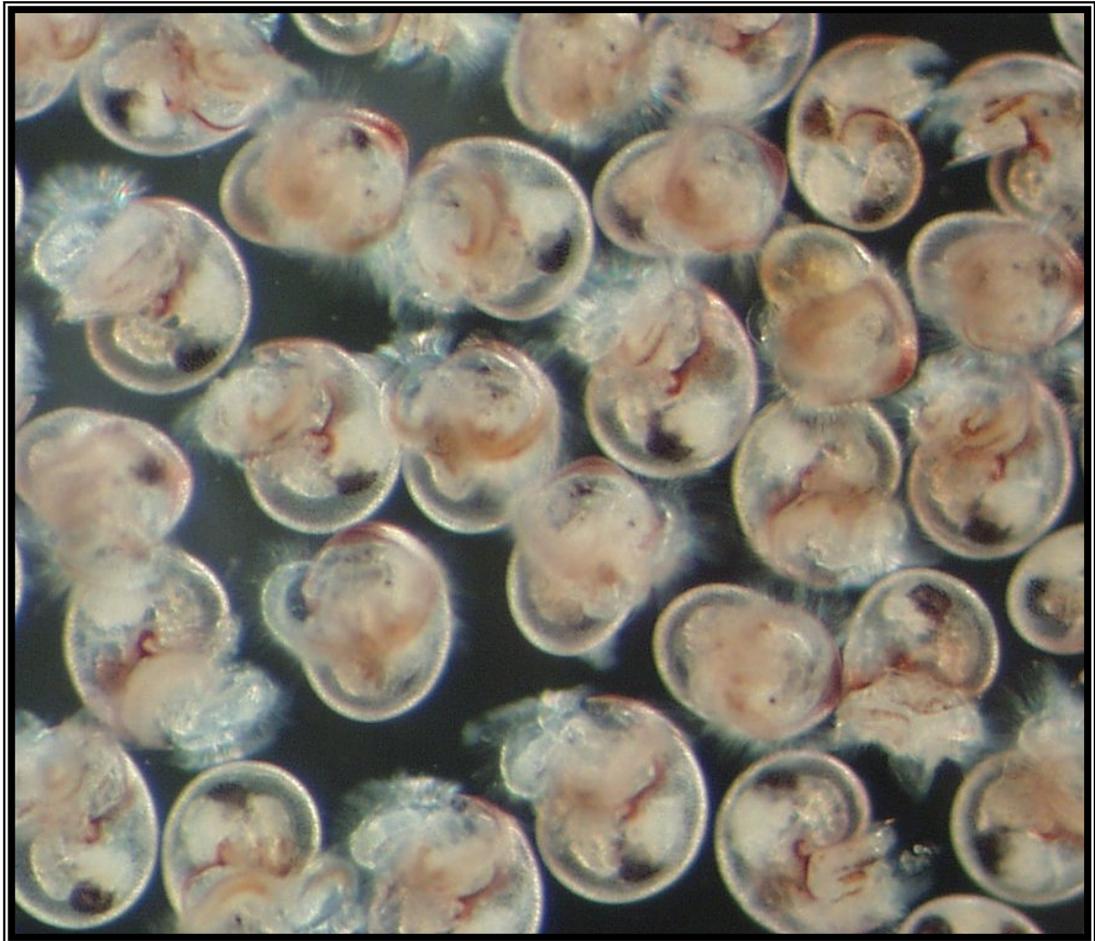


Chapter 6

**The origin of Tyrian purple precursors in egg masses:
maternal investment in the chemical defence of
encapsulated *Dicathais orbita* larvae (Neogastropoda:
Muricidae)**



6.0 Abstract

Bioactive Tyrian purple precursors occur in egg masses of the Muricidae, where they are thought to function in the chemical defence of encapsulated larvae. Although evidence suggests these brominated indoles are introduced as a form of maternal investment, the origin and biosynthetic capacity of muricid larvae is unknown. Histochemical techniques for the demonstration of compounds and enzymes essential for Tyrian purple synthesis were applied to the gonoduct and capsules, representing various phases of the reproductive cycle and larval development in *Dicathais orbita*. Liquid chromatography-mass spectrometry (LC-MS) was also employed to quantify the presence and concentration of the Tyrian purple prochromogen, tyrindoxyl sulphate, within capsule laminae, intracapsular fluid and larvae. Extracts of these egg mass constituents were also analyzed by thin-layer chromatography (TLC) to determine the distribution of associated bioactive choline esters within egg masses. The results of this investigation indicate that tyrindoxyl sulphate and the biosynthetic components for precursor and prochromogen synthesis are incorporated into capsule laminae and intracapsular fluid by specific capsule gland lobes. Tyrindoxyl sulphate and the components for prochromogen and indole precursor biosynthesis were also detected within larval vitellus. The absence of a hypobranchial gland in hatchling veligers suggests that *D. orbita* larvae rely on the passive synthesis of secondary metabolites from maternal investments within yolk granules. The distribution of choline esters coincided with that of the prochromogen in *D. orbita* egg masses. The complementary nature of the histochemical and chromatographic techniques applied has not only facilitated the localization of biosynthetic sites, but has provided insight

into the maintenance of bioactive indole synthesis during encapsulated and planktonic development in the Muricidae. The findings of this investigation strongly suggest that Tyrian purple precursors are incorporated into egg masses as a form of maternal investment in larval chemical defence. Potential functional roles for Tyrian purple precursors and choline esters in pathogen and predator defence are also discussed.

6.1 Introduction

The *de novo* synthesis or sequestration of dietary derived natural products by marine gastropods is a well documented phenomenon (reviewed in Fenical et al., 1979; Karuso, 1987; Faulkner, 1992; Pawlik, 1993; Garson, 1003, 2001; Avila, 1995, 2006; Marin and Ros, 2004; Bandaranayake, 2006; Wägele et al., 2006). However, reliable information on the significance of these compounds to molluscan reproductive and life history strategies is limited (Faulkner, 1992; Avila, 2006). It has been shown on several occasions that the anatomical distribution of secondary metabolites (Marin et al., 1991, 1999; Fontana et al., 1994; Avila and Paul, 1997) and their incidence during early life stages (Bandaranayake, 2006, Lindquist, 2002; Avila, 2006) can provide valuable insight into the ecological role of natural products. In fact, adopting a histological or ontogenetic approach has been recently highlighted as advantageous over functions inferred by bioactivity alone (Avila, 2006; Wägele et al., 2006), as *in situ* demonstration is often difficult to accomplish and typically lacking (Faulkner, 1992). Furthermore, this multifaceted perspective may be of particular use when investigating the selective benefit of compounds with heterogeneous bioactivity or intricate mixtures of secondary metabolites.

One family of marine gastropods renowned for their complex natural product biochemistry is the Muricidae. Tyrian purple is a historically important dye, formed external to the mollusc during enzymatic and photolytic cleavage of the hypobranchial metabolite, tyrindoxyl sulphate (Baker and Sutherland, 1968). Tyrian purple precursors constitute a suite of bioactive compounds (Westley et al., 2006). Tyrian purple prochromogens are choline esters of 6-bromoindoxyl and indoxyl, non-substituted and substituted with methylsulphonyl or methylthio (reviewed in Cooksey, 2001a). Prochromogen hydrolysis by arylsulphatase (Erspamer, 1946) produces brominated indoxyl intermediates, which oxidize to indoleninones and dimerize to give tyriverdin (reviewed in Cooksey, 2001a). Of these intermediates, tyrindoleninone, tyriverdin and the oxidation by-product, 6-bromoisatin are known to inhibit marine pathogens (Benkendorff et al., 2000).

The biosynthetic origin and ecological significance of these natural products has been long debated. Originally, Tyrian purple genesis was thought to be the inadvertent result of tryptophan catabolism (Fox, 1974) or part of a detoxification mechanism (Verhecken, 1989). However, detection of bromoperoxidase activity in hypobranchial extracts of the muricid, *Murex trunculus* (Jannun and Coe, 1987), revealed a biosynthetic capacity for precursor bromination. Furthermore, the presence of this enzyme together with arylsulphatase implies considerable metabolic energy is invested into the biosynthesis of secondary metabolites. A recent investigation into the hypobranchial gland of *Dicathais orbita* has confirmed that *de novo* synthesis of Tyrian purple prochromogens from dietary tryptophan occurs in the Muricidae (Chapter 5). Furthermore, histochemical evidence suggests that prochromogen hydrolysis and therefore, bioactive secondary metabolites genesis, may be regulated.

Together these findings suggest that the function of these natural products is both inducible and of selective benefit to the Muricidae.

The potential functions of muricid secondary metabolites have been recently reviewed by Westley et al. (2006). Of these, the isolation of bioactive indoles from egg masses representing three Muricidae subfamilies (Benkendorff et al., 2000, 2001, 2004) has prompted investigation into a role related to the antimicrobial defence of encapsulated juveniles. At present, the source of these egg mass metabolites and the significance of their presence to the evolutionary role of Tyrian purple genesis remain obscure. If encapsulated juveniles possess the capacity to synthesize precursors *de novo*, then it would appear that Tyrian purple genesis is of some antimicrobial benefit to the Muricidae throughout their life history. Alternatively, if prochromogens or intermediates are introduced during oogenesis or encapsulation, maternal investment in embryonic chemical defence may be an important component of this biosynthetic pathway.

Prochromogen hydrolysis by arylsulphatase also liberates the choline esters, murexine (Erspamer and Dordoni, 1947), seneciylcholine (Whittaker, 1957) and dihydromurexine (Roseghini, 1971), which display neuromuscular blocking and nicotinic action (Erspamer, 1948; Erspamer and Glässer, 1958; Keyl and Whittaker, 1958; Quilliam, 1957; Whittaker, 1963; Huang and Mir, 1971). Although these secondary metabolites have been implicated in adult prey capture, the lack of an effective delivery mechanism and failure to demonstrate ecologically relevant activity has rendered this hypothesis unlikely (Roller et al., 1995). To date, the presence and therefore, the functional role, of choline esters during early life stages and within capsule constituents have not been addressed. Thus, establishing the distribution of

choline esters within muricid egg masses may provide insight into their functional role in adults and the purpose of their activation in synchrony with antimicrobial intermediate genesis.

Although extracts of muricid egg mass have been shown to contain Tyrian purple precursors (Benkendorff et al., 2000, 2001, 2004), the presence of prochromogens has not been established. Furthermore, the distribution of these bioactive indoles within capsule laminae, intracapsular fluid and larvae is unknown. Until recently, relatively little consideration has been given to the incidence and biosynthesis of defensive compounds in early life stages of marine invertebrates (Lindquist, 2002). However, research has shown that natural product biosynthesis in the nudibranchs, *Dendrodoris limbata* (Avila, 1993) and *Doris verrucosa* (unpublished data in Avila, 2006) commences at an early larval stage. Thus, it is possible that biosynthesis is accomplished by encapsulated larvae, as the vitellus of *D. orbita* embryos has recently been shown to contain high concentrations of the indole precursor, tryptophan (Chapter 4) and may therefore provide for the synthesis of indoxyl sulphate prochromogens. However the life stage at which muricids develop a functional hypobranchial gland and the intracapsular availability of bromoperoxidase and arylsulphatase remains unknown.

Recent evidence suggests that bioactive indole synthesis may occur within the muricid pallial gonoduct for incorporation into egg masses (Chapter 3). The opisthobranch mollusc *Dolabella auricularia*, transfers antimicrobial and antifungal glycoproteins from their site of synthesis in the albumen gland to egg masses to protect developing embryos against infection (Iijima et al., 2003). Detection of the prochromogen, tyrindoxyl sulphate, in *D. orbita* albumen gland extracts and

additionally, bioactive intermediates in capsule gland extracts (Westley and Benkendorff, 2008) suggest a similar process may operate in the Muricidae. Failure to detect a mechanism for transporting precursors from the hypobranchial gland to the gonoduct (Chapter 3) further implies that biosynthesis occurs in reproductive glands. Tryptophan-positive material has been demonstrated within *D. orbita* albumen, capsule and pedal gland secretions, which comprise the perivitelline fluid, intracapsular fluid and the inner and outer capsule laminae, respectively (Chapter 4). Correlations between the distribution of tryptophan and tyrindoxyl sulphate indicate that prochromogen synthesis is possible within these reproductive glands, which would provide an effective means for incorporating precursors or intermediates into egg masses. Despite this potential, the presence of essential biosynthetic enzymes within these regions has not been confirmed.

To date, much of the principle research on Tyrian purple genesis has involved the Australian muricid, *D. orbita* (Baker and Sutherland, 1968, Baker, 1974; Baker and Duke, 1976; Roseghini et al., 1996, Benkendorff et al., 2000, 2001, 2004; Westley and Benkendorff, 2008). Furthermore, the process of encapsulation (Chapter 4) and the reproductive anatomy (Chapter 3) of this species has recently been described. Consequently, this investigation will address the origin of bioactive intermediates in the egg masses of *D. orbita*. Through the application of histochemical techniques for bromoperoxidase, tyrindoxyl sulphate and arylsulphatase, potential sites of tyrindoxyl sulphate and intermediate synthesis within the gonoduct will be determined. Liquid chromatography and mass spectrometry (LC-MS) will also be employed to establish the concentration and distribution of tyrindoxyl sulphate within encapsulated larval stages as a measure of

biosynthetic capacity and origin. Finally, larval anatomy will be examined for evidence of a hypobranchial gland and egg mass extracts will be analyzed by thin layer chromatography (TLC) to determine the presence and composition of choline esters. Through investigation into a maternal or embryonic source for egg mass natural products, it is hoped that further knowledge on the functional significance of Tyrian purple genesis in the Muricidae will be gained.

6.2 Methods and materials

6.2.1 Egg mass and specimen collection

D. orbita egg masses were collected during December, 2005 and 2006 from rocky intertidal platforms at Hallett Cove and jetty pylons at Brighton, South Australia. Care was taken to maintain basal membrane integrity to prevent the expulsion of encapsulated larvae. A total of 12 *D. orbita* specimens were collected from the rocky intertidal and subtidal regions of the metropolitan coast, Fleurieu and Eyre peninsulas of South Australia. To define periods of potentially heightened prochromogen synthesis and associated enzyme activity, three females representing each of four reproductive phases were collected over the annual cycle of 2006. These included 1) post-reproductive (March), 2) pre-reproductive (early July), 3) copulating (September), and 4) egg-laying (late November-December) females. As attempts to cryostat section capsules failed, egg-laying females were sampled directly from egg masses in the hope of obtaining capsule and embryo sections during formation within the female capsule gland.

6. 2. 2 Preparation of egg capsules and larvae

Three capsules from each egg mass were ruptured with a scalpel, and a wet-mount prepared of the encapsulated larvae. Larvae were examined under a compound light microscope (Olympus, BH-2), and the phase of intracapsular development established in accordance with Roller and Stickle (1988) and Romero et al. (2004). Anatomical observations and digital images were also taken to determine at what stage the hypobranchial gland becomes evident. This was also examined in paraffin sections of egg masses collected during 2005. A total of 12 capsules from three separately spawned egg masses representing stereoblastula, early veliger and veliger larval stages, were fixed in 10% neutral buffered formalin for 6hrs, dehydrated through an ethanol series, cleared in chloroform and embedded in paraffin.

Of the capsules collected in 2006, triplicate blastula, trochophore, early veliger and veliger hatchling egg masses were identified. To gain preliminary insight into the developmental stage and anatomical site of Tyrian purple synthesis, the larvae of one capsule from each egg mass was discharged into a petri-dish and set aside in ambient laboratory conditions. Observations of viability and pigmentation were made under a stereo-dissecting microscope (Olympus, SZH). To determine the presence and distribution of tyrindoxyl sulphate and choline esters, extracts of larvae, intracapsular fluid and egg capsule laminae were prepared separately. For each developmental stage, 5 egg capsules from each replicate mass were randomly selected, pierced with a scalpel and the larvae and intracapsular fluid removed by washing in a pre-weighed vial containing 1ml of distilled water sterilized by filtration (0.22 μ m). The liquid fraction, containing intracapsular fluid and distilled water, was

transferred to amber vials. After maceration, the encapsulated larvae, intracapsular fluid and empty capsules were extracted in 1ml dimethyl formamide (DMF, Sigma-Aldrich, 270547, HPLC Grade) for tyrindoxyl sulphate quantification. All samples were loaded onto a rotating platform to ensure adequate mixing of solvents and biological material during the 12h extraction period. Samples were sonicated (Galsonic Pty. Ltd. Vibron 08CD) for 5min before the solvent was removed with a pasture pipette and gravity filtered through glass wool. Extracts were evaporated overnight in a vacuum oven at 40°C, then weighed and re-dissolved in DMF to a concentration of 1mg/ml. Additional early veliger and veliger replicates were prepared in an identical manner, but extracted in absolute ethanol (Sigma-Aldrich, 459828, HPLC Grade) and concentrated under a stream of nitrogen gas for choline ester determination.

6. 2. 3 Biochemical analysis of egg mass extracts

Tyrindoxyl sulphate concentration was quantified by high performance-liquid chromatography (HPLC, Waters Alliance) couple to a mass spectrometer (MS, Micromass, Quatro microTM). HPLC separation was performed on a Phenomenex, Synergi, Hydro-RP C₁₈ column (250 x 4.6mm x 4µm) with parallel UV/Vis diode-array detection (DAD) at 300 and 600nm. The elution scheme employed a flow rate of 1ml/min of 0.1% formic acid and a gradient of acetonitrile in water starting at 30% for 1 min followed by 60% for 3 min, then 100% for 15min before returning to 30% for 15 min (Westley and Benkendorff, 2008). Tyrindoxyl sulphate was identified by the registration of major ions (m/z 338, 336) and fragment ions (m/z 240, 242; 224, 226) in electrospray ionization-mass spectrometry (ESI-MS) at a flow rate of

300 μ l/min (Westley and Benkendorff, 2008) and an injection volume of 20 μ l. Absolute prochromogen concentrations were obtained from the integrated peak area, calculated in the negative ion mode (ES-) using MassLynx 4.0 software.

Statistical analyses were performed in SPSS 14.0 for Microsoft Windows. Significant differences in prochromogen concentration with larval development and egg mass constituent (larvae, intracapsular fluid, capsule wall) were determined by a two-way ANOVA followed by a non-parametric Post Hoc Dunnett T3 analysis ($\alpha = 0.01$). Tyrindoxyl sulphate concentrations were $\log(x+1)$ transformed, but still failed to meet the assumption of the Levene Statistic for homogeneity of variances. Consequently, α was set at 0.01 to provide a more stringent threshold for significant differences.

Choline ester diversity was determined by TLC on aluminum-backed silica gel plates (Merck), employing an n-butanol-EtOH-acetic acid-water (8:2:1:3) solvent system (Roseghini et al., 1996). Dipping plates in Dragendorff Reagent (Fluka-44578) allows visualization of alkaloids and quaternary ammonium bases and has been used to detect choline esters in several muricid hypobranchial gland extracts (Roseghini et al., 1996). Development of yellow, rose or violet pigmentation in UV-active spots indicates the presence of senecierylcholine, murexine, and choline, respectively (Roseghini et al., 1996).

6. 2. 4 Preparation of adult tissue

The shell of each live 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 (0.22 μ m) seawater to reduce osmotic stress. The dorsal mantle and pallial gonoduct were separated from the rest of the visceral mass by an incision along the lateral margins of the columnar muscle. The mantle was then folded back and pinned with the ventral surface facing up. Longitudinal and transverse incisions were made along the junction between the ctenidium and branchial hypobranchial epithelium, and the ingesting and digestive glands, respectively. Integrity between the hypobranchial gland and gonoduct was maintained to allow histochemical examination the rectal gland and dorsal vascular sinus, as possible sites of bromoperoxidase acquisition and mobilization.

During the division of gonoduct tissue for cryostat embedding, the capsule gland of one egg-laying female was found to contain eggs and a near complete capsule. Embryos and intact capsule material expelled during incision of the capsule gland were successfully collected for independent sectioning. A second female also contained what appeared to be a small amorphous mass of capsule material in the anterior capsule gland, which along with the remaining capsule material from the first female, was sectioned within the lumen.

6.2.5 Histochemistry

Four serial transverse sections (5 μ m) from each egg mass replicate collected during 2005, were stained with Modified Harris Haematoxylin and Eosin Y with Phloxine B (Thompson, 1966), Toluidine Blue (Kramer and Windrum, 1954) and Periodic Acid Schiff (McManus, 1946) for morphological descriptions, mucopolysaccharide discrimination, and glycoprotein demonstration, respectively. The p-dimethylaminobenzaldehyde-nitrite method for tryptophan (Adams, 1957),

counterstained with nuclear fast red (C.I. 60760) was also applied as tryptophan sequestration is a prominent feature of muricid hypobranchial epithelium (Bolognani-Fantin and Ottaviani, 1981; Srilakshmi, 1991; Naegel and Aguilar-Cruz, 2006; Chapter 3).

A series of 15 transverse sections were obtained from each of five regions along the length of the gonoduct for each adult female specimen. The five regions included; 1) the anterior pallial gonoduct; 2) the medial capsule gland; 3) the posterior capsule gland; 4) the anterior ingesting gland; and 5) the posterior ingesting gland, which also contains the albumen gland. Post dissection, tissue was immediately fresh-frozen in O.C.T. compound (Tissue-Tek[®]) at -20°C, cryostat sectioned (15µm) and affixed to charged slides (ProSciTech, G311SF-W). Triplicate sections were stained with the post-coupling method for arylsulphatase (Rutenburg et al., 1952), the bromo-phenol red method for bromoperoxidase (Appendix II) and the acid-hydrolysis method for tyrindoxyl sulphate (Appendix III). Sections were also stained in Modified Harris Haematoxylin and Eosin Y with Phloxine B (Thompson 1966) and Toluidine Blue (Kramer and Windrum 1954). These stains were applied to assist with morphological descriptions where counterstaining was detrimental to enzymatic staining, and to allow biochemical comparisons with previously defined gonoduct secretions and capsule laminae. All egg mass and gonoduct sections were examined under a compound light microscope (Olympus, BH-2).

6.3 Results

6.3.1 Larval anatomy and biochemistry

Preliminary observations of stereoblastula, blastula, trochophore, early veliger and veliger larvae wet mounts failed to reveal the presence of a hypobranchial gland (Fig. 1). Similarly, secretory epithelia synonymous with that of an adult hypobranchial gland and intracellular tryptophan-positive structures were not detected within stereoblastula, early veliger and veliger tissue sections. However, extracellular yolk granules comprising the vitellus of larvae stained for high concentrations of tryptophan (Table 1). Yolk granules comprising the vitellus were observed to decline in number over the course of encapsulated development (Fig. 1), although a many were still evident just dorsal of the newly formed digestive gland at the veliger stage (Fig. 1). In addition, the inner (L3) and outer (L0) laminae of capsules and intracapsular fluid also stained for tryptophan.

Table 1. The distribution of precursors and biosynthetic enzymes for Tyrian purple precursor genesis within capsule laminae, intracapsular fluid and larval yolk granules. N = 2 for each capsule constituent (++, strong; +, weak; -, negative staining reaction).

Compound/enzyme	Capsule laminae					Intracapsular fluid	Yolk granules
	L0 ^δ	L1	L2	L3	L4		
Tryptophan	+	-	-	+	NA	++	++
Bromoperoxidase	NA	+	+	-	++	+	+
Tyrindoxyl	NA	-	+	-	-	+ [†]	+
Arylsulphatase	NA	+	+	-	-	-	+ [‡]

^δ L0 not present in the premature capsules examined. [†] Trace amounts detected by LCMS[‡].

Arylsulphatase staining was observed on the surface rather than within yolk granules.

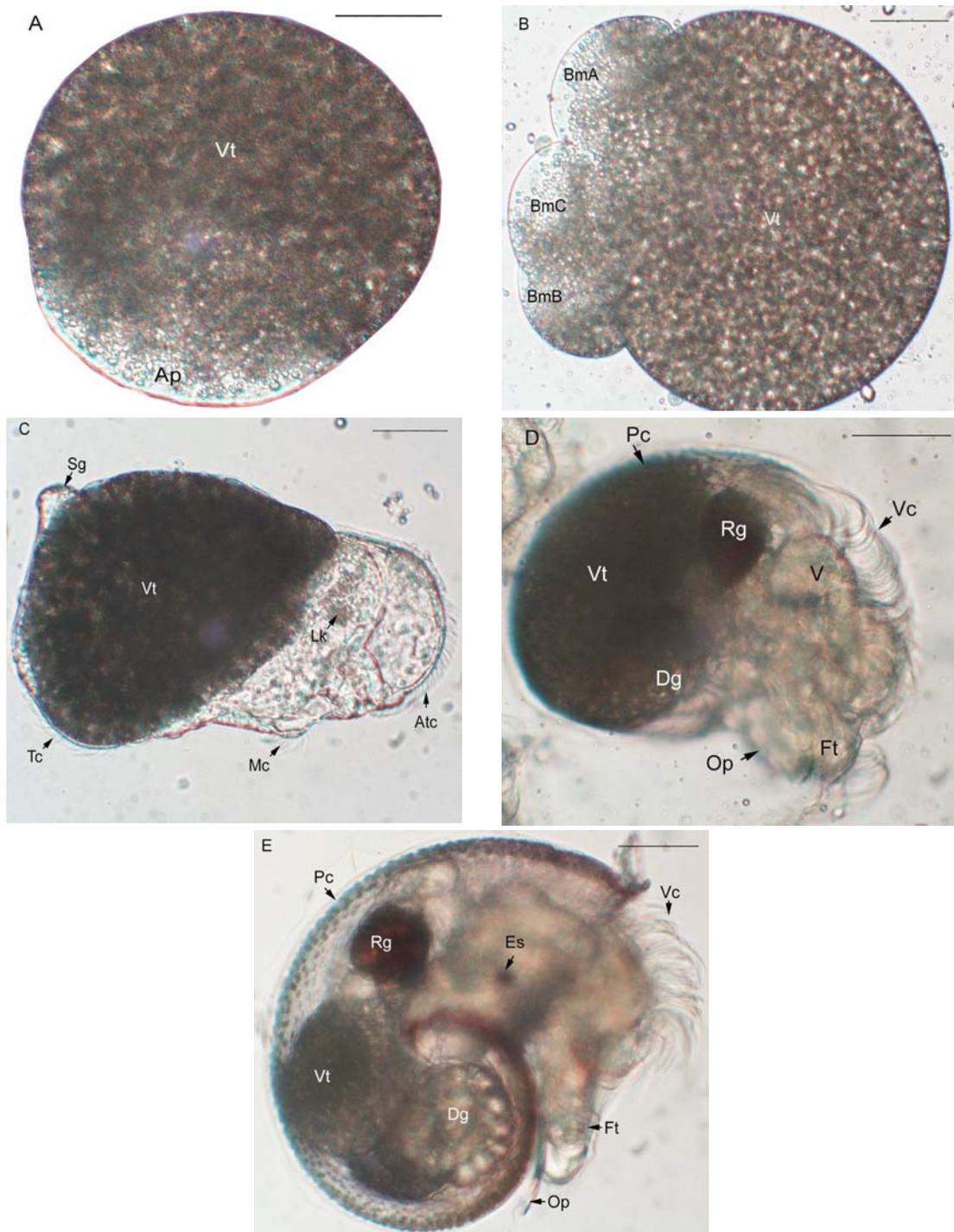


Figure 1. Wet mounts of encapsulated larvae over the course of development. A hypobranchial gland was not observed within (A) stereoblastula, (B) blastula, (C) trochophore, (D) early veliger, and (E) veliger larvae. Ap, animal pole; Atc, apical tuft cilia; Bm, blastomere; Dg, digestive gland; Es, eye spot; Ft, foot; Lk, larval kidney; Mc, metatrochal cilia; Op, operculum; Pc, protoconch; Rg, rectal gland; Tc, telotrochal cilia; V, velum; Vc, vellum cilia; Vt, vitellus (yolk). Scale bars = 100 μ m.

Encapsulated larvae set aside in ambient laboratory conditions developed purple pigmentation with a loss of viability. Pigmentation was localized to the vegetal pole of blastulas (Fig. 2a) and the vitellus of trochophores, while purple mucous additionally filled the mantle cavity and protoconch of early veligers and veligers (Fig. 2b).

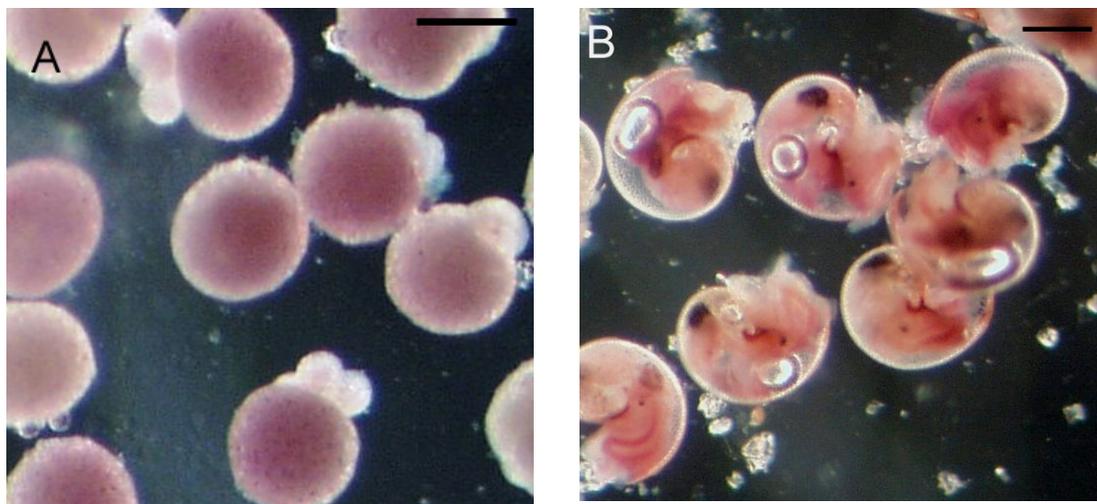


Figure 2. (A) Non-viable trochophores and (B) veliger hatchlings showing the purple pigmentation of vitellus and mantle cavity mucous, respectively. Scale bars = 500 μ m.

Tyrindoxyl sulphate was detected within all larval and capsule extracts through the registration of major ions (m/z 338, 336) and fragment ions (m/z 240, 242; 224, 226) in ESI mass spectrums. The highest concentrations of tyrindoxyl sulphate were detected in larvae, followed by capsule walls and intracapsular fluid, where only trace amounts could be detected in 58.33% of extracts (Fig. 3). Significant differences in prochromogen concentration with egg mass constituent were confirmed by a two-way ANOVA ($P < 0.001$, $F = 61.19$, $df = 2$). Post Hoc analysis revealed that tyrindoxyl sulphate concentrations were significantly higher in larval ($P < 0.001$) than capsule and intracapsular fluid extracts, and higher in capsule

($P < 0.001$) than intracapsular fluid extracts. Tyrindoxyl sulphate concentrations within larvae were observed to decline from blastula to veliger, while those within capsule wall extracts increased (Fig. 3). However, no significant interaction was detected between egg mass constituent and developmental stage ($P > 0.01$, $F = 0.66$, $df = 6$) or between tyrindoxyl sulphate concentration ($P > 0.01$, $F = 0.43$, $df = 3$).

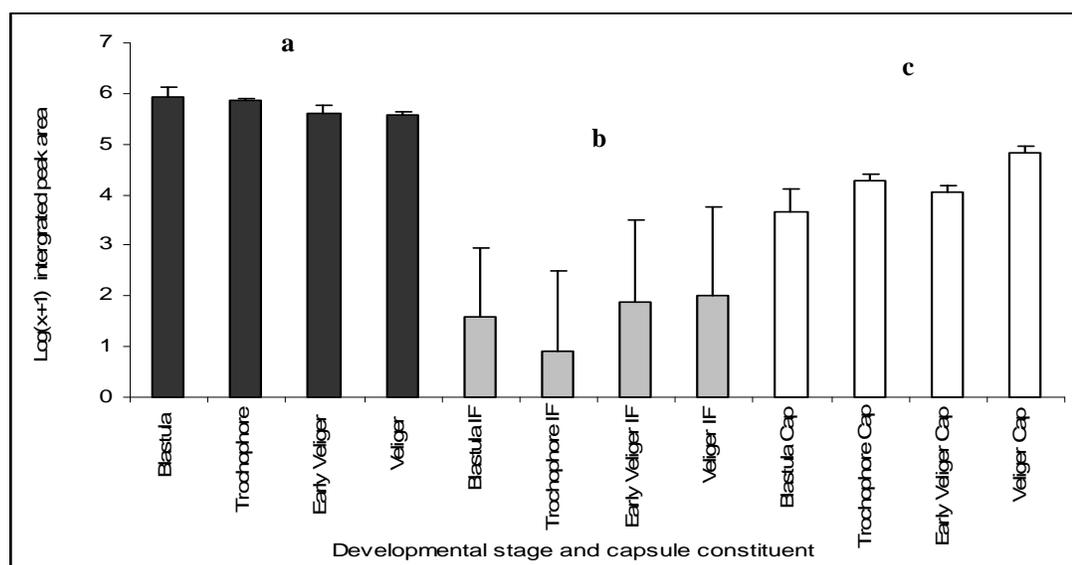


Figure 3. Tyrindoxyl sulphate concentration depicted as $\log(x+1)$ transformed integrated peak area in ES- obtained from DMF extracts (1mg/ml) of blastulas, trochophores, early veligers and veligers, corresponding intracapsular fluid fractions and capsules. $N=3$ for each developmental stage and capsule constituent. Error bars are \pm S. D. ■ = larval extracts; ■ = intracapsular fluid (IF) extracts; □ = capsule (cap) extracts. Letters indicate significant differences ($P < 0.001$).

Thin-layer chromatography of larval, intracapsular fluid and capsule ethanol extracts revealed two colourless UV-active spots, which developed rose (R_f 0.1) and violet (R_f 0.16) pigmentation after application of the Dragendorff reagent. These

staining reactions are indicative of murexine and choline, respectively (Roseghini et al., 1996).

6.3.2 Egg capsule and intracapsular fluid histochemistry

Cryostat sections of capsules within female capsule glands revealed weak bromophenol blue staining throughout the thick medial (L2) capsule lamina of the near complete capsule (Fig. 4a, Table 1). In contrast, intense staining was observed within the innermost eosinophilic lamina (L4), the vitellus of embryos and the intracapsular fluid (Fig. 4a, Table 1). Capsule material sectioned within the dorsal capsule gland lumen of the second female also stained strongly for bromoperoxidase activity. Application of the acid-hydrolysis method for tyridoxyl sulphate and the post-coupling method for arylsulphatase produced a feint purple (Fig. 4b) and red stain (Fig. 4b), respectively, within the L2 capsule lamina (Table 1). Distinct bands of feint purple and homogeneous red pigmentation were also observed within material remaining in capsule gland lumina of these respective females. In addition to capsule laminae, the vitellus of embryos also gained purple pigmentation for tyridoxyl sulphate (Fig. 4b), while only the surface of yolk granules developed red staining (Fig. 4c) for arylsulphatase (Table 1).

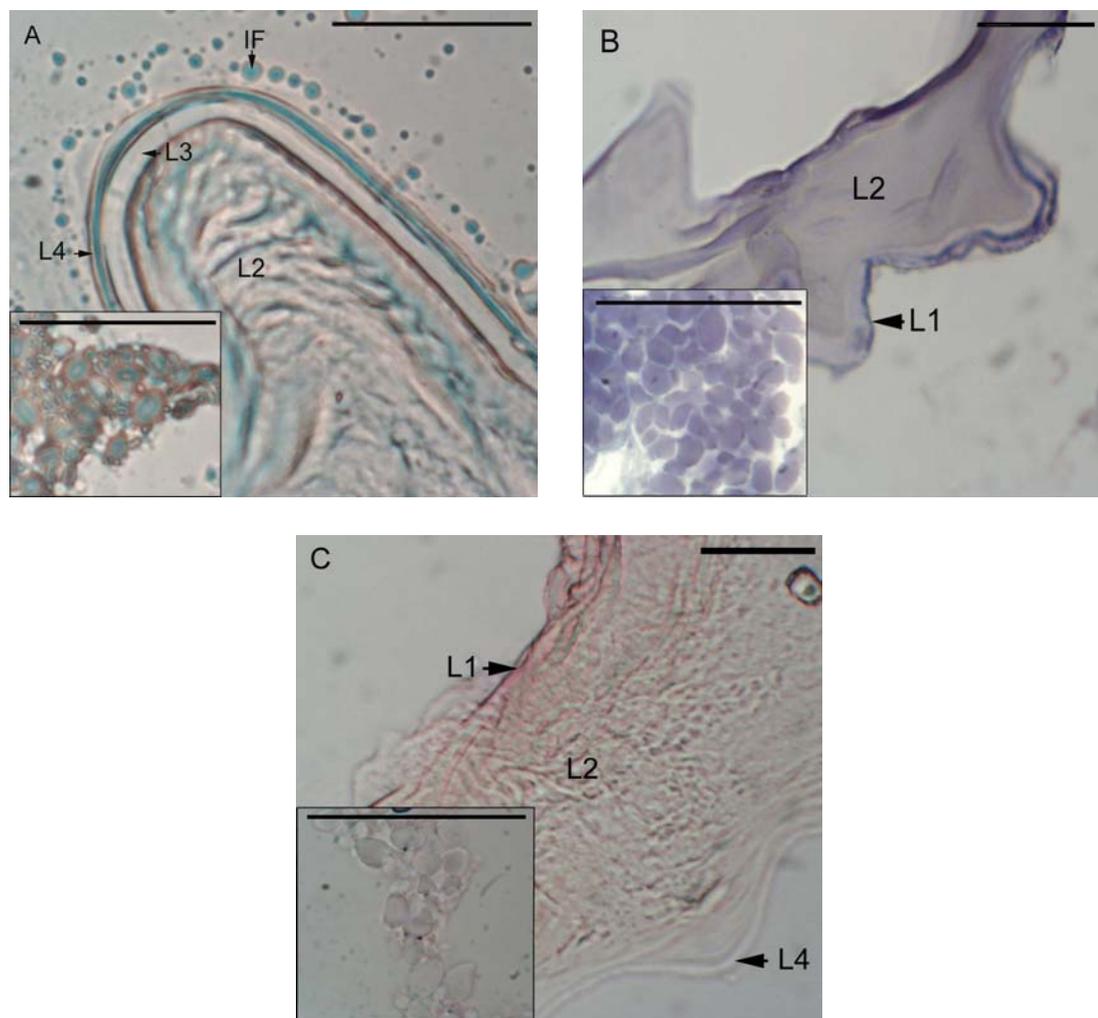


Figure 4. Transverse sections through the egg capsule wall stained with (A) the bromophenol-red method for bromoperoxidase, (B) acid-hydrolysis method for tyriodoxyl sulphate and (C) the post-coupling method for arylsulphatase showing positive capsule laminae. Inserts are of yolk granules comprising the vitellus of larvae. Scale bars = 50µm

6. 3. 3 Adult female gonoduct histochemistry

Sites of bromoperoxidase activity were identified within the capsule gland, the vascular sinus and rectal gland of *D. orbita* (Table 2). Feint bromophenol blue staining was observed amongst acini, epithelial cells and secretions within the capsule

gland of post-reproductive and egg-laying individuals (Table 2). Staining was observed within dorsal lobe acini in the anterior capsule gland (Fig. 5a), where the lobe extends ventrally to surround the vaginal opening. Weak staining for bromoperoxidase was also detected within proximal acini, epithelial cells and secretions of the lateral capsule gland lobes (Fig. 5b, Table 2). Activity was always restricted to the anterior portion of these lobes. Weak bromophenol blue staining was also noticed amongst acini and epithelial cells of the posterior capsule gland lobe on one occasion and within the ventral pedal gland epithelium (Fig. 5c) in two other individuals (Table 2). In addition to reproductive glands, a homogeneous material within the continuous subepithelial vascular sinus of the capsule, hypobranchial and rectal gland (Fig. 5d) displayed bromoperoxidase activity (Table 2). Furthermore, strong bromophenol blue staining was observed within rectal gland epithelial cells (Fig. 5d) in all egg laying specimens (Table 2). Unfortunately localization of bromoperoxidase within the albumen gland was not possible due to problematic sectioning of the posterior gonoduct combined with the diffuse nature of this staining method.

Table 2. The distribution of precursors and biosynthetic enzymes required for Tyrian purple intermediate genesis within the female pallial gonoduct and adjacent structures over the annual cycle. N = 3 for each reproductive phase.

Compound/ enzyme	Reproductive phase	Rectal gland	Vascular sinus	Albumen gland	Pedal glands	Capsule gland lobes			
						Dorsal	Lateral	Anteroventral	Posterior
Tryptophan *	Pre-reproductive	---	+++	+++	+++	-	-	+++	+++
	Copulating	---	+++	+++	+++	-	-	+++	+++
	Egg-laying	---	+++	+++	+++	-	-	+++	+++
	Post-reproductive	---	+++	+++	+++	-	-	+++	+++
Bromoperoxidase	Pre-reproductive	---	---	NA	+	-	-	-	-
	Copulating	---	---	NA	-	-	-	-	-
	Egg-laying	+++	++	NA	-	++	++	-	+
	Post-reproductive	---	---	NA	+	++	++	-	-
Tyrindoxyl sulphate	Pre-reproductive	---	++	---	-	---	---	-	-
	Copulating	+	+++	?	-	+	++	-	-
	Egg-laying	---	++	?	-	+	++	-	-
	Post-reproductive	+++	+++	?	-	---	+++	-	-
Arylsulphatase	Pre-reproductive	++	+++	+++	-	+++	+++	-	-
	Copulating	++	+++	+	-	+++	+++	-	-
	Egg-laying	+++	+++	+	+	+++	+++	-	-
	Post-reproductive	+	+++	+++	-	+++	+++	-	-

* Tryptophan distribution reproduced from Chapter 4.

+++ , present in three; ++ , present in two; + , present in one; - absent from all replicates; ? , questionable staining, NA, not available (tissue damage).

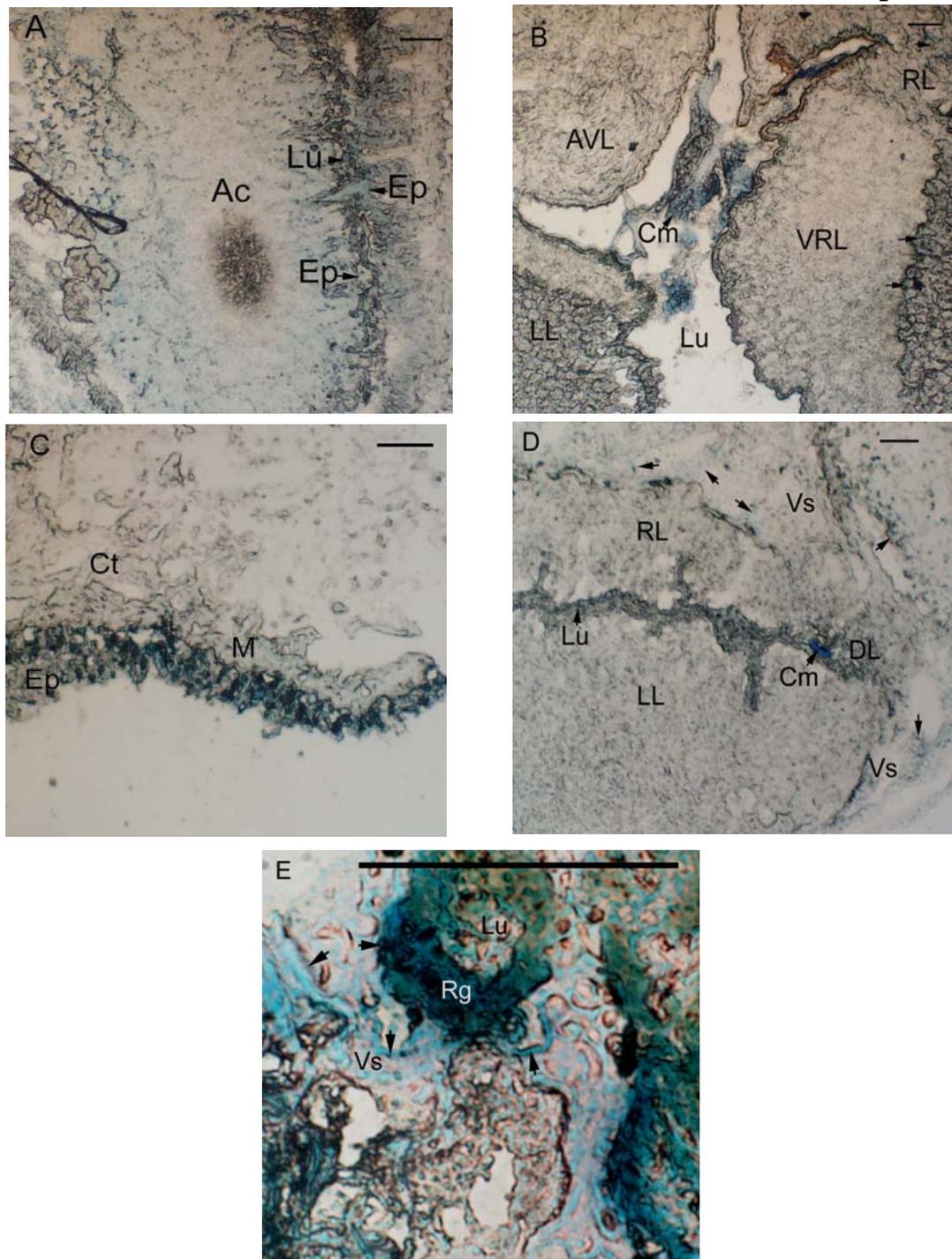


Figure 5. Transverse sections through the (A) anterior and (B) medial capsule gland, the (C) pedal gland, (D) the dorsal vascular sinus (Vs) of the capsule gland and the rectal gland (Rg) stained with the bromophenol-red method for bromoperoxidase (arrows). Ac, acini; AVL, anteroventral lobe; Cm, capsule material; DL, dorsal lobe; Ep, epithelium; LL, left lobe, Lu, lumen; M, muscle; RL, right lobe; VRL, ventral right lobe. Scale bars = 100 μ m.

Application of the acid-hydrolysis method identified sites of tyrindoxyl sulphate within the capsule gland, surrounding vascular sinus and possibly within the albumen gland (Table 2). Medial and anterior portions of lateral capsule gland lobes gained purple pigmentation (Fig. 6) in the majority of individuals (Table 2). Staining was localized to minute inclusions within proximal medial and all anterior acini (Fig. 6a). Columnar epithelial cells and secretions within the lumen also stained purple, and in the case of two egg-laying and post-reproductive females, pink crystals were observed within apical cilia and secretions immediately posterior of the vaginal opening, respectively (Fig. 6b).

Detection of tyrindoxyl sulphate within dorsal lobe acini was hindered by the use of haematoxylin as a counter stain. As acini of this lobe are basophilic, discrimination between purple pigmentation resulting from low prochromogen concentrations and haematoxylin staining was difficult discern. Nevertheless, dorsal lobe acini of two females gained purple pigmentation of visually increased concentration and intensity to control sections (Table 2). Purple staining was also observed within perivitelline secretions of the ventral albumen gland lobe within three reproductively distinct females (Table 2). However, the increase in purple pigmentation of inclusions in comparison to negative controls was negligible. In addition to reproductive structures, pink crystals were observed within the vascular sinus adjacent to the left (Fig. 6c) and dorsal capsule gland lobes and subepithelial vascular spaces of the medial, branchial and rectal hypobranchial epithelium (Table 2). Lastly, material within rectal gland lumina of all post-reproductive and one copulating female (Table 2) also developed a pink crystalline appearance (Fig. 6d) after application of the acid-hydrolysis method.

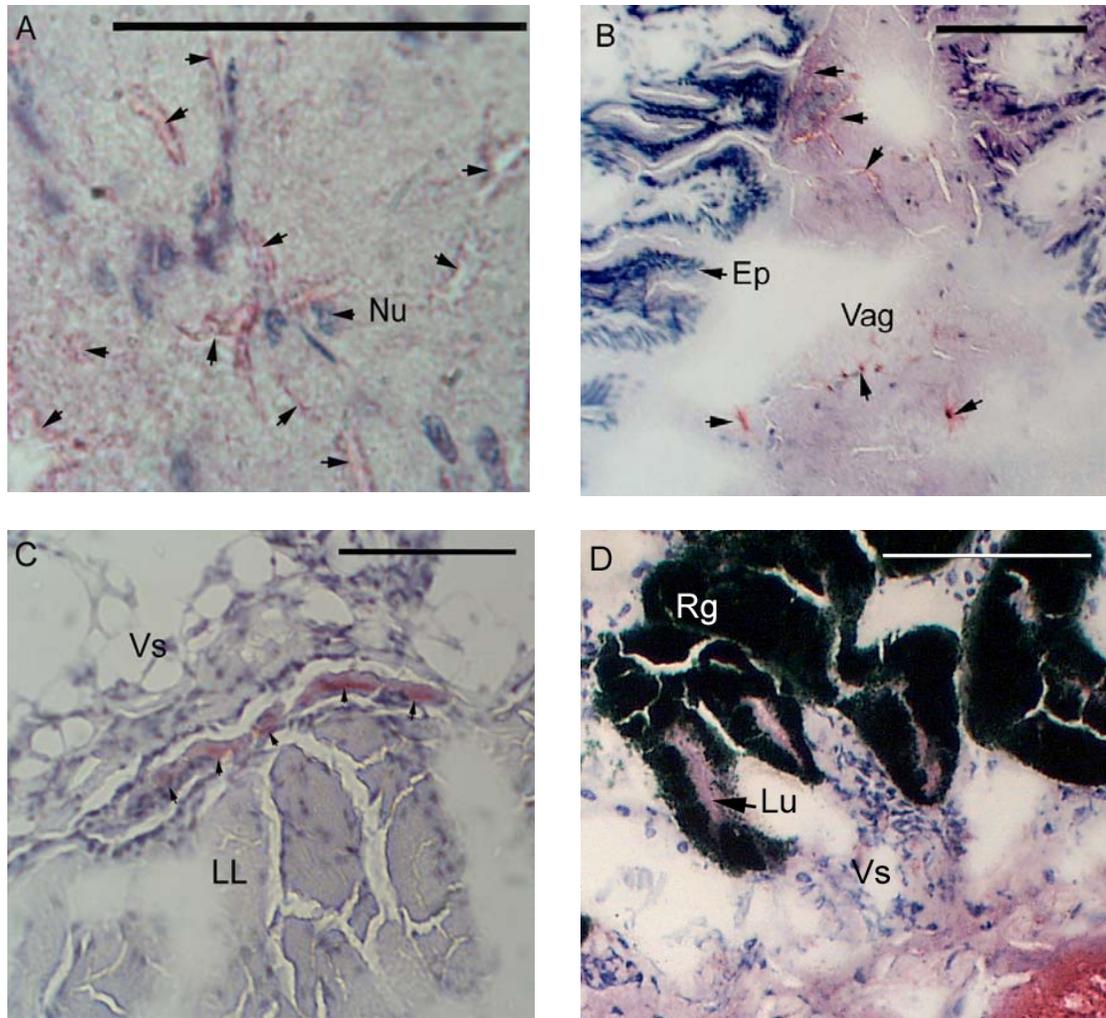


Figure 6. Transverse sections of the (A-C) capsule gland and (D) rectal gland (Rg) stained with the acid-hydrolysis method showing tyrindoxyl sulphate-positive (arrows) inclusions within (A) capsule gland acini, (B) secretions in the vagina (Vag), (C) the capsule gland vascular sinus (Vs), (D) rectal gland lumina (Lu) and the rectal-hypobranchial gland subepithelial vascular sinus. Ep, epithelium; LL, left lobe; Nu, nucleus. Scale bars = 50 μ m (A) and 100 μ m (B-D).

Arylsulphatase was invariably detected within the dorsal and lateral capsule gland lobes of *D. orbita* (Table 2), although the concentration and staining reaction varied considerably. Isolated inclusions within dorsal lobe acini stained red in all pre-

reproductive and one copulating female, while blue azo dye deposits were observed in the remaining copulating individuals. Inclusions within the dorsal lobe of all egg-laying females developed blue pigmentation (Fig. 7a), although the concentration of positive sites remained comparatively low. In contrast, dorsal lobe acini of all post-reproductive females gained an intense blue stain throughout (Fig. 7b). Secretions within the dorsal lumen stained in an identical manner to adjacent acini. Inclusions within acini of the lateral lobes typically developed red pigmentation after application of the post-coupling method for arylsulphatase (Fig. 7c), although purple staining was evident in some females. The number of arylsulphatase-positive inclusions within lateral capsule gland lobes was low in comparison to those of dorsal lobe secretions. Secretions within the dorsoventral lumen between these lobes stained similarly to lateral acini, although enzyme concentration was noticeably elevated.

Albumen gland acini were positive for arylsulphatase (Fig. 7d) and staining reactions appeared to be correlated with those of the dorsal capsule gland lobe for each individual. The cytoplasmic contents of various pedal gland epithelial cells in a female with a capsule under manufacture, also gained dark blue pigmentation (Table 2). Apart from the gonoduct, many subepithelial vascular spaces contained a homogeneous substance, which produced a weak purple stain (Fig. 7e, Table 2). Granules in the lumina of rectal gland acini of five reproductively distinct females also gained purple pigmentation and in the case of egg-laying females, cytoplasmic pigment granules stained bright blue (Fig. 7e, Table 2).

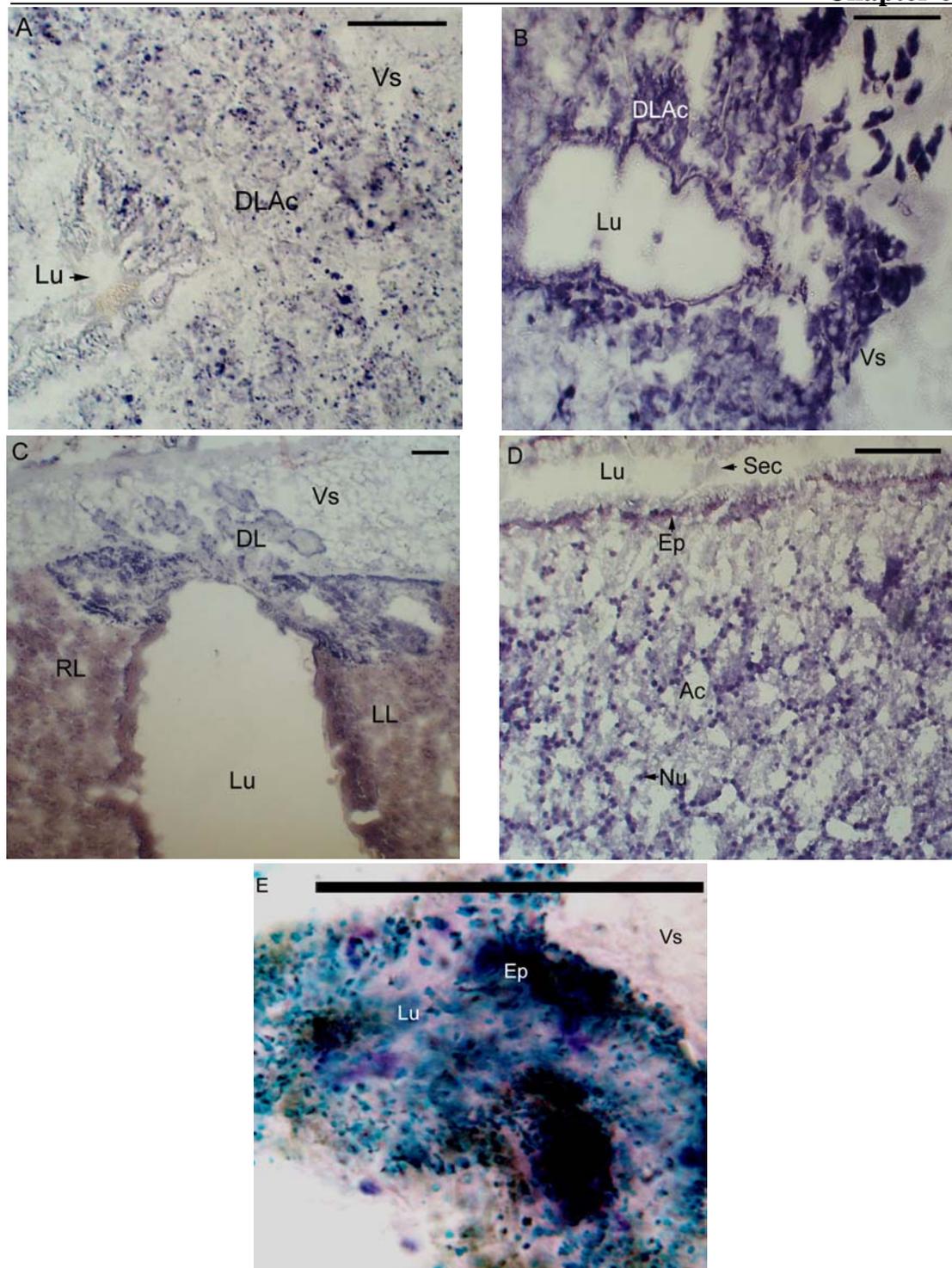


Figure 7. Transverse sections of the (A-C) capsule, (D) albumen and (E) rectal gland stained for arylsulphatase. Differences in dorsal lobe (DL) enzyme concentration between (A) egg-laying and (B) post-reproductive females are shown, along with the (C) low enzyme activity (red) of left (LL) and right (RL) lateral lobe secretions, (D) the high activity of albumen gland sections (Sec) and (E) the strong and weak staining of rectal gland pigment granules and material within the vascular sinus (Vs), respectively. Ac, acini; Ep, epithelium; Lu, lumen; Nu, nucleus. Scale bars = 100 μ m (A-D) and 50 μ m (E).

6.4 Discussion

The combined application of liquid chromatography-mass spectrometry (LC-MS) and histochemistry has enabled detection of the primary precursor, prochromogen and biosynthetic enzymes required for bioactive indole synthesis within the egg masses and gonoduct of *D. orbita*. LC-MS analysis of egg capsules, intracapsular fluid and larvae has provided a quantitative comparison, complementary to tyrindoxyl sulphate localization by histochemical methods. Histochemical information on the distribution of biosynthetic components within the female gonoduct has also identified potential sites of prochromogen synthesis and mechanisms of incorporating precursors into egg capsules for secondary metabolite synthesis.

LC-MS revealed the presence of tyrindoxyl sulphate within muricid egg capsules for the first time. This suggests that bioactive indoles are generated within the capsule, rather than introduced in their hydrolyzed bioactive form during encapsulation. Significantly greater concentrations ($P < 0.001$) of tyrindoxyl sulphate within larval extracts in comparison to those of intracapsular fluid and capsules (Fig. 3), coupled with the purple pigmentation of yolk granules after acid hydrolysis (Fig. 4b), implies that the prochromogen is primarily associated with larval vitellus. Examination of stereoblastula, blastula, trochophore, early veliger and veliger wet mounts failed to reveal the presence of a hypobranchial gland and therefore a capacity to *de novo* synthesize brominated indole precursors. Furthermore, tryptophan sequestration within secretory epithelial cells was not detected during histochemical analysis of larval sections. Declines in larval prochromogen concentration (Fig. 3) and vitelline content (Fig. 1) during encapsulated development indicate that

tyrindoxyl sulphate in larval vitellus is metabolized over the course of encapsulated development. These findings imply that bioactive intermediates within the egg masses of *D. orbita* are produced from biosynthetic components introduced from a maternal source.

The coincidence of tryptophan and bromoperoxidase suggests that prochromogen synthesis may actually occur within yolk granules of *D. orbita* larvae. Furthermore, histochemical analysis revealed the presence of bromoperoxidase and tryptophan within subepithelial vascular spaces of the gonoduct in egg-laying females (Table 2). Although muricid yolk proteins are synthesized within follicle cells or the oocyte (Amor et al., 2004), the vitellin precursor vitellogenin in other molluscs (Suzuki et al., 1992; Eckelbarger and Young, 1997) and arthropods (Pal and Hodgson, 2002) is acquired from the haemolymph via receptor-mediated endocytosis. Thus it is possible that essential amino acids and biosynthetic enzymes are also endocytosed from the haemolymph for incorporation into yolk granules in the Muricidae. Overall these findings suggest that tyrindoxyl sulphate is synthesized within the vitellus of *D. orbita* larvae from biosynthetic components introduced during yolk granule formation in the maternal ovary.

Observations of Tyrian purple production within the vitellus of non-viable larvae indicate that arylsulphatase is also incorporated during ovarian yolk granule synthesis. Histochemical analyses of embryos within a near complete capsule indicate that arylsulphatase occurs on the yolk granule surface (Table 1). Arylsulphatase has previously been detected within the ooplasm of starfish, where it is thought to aid in the conversion of yolk granules to multivesicular bodies (Aisenshtadt and Vassetzky,

1986). Thus, it is possible this enzyme has evolved a secondary role to yolk catabolism, in facilitating bioactive intermediate synthesis in the Muricidae.

Correlations between Tyrian purple evolution and larval mortality imply that the prochromogen is stored separately from arylsulphatase to prevent spontaneous dye synthesis within viable larvae. As arylsulphatase was restricted to the surface of yolk granules (Fig. 4c), the integrity of the plasma membrane must be degraded in dying larvae by lysosomal enzymes released during autolysis (Moore, 1994), to unite arylsulphatase and tyrindoxyl sulphate. In viable larvae, the prochromogen must be actively transferred to the yolk exterior to facilitate bioactive intermediate synthesis. Research on the mechanisms facilitating yolk exploitation in insect embryos suggests that cleavage products are divided into discrete granules and transported across the plasma membrane by vitellophages (Fausto et al., 1994, 2001). Thus, it is possible that tyrindoxyl sulphate is transported to the yolk surface in a similar manner, which would theoretically regulate prochromogen hydrolysis by arylsulphatase and subsequent natural product genesis in muricid larvae.

Although not histochemically detectable (Table 2), LC-MS revealed trace amounts of tyrindoxyl sulphate within the intracapsular fluid (Fig. 3) of >50% of capsules sampled. Intracapsular fluid is secreted by the posterior capsule gland lobe in *D. orbita*, which is also known to function in tryptophan sequestration (Chapter 4). Bromoperoxidase was observed amongst acini and epithelial cells of this lobe in one egg-laying female and the intracapsular fluid of a partially formed capsule (Table 2). Consequently, seasonal secretion of these biosynthetic components may facilitate prochromogen synthesis within the intracapsular fluid of *D. orbita* capsules. Although albumen secretions do not directly contribute to the intracapsular fluid of

D. orbita capsules (Chapter 4), the perivitelline surrounding embryos may ultimately combine with intracapsular fluid. Histochemical detection of the prochromogen in albumen gland sections was questionable, which may be due to low concentrations as proposed for capsule sections (Table 2). Nevertheless, tyrindoxyl sulphate has previously been identified within albumen gland extracts by LC-MS (Westley and Benkendorff, 2008). Thus, perivitelline secretions may in part be responsible for the presence of tyrindoxyl sulphate within intracapsular fluid. Furthermore, these secretions also stained for arylsulphatase (Table 2), which was of higher activity in breeding, egg-laying and immediately post-reproductive females than pre-reproductive individuals. Thus, the coincidence of these biosynthetic components should promote some level of constitutive bioactive intermediate synthesis within the intracapsular fluid and perivitelline surrounding early larval stages.

Over the course of development, intracapsular fluid is thought to be consumed by muricid larvae and replaced by seawater (Roller and Stickle, 1988; Middelfart, 1993). However, prochromogen concentrations in blastula capsules failed to vary significantly ($P > 0.01$) from those containing veligers (Fig. 3). As yolk cleavage products are known to enter the perivitelline fluid via transcytosis across the serosa membrane in insect embryos (Fausto et al., 2001), it is possible that prochromogen within yolk granules supplements intracapsular fluid reserves. Overall, this mechanism may be responsible for the maintenance of constant tyrindoxyl sulphate concentrations and hence, the potential for bioactive intermediate synthesis within the intracapsular fluid.

Tyrindoxyl sulphate within the capsule wall (Fig. 4b) appears to originate from several regions within the maternal capsule gland. Like many Muricidae

(D'Asaro, 1988), the capsules of *D. orbita* are composed of four structural lamina, (L4-L1) and an additional non-structural surface lamina (L0). Purple staining indicating low prochromogen concentrations were observed within the middle capsule lamina (L2) of near-complete capsule sections (Table 2). Similar staining was also observed within lateral capsule gland lobes (Table 2), which are known to function in the secretion of L2 in *D. orbita* capsules (Chapter 4). Furthermore, pink crystals indicative of high prochromogen concentrations were present within epithelial cilia and secretions immediately posterior of the vaginal opening in egg-laying and post-reproductive females. These findings not only expand on previous reports of tyrindoxyl sulphate within capsule gland extracts (Westley and Benkendorff, 2008), but suggest that the prochromogen is concentrated upon exocytosis from lateral capsule gland acini and incorporated into the middle capsule lamina.

Prochromogen concentration within the capsule wall was observed to increase over the course of intracapsular development (Fig. 3). This is unusual as muricid capsules degrade over time (Roller and Stickle, 1988; Lim et al., 2007; Chapter 4). Ultrastructural examination of mature *D. orbita* capsules has previously documented the presence of unidentified droplets ranging from 8-20 μ m on the capsule surface (Lim et al., 2007). The morphology of these droplets is remarkably similar to that of the yolk granules described in this and previous investigations (Chapter 4). At later stages of larval development, the loosely woven fibrous middle lamina is exposed to the environment due to prior shedding of L0 and L1 (Lim et al., 2007; Chapter 4). Thus, it is possible that yolk granules pass through this lamina to the exterior and

accumulate on the surface, which would explain the observed increase in prochromogen concentration in mature capsule extracts.

Although application of the acid-hydrolysis method failed to demonstrate tyridoxyl sulphate presence within any other capsule laminae (Table 2), synthesis from tryptophan may be possible within other regions of the capsule. Bromoperoxidase was demonstrated within L1, L2 and L4, as well as the dorsal and lateral capsule gland lobes (Table 2), which secrete these laminae (Chapter 4). Detection of this enzyme with pedal gland secretions (Table 2) further suggests that tryptophan is incorporated into L0 during pedal molding (Sullivan and Mangel, 1984; Chapter 4). As L1, L2 and L4 border L0 and L3, which are known to contain tryptophan (Table 1), the spatial association of these laminae may facilitate prochromogen synthesis within or at the interface depending on the potential for interlaminae diffusion.

Low activity arylsulphatase was demonstrated within L2 and inclusions of lateral lobe secretions (Table 2), which suggests that this enzyme is introduced to *D. orbita* capsules in unison with the prochromogen. Arylsulphatase was also histochemically demonstrated within the dorsal capsule gland lobe and L1, which is secreted by these acini. The ventral pedal gland of one individual also stained for high arylsulphatase activity, which further suggests that L0 may contain this enzyme. Theoretically, this would provide a means for bioactive intermediate synthesis within L2, L0 and possibly between L1 and adjoining laminae.

The evidence presented in this investigation strongly indicates that tryptophan and the biosynthetic components required for prochromogen and bioactive precursor synthesis within capsule laminae are of material origin. However, it is unclear

whether the prochromogen is actually synthesized within the gonoduct or acquired from the adjacent haemocoel. Although histochemical analysis of the female gonoduct revealed several sites of prochromogen, bromoperoxidase and arylsulphatase storage (Table 2), regions of tyrindoxyl sulphate failed to coincide with those of bromoperoxidase and previously identified sites of tryptophan sequestration (Chapter 4). This suggests that prochromogen evolution requires the combining of biosynthetic components from discrete regions, which may provide a regulatory mechanism for the genesis of bioactive precursors. Tyrindoxyl sulphate and bromoperoxidase were detected within lateral and dorsal capsule gland lobe (Table 2). However, tryptophan is known to be restricted to the posterior and anteroventral lobes (Chapter 4). Detection of bromoperoxidase, tyrindoxyl sulphate (Table 2) and tryptophan (Chapter 4) in capsule gland subepithelial vascular spaces implies the potential for prochromogen synthesis within this vascular sinus. Similarly, crystals were also observed within vascular spaces (Table 2) adjacent to the left lateral and dorsal capsule gland lobes in this investigation. Synthesis within subepithelial vascular spaces of the hypobranchial gland has been previously demonstrated by the formation of pink crystals indicating high concentrations of tyrindoxyl sulphate (Chapter 5). Thus, endocytosis of tyrindoxyl sulphate by capsule gland acini from the subepithelial vascular sinus is possible.

Alternatively, tryptophan may be acquired from the vascular sinus for prochromogen synthesis within lateral lobes. The rectal gland commences and terminates with the capsule and medial hypobranchial gland where prochromogen synthesis is readily observed (Chapter 5). Due to the presence of tyrosine deposits within rectal gland epithelial cells, it has been suggested that haemolymph

macromolecules are catabolized within this gland (Andrews, 1992). As the respiratory pigment haemocyanin contains tryptophan residues (Waxman, 1975; Avissar et al., 1986), it is possible that the amino acid is liberated into the vascular sinus for tyrindoxyl sulphate synthesis within the adjacent capsule gland. Furthermore, cytoplasmic granules within rectal gland epithelial cells stained bright blue for high arylsulphatase activity within females engaged in capsule manufacture. Detection of this enzyme is most likely due to the predominance of lysosomes, which facilitate polypeptide degradation (Andrews, 1992). Elevations in enzyme activity during this period may indicate an increased demand for tryptophan to enable prochromogen synthesis within the gonoduct prior to incorporation into capsule laminae. It has also been suggested that bromoperoxidase may originate from the rectal gland (Chapter 5). Bromoperoxidase activity was heightened within the rectal glands of all egg-laying females in this investigation and occurred within the subepithelial vascular sinus of the capsule gland (Table 2). Together, these findings suggest that supplementary tryptophan and bromoperoxidase may be sourced from the rectal gland and mobilized in the haemolymph for prochromogen synthesis within or adjacent to the capsule gland. Similar modes of tyrindoxyl sulphate synthesis may occur within and surrounding the albumen gland, although supporting evidence could not be obtained due problematic cryostat sectioning and staining within the posterior gonoduct.

The distribution of tyrindoxyl sulphate and arylsulphatase within capsule lamina coupled with the antimicrobial activity of intermediate precursors, strongly suggests a function in the protection of muricid larvae against pathogen infection. Furthermore, the inclusion of tryptophan and bromoperoxidase appears to ensure

prochromogen availability for continued natural product synthesis from oviposition through to hatching. The primary line of protection for developing larvae against potentially harmful microbes is the physical presence of a multi-laminated capsule (Pechenik et al., 1984; Lord et al., 1986; Garrido and Gallardo, 1993, Rawlings, 1995). However the two outer laminae (L0-L1) of *D. orbita* capsules sequentially delaminate in response to biofilm formation, presumably to prevent influx of toxic microbial metabolites (Lim et al., 2007) and maintain gas exchange (Chapter 4). In comparison to the bacterial loading of chemically undefended cephalopod capsules, those of *D. orbita* remain relatively free of microbes until just prior to these shedding events (Lim et al., 2007). Thus, it appears that the eventual proliferation of fouling organisms and subsequent delamination may reflect the exhaustion of chemical defence mechanisms within a particular lamina.

Prochromogen and subsequent cytotoxic intermediate synthesis within L0 is likely to prevent initial bacterial colonization. However, once amino acid and prochromogen reserves become depleted on the L0 surface, the biosynthetic pathway becomes suppressed, which may trigger delamination. Although tyrindoxyl sulphate is absent from the forthcoming L1, the potential for prochromogen and intermediate synthesis at the L2-L1 interface (Table 2) would prevent microbial invasion into the capsule interior. Furthermore, L1 microtopography is unfavorable for bacterial attachment (Lim et al., 2007), which may supersede the need for chemical defenses until the physical structure degrades. Subsequent delamination of L1 would restore chemical defence mechanisms during the final weeks of larval development as L2 is laden with tyrindoxyl sulphate, arylsulphatase and the biosynthetic components for supplementary synthesis (Table 2). In combination with various physical processes,

the division of precursors and enzymes between capsule laminae appears to limit microbial settlement and prolong the effectiveness of this intricate defence mechanism.

Intracapsular fluid and larval vitellus are usually considered to be of nutritional value alone (Roller and Stickle, 1988; Middelfart, 1993; Naegel, 2004). However, previous research into the pulmonate gastropod, *Biomphalaria glabrata* has shown that precursors to the prophenoloxidase immune response are transferred from the albumen gland to the perivitelline fluid of eggs (Bai et al., 1996), which may provide antimicrobial defence during development. Similarly, the results of this investigation suggest that precursors to bioactive indoles are also incorporated into muricid eggs and intracapsular fluid from a maternal source. Previous investigations have shown the contents of capsules from the muricid, *Nucella lapillus* to be axenic (Lord et al., 1986). However, the presence of yolk granules on the surface of *D. orbita* capsules suggests that passage to the interior through fissures in the L2 matrix is possible. Although, the intracapsular fluid of *D. orbita* capsules contains the biosynthetic components for prochromogen synthesis, arylsulphatase and therefore the potential for bioactive indole synthesis, is lacking. As some marine bacteria are known to contain arylsulphatase (Dodgson et al., 1954; Barbeyron et al., 1995), pathogens which penetrate L2 or reach the capsule interior, may inadvertently instigate local prochromogen hydrolysis. Furthermore, the transport of intermediates generated within larval vitellus to intracapsular fluid may act as a last line of antimicrobial defence.

The presence of components for sustained prochromogen and bioactive intermediate synthesis in larval vitellus suggest yolk granules may provide for the

chemical defence of larvae prior to settlement and the formation of a functional hypobranchial gland. The presence of yolk granules in *D. orbita* veliger hatchlings has previously been suggested to provide nourishment during the 20day pelagic life of this species (Phillips, 1969). However, as these veligers are planktotrophic (Phillips, 1969), it is possible that prochromogen laden granules are not intended solely for nutrition. Alternatively they may be retained to combat bacteria internalized during feeding or respiration, whereby pathogen entry to the mantle cavity elicits bioactive indole synthesis via the transcytosis of yolk prochromogen to the plasma membrane where arylsulphatase occurs. *D. orbita* invests considerable metabolic energy in the chemical defence of encapsulated larvae and the maternal provision of defensive metabolites ensures there is no trade-off between growth and defence for veliger larvae (Lindquist, 2002). Thus, it would be unusual for veligers to forfeit the selective advantage bioactive yolk constituents may confer whilst in the plankton.

In addition to defence against pathogens, this biosynthetic pathway may also reduce predation during encapsulation and vulnerable pelagic life stages. Murexine and choline also occur in larvae, intracapsular fluid and capsule laminae. The presence of choline within these constituents is partly expected, as choline is a major source of methyl-groups for amino acid synthesis and is essential to trans-membrane cell signaling, lipid transport and metabolism (Zeisel and Blusztajn, 1994). However, the significance of murexine to ontogenesis is less obvious. Murexine displays neuromuscular blocking activity (see Roseghini et al., 1996) and is liberated during prochromogen hydrolysis by arylsulphatase. As autolysis may also induce hydrolysis, it is possible that murexine within encapsulated and pelagic larvae functions in

defence against predators. Once taken by a predator, stress or death induced liberation of paralyzing murexine may deter predators capable of learned aversion or elicit decreased fitness in predators incapable of such learned recognition (Lindquist and Hay, 1995). This would ultimately drive selection towards predators that recognize and reject muricid egg masses or veligers, therefore increasing larval survival.

The Muricidae have diversified extensively in the rocky intertidal and are important trophic members throughout the world (Taylor, 1976). It seems possible that the proliferation of this family is due in part, to the chemical defence of larvae by maternal secondary metabolites. Bioactive Tyrian purple precursors have been detected in the egg masses of muricid species across three subfamilies (Benkendorff et al., 2001, 2004), which suggests that larval chemical defence may be generic to the Muricidae. The results of this investigation imply that Tyrian purple prochromogens and the biosynthetic components for intermediate and supplementary prochromogen synthesis are introduced to egg masses from a maternal source. However, it is unknown whether maternal investment in chemical defence is also a common phenomenon. Only one other attempt has been made to determine whether Tyrian purple prochromogens and arylsulphatase occur within the muricid gonoduct. On this occasion, neither was detected within gonoduct extracts of *Murex trunculus* and *M. brandaris* (Erspamer, 1946). However quantification through the exposure of crude extracts to sunlight may not yield a visible colour change if prochromogen concentrations are as low as those detected in the capsule and albumen glands of *D. orbita*.

Research on muricid egg masses suggest that precursor distribution and synthesis may vary with subfamily or developmental mode. In contrast to Rapaninae

species such as *D. orbita*, tryptophan is absent from the larval vitellus and intracapsular fluid of *Nucella lapillus* (Bayne, 1968) and *Ocenebra erinacea* (Hawkins and Hutchinson, 1988), which belong to the Ocenebrinae subfamily. Although the prochromogen may still be incorporated from a maternal source, the absence of tryptophan would prevent supplementary prochromogen synthesis. This may explain the lower concentration of Tyrian purple precursors in Ocenebrinae capsules in comparison to the Rapaninae (Benkendorff et al., 2001) and provide evidence for an evolutionary divergence in chemical defence investments. Alternatively, *N. lapillus* and *O. erinacea* are both direct developing species (Spight, 1976). Consequently, larvae emerge from the capsule as metamorphosed juveniles, presumably with a functional hypobranchial capable of dietary tryptophan sequestration. Thus, the absence of a planktonic phase may reduce the need for passive prochromogen synthesis within vitelline reserves. Furthermore, the capsules of direct developing species are typically thicker than planktotrophic species (Chapter 4), which may eliminate the need for bioactive indole synthesis in the intracapsular fluid as pathogens are less likely to penetrate the interior.

Despite the lack of evidence for the presence of Tyrian purple precursors in gonoduct tissues, the evolution of caenogastropod gonoducts from an ancestral right hypobranchial gland (Kay et al., 1998) suggests that a retained capacity for bioactive indole synthesis in the muricid gonoduct is likely. Egg masses of the Littorinidae, Naticidae, Ranellidae, Mitridae, Conidae, (Benkendorff et al., 2001), Cassidae, Cypraeidae, and Buccinidae (Santhana Ramasamy and Murugan, 2005) also display antimicrobial activity. Furthermore, hypobranchial gland secretions of Volutidae (Weaver and Du Port, 1970) and Olividae (Marcus and Marcus, 1959) neogastropods

are also purple, and members of the Olividae have chemically defended egg masses (Santhana Ramasamy and Murugan, 2005). Thus, it is possible that hypobranchial gland natural product synthesis also occurs in the gonoducts of these related families. Further investigation into potential correlations in caenogastropod hypobranchial, gonoduct, egg mass and larval secondary metabolite composition may reveal an interesting ancestral linkage driving the success of this diverse superorder.

6.5 Conclusion

The distribution of precursors and biosynthetic enzymes within *D. orbita* capsules appears capable of defending larvae during encapsulated and planktonic development. Chemical defence is an important determinant in adult benthic invertebrate survival (Lindquist, 2002). However, due to the small size and handling difficulties of invertebrate larvae, little is known on the storage and synthesis of secondary metabolites and hence, chemical defence in early life stages (Lindquist, 2002). Reports of marine invertebrates with chemically defended eggs and larvae have only recently begun to accumulate (reviewed by Lindquist, 2002). This is the first account of chemical defenses within shelled gastropod larvae, and the first comparison of secondary metabolites across all life stages in this subclass. The combined use of histochemical and LC-MS has not only identified sites of secondary metabolite storage, but the origin and division of essential biosynthetic components and changes in natural product distribution and concentration with larval development. Together these findings provide insight into the ecological significance of Tyrian purple genesis in the Muricidae and the factors operating to sustain such a complex chemical defence mechanism from embryo to juvenile. Overall, it is hoped

that this approach will prove beneficial for future investigations into the contribution of secondary metabolites to reproductive and life history strategies in marine invertebrates.

Acknowledgments

I would like to thank Ms. M. Lewis for technical advice throughout histochemical analyses, Mr. W. Nobel and Miss A. Glavinic for assistance during field collections, and Mr. D. Jardine for assistance with LC-MS analyses. Many thanks are due to Dr. K. Benkendorff for assistance with analyses and valuable feedback on the manuscript. The provision of a Flinders University Postgraduate Scholarship is greatly appreciated. This research was supported by a Philanthropic research grant to Dr. K. Benkendorff.