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Observations on the production of purple pigments in the egg capsules, hypobranchial and reproductive glands from seven species of Muricidae (Gastropoda: Mollusca).

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SUMMARY

Tyrian purple is a well-known colourant that can be obtained from the hypobranchial glands of Muricids. Here we investigate the presence of purple and related pigments in the hypobranchial glands, reproductive glands and egg capsules of three Rapaninae, three Ocenebrinae and one Muricinae species. Observations on the dissected specimens revealed the presence of purple pigmentation in the hypobranchial glands of all species. All of the Rapaninae and the Muricinae, but only some species of Ocenebrinae, appear to transfer the pigment precursors to their egg capsules. This provides evidence that the precursors of Tyrian purple are not produced for the sole purpose of protecting the egg masses as has been previously suggested (Benkendorff *et al* 2000). In all subfamilies, the hypobranchial and reproductive (capsule and prostate) glands lie adjacent to one another. Colour changes in the reproductive glands, indicating the presence of dye precursors, were observed in two species of Rapaninae. In *Dicathais orbita*, colour changes could be seen in both the capsule glands of females and prostrate glands of males. The final colour was more red in the reproductive glands than the purple observed in the hypobranchial glands. Preliminary observations of detached hypobranchial gland sections in both *D. orbita* and *Pterynotus triformis* indicate a different suite of colour reactions occur when compared to sections that are intact and attached to the reproductive glands. This demonstrates that the reproductive glands can influence the chemical conversions of dye precursors synthesized by these species. Further studies on the secondary metabolism occurring within the hypobranchial glands and reproductive organs of Muricidae are ongoing, including histological sectioning and confirmation of the chemistry behind the colours observed, and functional analysis of Tyrian purple precursors.

INTRODUCTION

The Muricidae are an economically important family of predatory marine gastropods. Many species are relatively large and harvested commercially, either as a source of food (Gallardo 1973, 1979, González and Gallardo 1999), for their decorative shells (Radwin and D'Attilio 1976, Bech, 1994), or as a source of purple dye (Baker 1974, Cooksey 2001, Naegel and Cooskey 2002). Tyrian purple, also known as Royal purple, shellfish purple and Purple of the Ancients, is a well-known colourant obtained solely from muricids and has been used since antiquity on at least three Continents (Europe, Asia and the Americas). The religious significance of Tyrian purple and its importance in many ancient civilizations has triggered much interest in the chemistry of its biosynthesis.

Tyrian purple does not exist in the adult molluscs itself, but is generated from tryptophan derivatives called chromogens found in the hypobranchial gland, upon exposure to oxygen and light (Baker 1974, Cooksey 2001, Scheme 1). Traditionally, Tyrian purple was obtained from the hypobranchial glands of large snails, with the whole bodies of smaller species being crushed and processed (Cooksey 2001). A range of blue, through to deep red colourants can be obtained, depending on which species and the extraction conditions used (Baker 1974). Subsequent studies have revealed that the number and nature of chromogens involved in the production of Tyrian purple differs between species (Fouquet and Bielig 1971, Baker 1974, Prota 1980) and that muricids can produce a wide range of indole derivatives (e.g. Fouquet and Bielig 1971, Baker 1974, Clark and Cooksey 1997, Benkendorff *et al*. 2000, 2001, Cooksey, 2001).

The main component of the dye Tyrian purple is 6,6' dibromoindigotin (**1,** Scheme 1) and appears to be found in hypobranchial gland secretions of all purple producing species studied to date (reviewed by Cooksey 2001). The related non-brominated compound, indigotin (**2,** Scheme 1), is also produced by some species (e.g. *Trunculariopsis trunculus* Elsner and Spanier 1985, Michel *et al*. 1992). Indigoid isomers, the indirubins (**3, 4,** Scheme 1), have been identified in the secretions of several species (Wouters and Verhecken 1992, Cooksey 2001, Michel *et al.* 1992, Clark and Cooksey 1997). Bromoindirubin (**3**) is thought to arise from the condensation of tyrindoxyl (**5**) with 6-bromoisatin (**6**) (Clark and Cooksey 1997). Likewise, indirubin (**4**) is expected to form from the condensation of the equivalent non-brominated indoles. As 6-bromoisatin is likely to be an oxidation artifact (Baker 1974), a greater yield of indirubins could be expected under aerobic conditions. Hence the final dye composition can be affected by photochemical and oxidative reactions of the dye precursors, resulting from the extraction conditions.

Until recently there has been no known function for Tyrian purple and in fact, several researchers simply regard it as an artifact or waste product (Fox 1966, Prota 1980, Naegel and Cooksey 2002). However, Benkendorff *et al.* (2000) found that some of the intermediate precursors have potent antimicrobial properties and suggested a functional role in the sterilization of the egg masses. These intermediate precursors, along with Tyrian purple, have now been positively identified as natural products in the egg masses of seven species from three subfamilies of Muricidae (Table 1; Palma *et al*. 1991; Benkendorff *et al*. 2001). However, extracts from the egg masses of a further two species from the Ocenebrinae subfamily were not found to contain any brominated metabolites, including Tyrian purple (Benkendorff *et al*. 2001). These previous studies indicate that some species of Ocenebrinae either; 1) do not biosynthesize Tyrian purple or; 2) they can produce the purple pigment but do not transfer these across to the egg masses. In order to establish which of these hypotheses holds true, further observations are required on the hypobranchial glands of these species.

The principal aim of this study was to resolve whether some species of Muricidae do produce Tyrian purple without transferring the precursors to their egg masses. For those species that do produce purple egg masses the question remains as to how they get there. No previous studies on the anatomy of muricids have recorded any relationship between the hypobranchial gland and the reproductive glands (see Andrews *et al.*

1991, Jaramillo 1991, Middelfart 1992, Kool 1993b, Roller *et al.* 1995, Aungtonya 1997, Lindberg & Ponder 2001). Thus, this study also aims to present a preliminary report on the relationship between the reproductive glands (capsule and prostate) and the expression of pigments within the hypobranchial glands. The results presented here are based solely on observations on the pigmentation observed in seven species of Muricidae and thus, this study should be regarded as a work in progress.

METHODS

Mollusc specimens from three species were collected from along the south-eastern coast of Australia and a further four were obtained from the south-west coast of Chile (Table 2). Specimens from Metri Bay in Chile were obtained from a recirculating seawater system, whereas all others were obtained from their natural environment. Species endemic to Australia were preserved with shell intact either in 70% ethanol or frozen at -20°C (Table 2). Specimens originating from Chile were removed from their shells and preserved in 70% ethanol prior to importation into Australia. Prior to dissection, the sex of the animal was recorded by documenting the presence or absence of a penis alongside the right eyestalk. One specimen of *Concholepas concholepas* was suspected to have imposex, as determined by the simultaneous presence of a capsule gland and penis. The maturity of each specimen was determined according the presence of a relatively large, well-formed capsule or prostate gland.

When required, the animals were removed from their shells by cracking with a hammer at the primary body whorl-spire junction and the soft body removed by severing the collumellar muscle with a scalpel. The hypobranchial gland is an anteroposteriorly elongated structure (Roller *et al.,* 1995) located just ventral to the dorsal mantle, lying over the buccal mass. The gland occupies the right medial and lateral sides of the mantle cavity and surrounds the rectum on its ventral surface (Freter & Graham, 1994; Roller *et al.,* 1995). Consequently, the mantle cavity was opened mid-dorsally and pinned back to real the visceral mass. Muricid dissections were carried out using Fretter and Graham (1994, fig 10, pp. 24) and Roller *et al.,* 1995) as an anatomical guide. Due to the distortion of various anatomical features by preservation in ethanol, the rectum, (black in colour), was used to locate the hypobranchial gland. The generation of Tyrian purple within the hypobranchial gland was determined by the observed production of pigmented compounds. Thus, any colour changes within the hypobranchial gland, capsule gland, prostrate gland or adjoining features were documented. Digital photographs were also taken to increase the observational power of this investigation and to facilitate subsequent comparisons. In many cases, specimens preserved in ethanol with their shell removed (i.e those imported from Chile) already displayed evidence of Tyrian purple production, supposedly due to prior sunlight and oxygen exposure. However, in the case of those that remained intact and frozen, the intervals between the chemical conversion of pigmented precursors and finally the generation or Tyrian purple were timed and recorded.

Additional observations were undertaken on freshly collected specimens of two Australian species (*Dicathais orbita*, three males specimens; and *Pterynotus triformis* one female specimen) to assess the influence of the reproductive organs on the resulting colour production in the hypobranchial gland. This involved detaching a section of the hypobranchial gland away from the prostate or capsule gland, whilst leaving a further section still intact. A timed series of the color reactions was recorded simultaneously in the sections that were both detached and still attached to the prostrate gland.

Further observations on the presence or absence of purple pigments associated with the intracapsular fluid of Muricidae egg capsules were also made on each species; both in the field and from samples collected and exposed to direct sunlight in the laboratory. Overall, thousands of egg capsules derived from hundreds of individual females were observed from each species over several years. It should be noted that some pink or “purple” pigment is typically associated with the visceral mass of dead muricid embryos and has been used indicator of embryonic mortality by many previous researchers (Spight 1975, Gallardo 1979,

Pechenik 1982, 1983, Rawlings 1996). This colour is likely to stem from the breakdown of the embryonic hypobranchial glands and the resulting faint pink tinge should be distinguished from the bright purple colour associated with the intracapsular fluid. Thus a positive score for pigmentation in the egg capsules, as reported here, requires the development of a bright purple colour in the intracapsular fluid and not just the eggs, embryos or larvae. This pigment will also stain the insides of the capsule walls after hatching.

RESULTS

Purple Pigments in the Egg Masses

Observations on the presence of purple pigmentation in the intracapsular fluid of muricid egg capsules produced results that were consistent with previous chemical studies on the presence of Tyrian purple precursors (Table 1). Two additional species were also examined and these were consistent with previous findings in closely related species. All of the Rapaninae and Muricinae examined to date have the capacity to transfer purple pigments to the egg capsules, whereas only some species of Ocenebrine appear to do this (Table 1).

Purple Pigments in the Hypobranchial Glands

Purple pigmentation was observed in the hypobranchial glands of every species of muricid examined (Table 2), irrespective of whether or not purple pigments occur in the egg masses (Table 1). The purple pigment was observed in most specimens from all species, with the exceptions of one male specimen of *Chorus giganteus* and one immature specimen of *Xanthochorus cassidiformus* (Table 2), however there is no replication for either of these species types. Nevertheless, male specimens from several other species were found to produce purple pigments (Table 2). Furthermore, purple colouration was observed in the hypobranchial glands of immature *Dicathais orbita* (Table 2).

Generally, a more intense purple pigment was observed in the hypobranchial glands of Rapaninae, compared to a more pinkish tinge observed in the Ocenebrinae. Furthermore, the purple coloration extended down the entire length of the hypobranchial gland in the Rapaninae (e.g. Figure 1) and Muricinae, but was restricted to the anterior end in the Ocenebrinae *C. giganteus* (Figure 2b). Purple pigmentation was only observed in association with the posterior mantle protrusion in another species of Ocenebrinae, *Acanthina monodon* (Figure 2a). Some blue pigment was also observed on a distinct section of the hypobranchial gland in specimens of *Concholepas concholepas* and some *D. orbita*.

Relationship between the hypobranchial and reproductive glands

In all species examined, except *Acanthina monodon*, the hypobranchial gland and reproductive glands lie adjacent to one another in both males and females (e.g. Figure 1). The hypobranchial gland extends along the entire length on the right hand side of the capsule or prostate gland, with the rectum lying on the ventral surface. In the case of *A. monodon*, a cartilaginous protrusion containing the rectum and hypobranchial gland was observed (Figure 2a). The structure was positioned at the posterior of the capsule or prostrate gland in both female and male specimens, respectively. It was unclear whether the gonads extended into this unidentified structure.

Colour changes and/or purple pigmentation were only observed in the reproductive glands of two species of Rapaninae (Table 1). This occurred in all mature specimens of *D. orbita*, but in only one of two mature females of *C. concholepas*. In *D. orbita*, the colouration observed in both the capsule and prostate glands was much more red than the purple observed in the hypobranchial glands (e.g. Figure 1).

Hypobranchial gland detachment experiments

In one female specimen of *Pterynotus triformis* a section of the hypobranchial gland become isolated from the specimen during dissection. Upon removal from shell, both the intact and isolated sections of the

hypobranchial gland immediately turned golden yellow. Simultaneously, sections of the hypobranchial gland that remained intact began turning dark green and after 5 min the whole gland and surrounding tissue was green in colour. After 24 hrs in 99.9% ethanol, the majority of green pigment had been converted to purple. However, the section of the hypobranchial gland that had been detached from capsule gland remained yellow, while the connected tissue displayed some green colouration.

A controlled detachment experiment was then conducted using a fresh male specimen of *D. orbita*. Both attached and detached sections of the hypobranchial gland displayed yellow pigmentation upon removal from the shell (Fig. 1a). After 10 min exposure to light and oxygen, the section of hypobranchial gland complete with prostate gland (left panels), simultaneously began to display red and purple pigmentation respectively (Fig. 1b). By time 30 min, the purple pigment of the connected hypobranchial gland had increased in both intensity and magnitude and continued until approximately 60 min (Fig. 1c). By comparison, the detached section of hypobranchial gland (right panels) displayed a change in pigmentation unlike that of the connected section, with the development of some green pigmentation at time 10 min (Fig. 1b). This section remained predominately green until time 30 min, where the gland began to simultaneously generate blue, and to a lesser extent purple, with pigment generation increasing until time 50 min (Fig. c). These observations were repeated in several further specimens of *D. orbita* and the results reported here are representative of those observed when the anterior end of the hypobranchial gland is cleanly detached. In specimens that had been stored for a prolonged period (> month), the timing of colour changes was delayed and the intensity of the final colour was reduced, although consistent patterns were observed on all occasions.

DISCUSSION

Observational studies on the hypobranchial glands of species from two subfamilies of Muricidae indicate that all do have the capacity to produce purple pigmentation (Table 2). This is consistent with previous chemical studies, for which Tyrian purple and/or it's precursors have been detected in hypobranchial gland secretions from all species examined, encompassing at least three subfamilies of Muricidae (Baker and Sutherland 1968,Fouquet and Bielig 1971, Baker 1974, Michel *et al.* 1992, Wouters and Verhecken 1992, Clark and Cooksey 1997, Cooksey 2001,). Muricidae are the only molluscs currently known to be capable of biosynthesizing brominated metabolites, with homogenates from their hypobranchial glands containing a brominating enzyme (bromoperoxidase) (Jannun and Coe, 1987*).* Other molluscs reported to contain brominated compounds are more likely to accumulate these from a dietary source (see Okuda *et al*. 1982, Carte *et al.* 1986, Faulkner 1992). Thus, the biosynthesis of Tyrian purple precursors appears to be restricted to this one family of predatory marine molluscs (Benkendorff *et al*. 2001, Cooksey, 2001) and may be a useful characteristic of this family for future evolutionary and taxonomic studies.

Considering the energetic costs that can be associated with the synthesis of secondary metabolite (Williams *et al*. 1989), it seems likely that these brominated compounds provide some selective advantage to the Muricidae. Benkendorff *et al*. (2000) proposed a functional role for these compounds in the egg masses by demonstrating that they could prevent bacterial infection. However, this study shows that these compounds do not occur in the egg masses of at least three Ocenebrinae (Table 1), despite the fact that purple pigment can be observed in the hypobranchial glands of the adult specimens (Table 2). This, along with the presence of purple pigments in males (Table 2), indicates that the biosynthesis of Tyrian purple precursors has not evolved solely for the protection of the egg masses. Broad spectrum antimicrobial activity has been reported from the hypobranchial gland of some muricids from India (Murugan *et al.* 1992, Prem Anand *et al*. 1997), suggesting that these compounds may have originally evolved as part of the immune defense system of the adult molluscs.

The ability to transfer the precursors of Tyrian purple to the reproductive material is likely to represent a later step in the evolution of Muricidae chemistry, since this does not occur in all species unlike production in the hypobranchial glands. This trait is unlikely to have been lost in more derived groups due to the apparent selective advantage of protecting vulnerable eggs and embryos from infection. Of the species examined to date, all of the Rapaninae and some species from at least two other subfamilies transfer the pigment precursors to the egg masses (Table 1). However, within the Ocenebrinae an inconsistent pattern was observed with no purple pigmentation observed in three out of the five species examined (Table 1). Genetic studies by Marko and Vermeij (1999) have demonstrated that the Eastern Pacific Ocenebrinae form two large sister clades; one which includes the genera *Acanthina*, whereas the other contains *Ceratostoma*. Species from these two genera are shown here to be different with respect to the presence of purple in their egg masses (Table 1). The clade containing *Ceratostoma* also includes *Nucella* and observations on the egg capsules of *N. lapillus* show that these also contain purple pigments (unpublished data). The lack of purple pigment in the egg masses of *Chorus giganteus* and *Xanthochorus cassidiformus* suggests that these genera should occur within the Acanthina clade. Observations on the embryonic development of *A. monodon* and *C. giganteus* provide further support their close relatedness (Gallardo, in prep). Phylogenetic studies are currently underway in our lab to ascertain whether the lack of pigments in the egg masses of species such as *A. monodon* and *C. giganteus* could represent that ancestral condition in the Muricidae. Despite their ecological and economic importance, the phylogenetic relationships within the Muricidae family remain confused (Kool 1993a,b, Ponder 1998, Romero et al. 2004) and characters such as secondary metabolites in the hypobranchial glands and egg masses could provide additional taxonomic markers.

The transfer of secondary metabolites to the egg masses in some muricids would be facilitated by the close proximity of the hypobranchial gland in relation to the reproductive glands. Surprisingly however, colour changes indicative of the dye precursors were only observed in the reproductive glands of two of the seven species examined (Table 2). Specimens from one Rapaninae and one Muricinae showed no pigmentation in their reproductive glands, despite the presence of purple pigments in their egg capsules (Table 1). The specimens of *Pterynotus triformis* were collected alongside some recently spawned egg capsule, suggesting that the supply of purple precursors may have been exhausted in the reproductive glands prior to sampling this species. The *Morula marginabla* specimen used here was also collected during breeding season, although the spawning status is unknown. There is a clear need for more temporal replication in the collections, particularly in and around breeding season. Nevertheless, it can be concluded that the absence of colour changes in the reproductive glands of adult muricids should not be taken as a reliable indicator for the presence or absence of purple pigment in the egg capsules.

There have been previous indications that both the season of collection and the sex of the specimens can influence dye composition in muricids. Here we found purple pigmentation in the hypobranchial glands of both males and females of two out of three species for which both sexes were available (Table 1), thus excluding any general sex specific differences. However, Elsner and Spanier (1985) found that male *Trunculariopsis trunculus* secrete mainly indigo, whereas females secrete the brominated Tyrian purple. Conversely, Michel *et al*. (1992) found no statistical difference in the final dye colouration between male and female *T. trunculus*, unless the samples were stored in the dark, in which case the male secretions were found to be predominately purple. These conflicting results may be related to the season of collection since Michel *et al*. (1992) obtained all their specimens at the start of breeding season. Elsner and Spanier (1985) suggest that the high fluctuations in the ratio of indigo to dibromoindigo in their study could be related to the month of collection. Interestingly, this is the first study to examine the possibility of purple production in immature specimens and this was observed in one out of the two species (Table 1). Thus, purple production can commence prior to reaching reproductive maturity in the Muricidae. However, the influence

of reproductive maturity and gonad ripening may be species specific, and further replicated sampling is required to confirm patterns in the timing of production.

Dye production was only consistently observed in the capsule and prostate glands of one species *Dicathais orbita* (Table 1). Interestingly these reproductive glands appeared to turn deep red as opposed to the bright purple observed in the hypobranchial glands (Figure 1). This red coloration could be explained by the formation of bromoindirubin (**3**), as opposed to Tyrian purple (**1**). The formation of bromomindirubins is likely to be favoured under oxidative conditions since they are derived from the condensation of the oxidative artefact bromoisatin (**6**, Scheme 1). By comparison, the hypobranchial glands of *D. orbita* may provide a relatively reducing environment due to an abundance of methane thiol and/or dimethyl disulfide which is also required for the biosynthesis of the ultimate precursor of Tyrian purple in this species (Scheme 1; Baker and Sutherland 1968). We are yet to confirm the chemistry behind the colours observed here, but there does appear to be an interesting relationship between the hypobranchial and reproductive glands, with some important differences in the apparent chemical reactions taking place.

The necessity for preserving membrane integrity between the hypobranchial gland and the reproductive glands for normal colour production was evident from detachment experiments in two species of Muricidae. For *Pterynotus triformis*, ample exposure to light and oxygen in detached sections of the hypobranchial glands did not result in the production of Tyrian purple. Similarly, production of pigmentation in *D. orbita* was noticeably slow in the detached section and resulted in a predominance of blue as opposed to purple colouration (Figure 1). The blue pigmentation could be explained by the formation of the non-brominated indigotin (**2**), as opposed to 6,6'-dibromidigotin (**1**). This suggests that in *D. orbita*, either bromine ions or the bromoperoxidase enzyme could be associated with the prostate gland rather than the hypobranchial gland itself. Again it can only be assumed at this stage that the observed pigments are due to the precursors of the dyes indigo and Tyrian purple. Consequently, future work (in progress) will involve investigating extracts of the various glands by liquid chromatography/mass spectrometry (LC/MS) to confirm the chemical composition.

Standardized methods of preparation are important for these types of experiments on muricid hypobranchial glands. We have found that the best approach is to use freshly collected specimens that can be sacrificed either in the freezer or in 70% ethanol, but preferably no more than a fortnight prior to dissection. Prolonged storage may affect some of the biosynthetic enzymes, as we observed delays in the timing of the colour reactions in specimens that had been preserved for months. Furthermore, removal of the shell will result in exposure of the hypobranchial gland to light and thus only the final pigments can be observed, as opposed to the whole sequence of colour reactions. Despite the need to import specimens using different methods of preparation in this study, we were still able to observe pigments in the hypobranchial glands of all species examined. There is clearly some within species variation in the present of pigment precursors, but male, females and immature adults do all have the capacity to produce these compounds. Conversely, the transfer of the pigment precursors to the egg masses differs between species of Ocenebrinae, but is stable within species of Rapaninae and Muricinae. The reproductive glands do influence the production of pigments in the hypobranchial glands of at least one species, *Dicathais orbita*. In conclusion, the biosynthesis of Tyrian purple and related compounds in the Muricidae may be even more complex than previously envisioned. Further research is currently being conducted by C. Westely as part of a Ph.D. project on the chemical changes and anatomical connections between the reproductive and hypobranchial glands. As another component of this project, *Dicathais orbita* is being used as a model species with the hope of establishing a biological function for these compounds in the adults.

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Table 1: The presence of purple pigments in the egg masses of Muricidae from three subfamilies. Literature review was used to establish the presence of Tyrian Purple based on chemical studies of organic extracts from the egg masses. Observations on the purple pigment this study were based on a colour change associated with the intracapsular fluid, rather than eggs or embryos and persistent purple staining of the empty or hatched capsules.

Subfamily	**Species**	**Tyrian Purple Precursors**	**Purple pigmentation observed**
Rapaninae	*Dicathais orbita*	Yes[1,2]	Yes
	Agnewia tritoniformis	Yes[2]	Yes
	Morula marginabla	Yes[2]	Yes
	Concholepas concholepas	Yes[3]	Yes
Muricinae	*Pterynotus triformis*	NA[4]	Yes
	Trunculariopsis trunculus	Yes[2]	NA
Ocenebrinae	*Lepsiella reticulata*	Yes[2]	Yes
	Ceratostoma erinaceum	Yes[2]	NA
	Acanthina monodon	No[2]	No
	Chorus giganteus	No[2]	No
	Xanthochorus cassidiformus	NA	No

[1] Benkendorff *et al*. 2000
[2] Benkendorff *et al*. 2001
[3] Palma *et al.*, 1991
[4] NA = Not applicable – no prior reports in the literature.

Table 2: Muricid specimens used for observation of purple colouration in the hypobranchial glands and reproductive glands; male (M) = prostate; female (F) = capsule gland. The method of preservation prior to dissection in recorded for each specimen. Observation of the presence of purple pigments in the egg capsules is also provided for each species. Presence or absence of the purple pigment is recorded after at least one-hour exposure to sunlight.

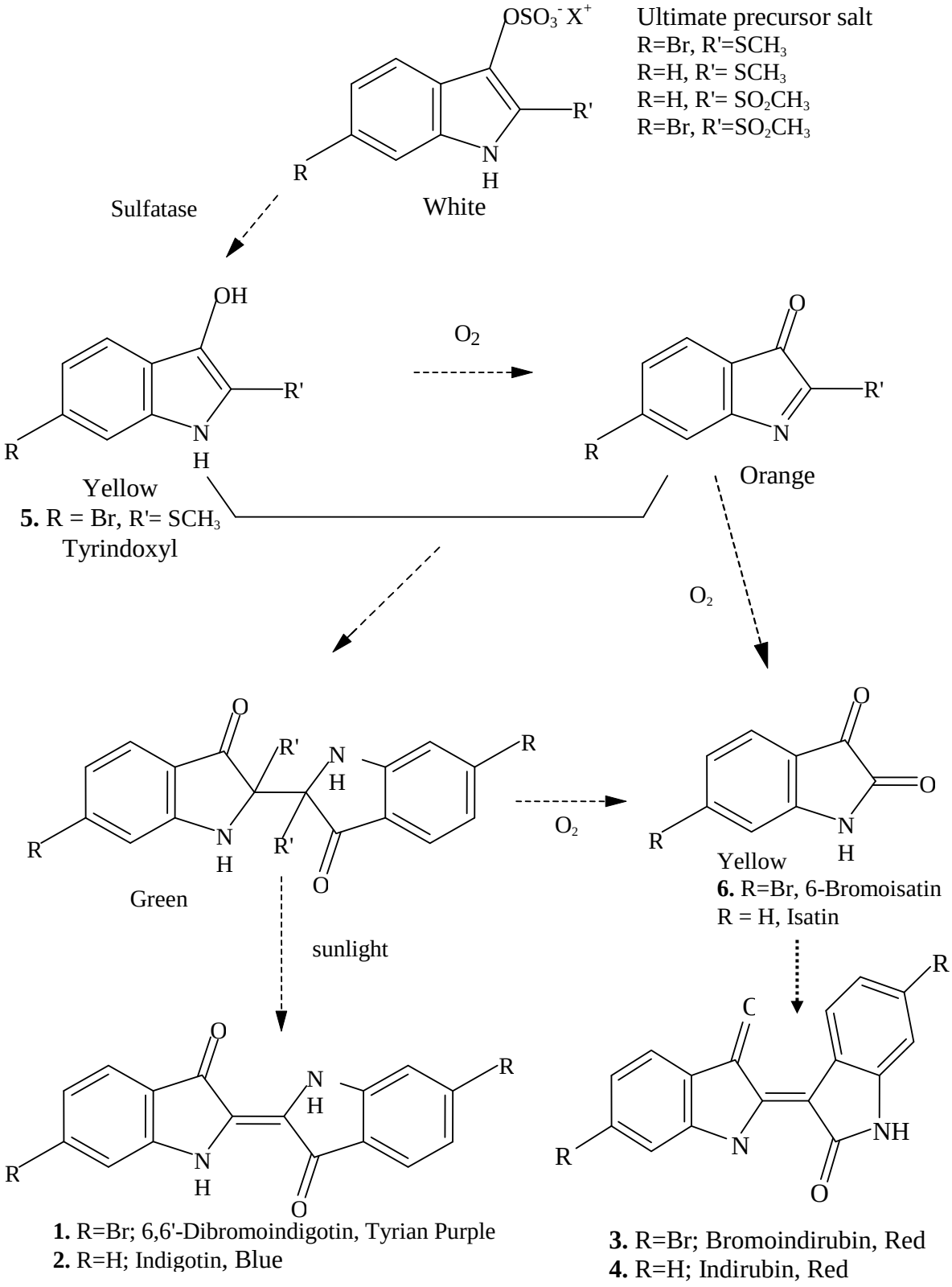
Subfamily	**Species**	**Location/Reference**	**Date**	**Preparation**	**Sex & Maturity**	**Hypobranchial glands**	**Reproductive glands**
Rapaninae	*Dicathais orbita*	Seaford, SA, Australia	10/2003	Frozen	F (mature)	present	present
	Dicathais orbita	Seaford, SA, Australia	10/2003	Frozen	F (mature)	present	present
	Dicathais orbita	Seaford, SA, Australia	10/2003	Frozen	F (mature)	present	present
	Dicathais orbita	Port Lincoln, SA, Australia	12/2003	Ethanol	M (mature)	present	present
	Dicathais orbita	Port Lincoln, SA, Australia	12/2003	Ethanol	M (mature)	present	present
	Dicathais orbita	Port Lincoln, SA, Australia	12/2003	Ethanol	F (immature)	present	absent
	Dicathais orbita	Port Lincoln, SA, Australia	12/2003	Ethanol	M (immature)	present	absent
	Concholepas concholepas	Mehuin Bay, Chile	10/2003	Ethanol	F (mature)	present	present
	Concholepas concholepas	Mehuin Bay, Chile	10/2003	Ethanol	Imposex	present	absent
	Concholepas concholepas	Mehuin Bay, Chile	20/2003	Ethanol	F (mature)	present	absent
	Morula marginalba	Bass Point, NSW, Australia	11/2003	Ethanol	F (mature)	present	absent
Muricinae	*Pterynotus triformis*	Seacliff, SA, Australia	10/2003	Frozen	F (mature)	present	absent
	Pterynotus triformis	Seacliff, SA, Australia	10/2003	Frozen	F (mature)	present	absent
Ocenebrinae	*Chorus giganteus*	Metri Bay Marine Station, Chile	10/2003	Ethanol	F (mature)	present	absent
	Chorus giganteus	Metri Bay Marine Station, Chile	10/2003	Ethanol	M (mature)	absent	absent
	Chorus giganteus	Metri Bay Marine Station, Chile	10/2003	Ethanol	F (mature)	present	absent
	Acanthina monodon	Mehuin Bay, Chile	11/2003	Ethanol	F (mature)	present	absent
	Acanthina monodon	Mehuin Bay, Chile	11/2003	Ethanol	M (mature)	present	absent
	Acanthina monodon	Mehuin Bay, Chile	11/2003	Ethanol	M (mature)	present	absent
	Acanthina monodon	Mehuin Bay, Chile	11/2003	Ethanol	F (mature)	present	absent
	Acanthina monodon	Mehuin Bay, Chile	11/2003	Ethanol	F (mature)	present	absent
	Xanthochorus cassidiformus	Metri Bay Marine Station, Chile	11/2003	Ethanol	M (mature)	present	absent
	Xanthochorus cassidiformus	Metri Bay Marine Station, Chile	11/2003	Ethanol	F (mature)	present	absent
	Xanthochorus cassidiformus	Metri Bay Marine Station, Chile	11/2003	Ethanol	F (immature)	absent	absent

FIGURE LEGENDS

Scheme 1: The formation of coloured compounds from ultimate precursors in the hypobranchial glands of Muricidae. Tyrian purple (**1**) is formed under normal circumstances from the brominated precursors and likewise, blue Indigotin is formed from the non-brominated precursors. However, under relatively oxidizing conditions formation of the yellow isatins (**6**) and red indirubins (**3, 4**) is enhanced.

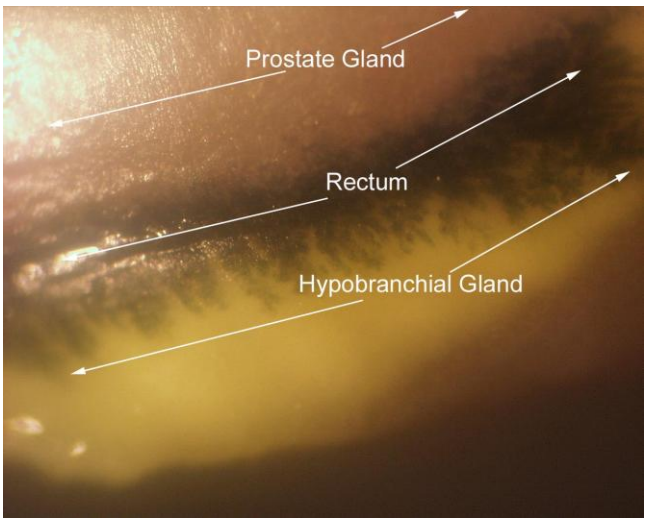
Figure 1: Timed series of colour generation in the hypobranchial glands and prostate glands from the Rapaninae *Dicathais orbita*; a) 0 min, b) 10 min, and c) 60 min after dissection. Left panels = hypobranchial gland attached to the prostate gland; Right panels = hypobranchial gland detached from the prostate gland.

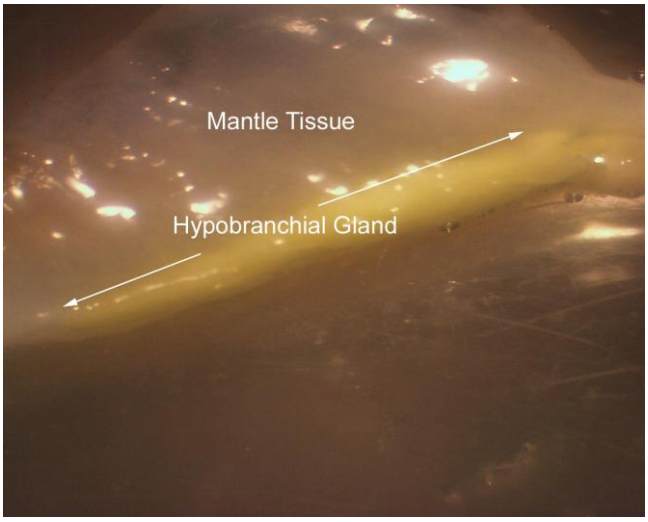
Figure 2: The association of purple colouration in two Ocenebrinae, with a) an unidentified protrusion in the mantle of *Acanthina monodon*, and b) the anterior end of the hypobranchial gland of *Chorus giganteus.*


OSO3- X+
R
R'
N
H
Ultimate precursor salt
R=Br, R'=SCH3
R=H, R'= SCH3
R=H, R'= SO2CH3
R=Br, R'=SO2CH3
Sulfatase
White
OH
O2
O
Yellow
5. R = Br, R'= SCH3
Tyrindoxyl
Orange
Green
Yellow
6. R=Br, 6-Bromoisatin
R = H, Isatin
sunlight
1. R=Br; 6,6'-Dibromoindigotin, Tyrian Purple
2. R=H; Indigotin, Blue
3. R=Br; Bromoindirubin, Red
4. R=H; Indirubin, Red

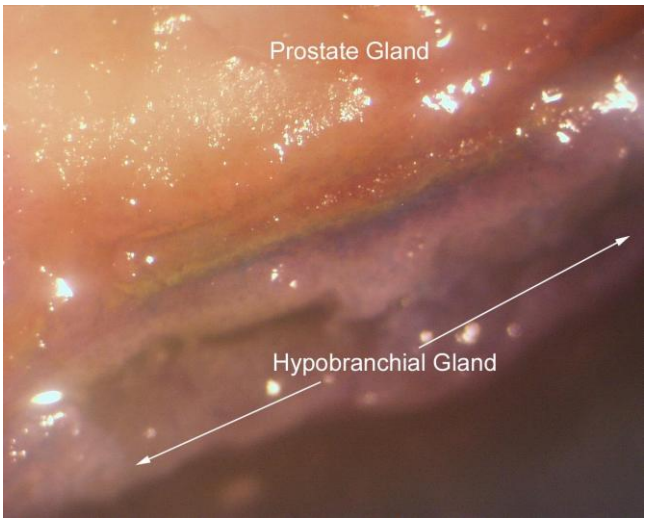
Scheme 1

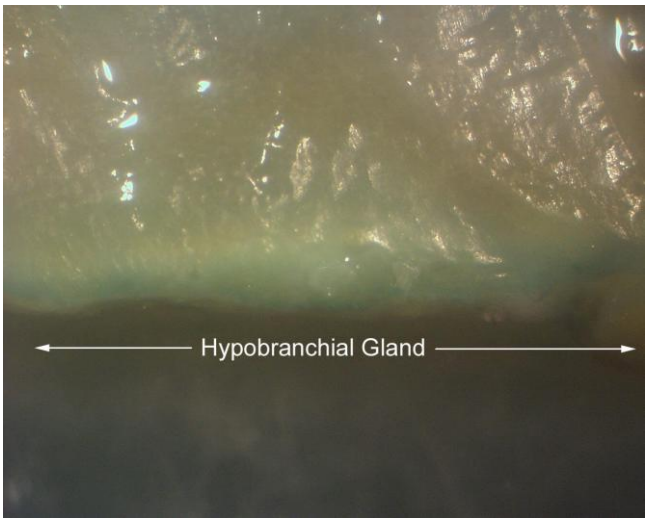
a)


Prostate Gland
Rectum
Hypobranchial Gland

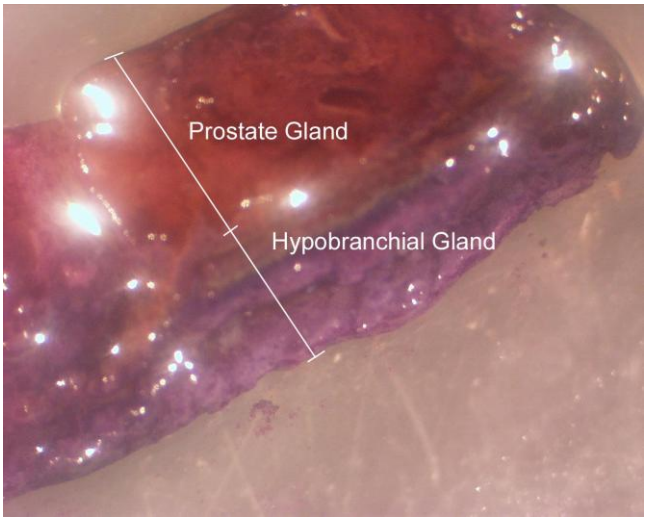

Mantle Tissue
Hypobranchial Gland

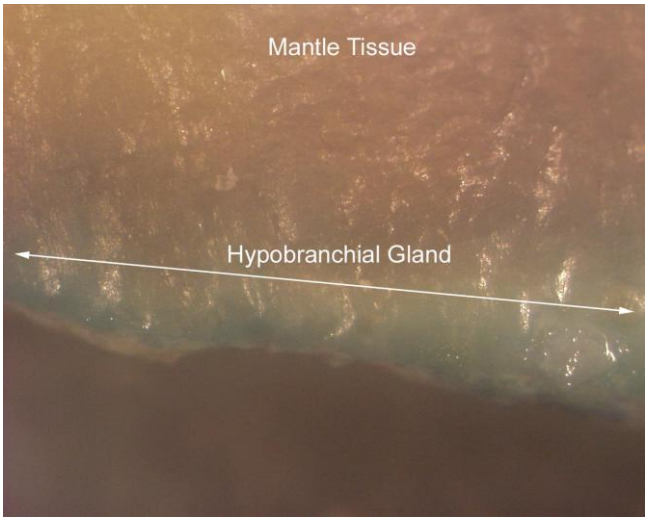
b)


Prostate Gland
Hypobranchial Gland

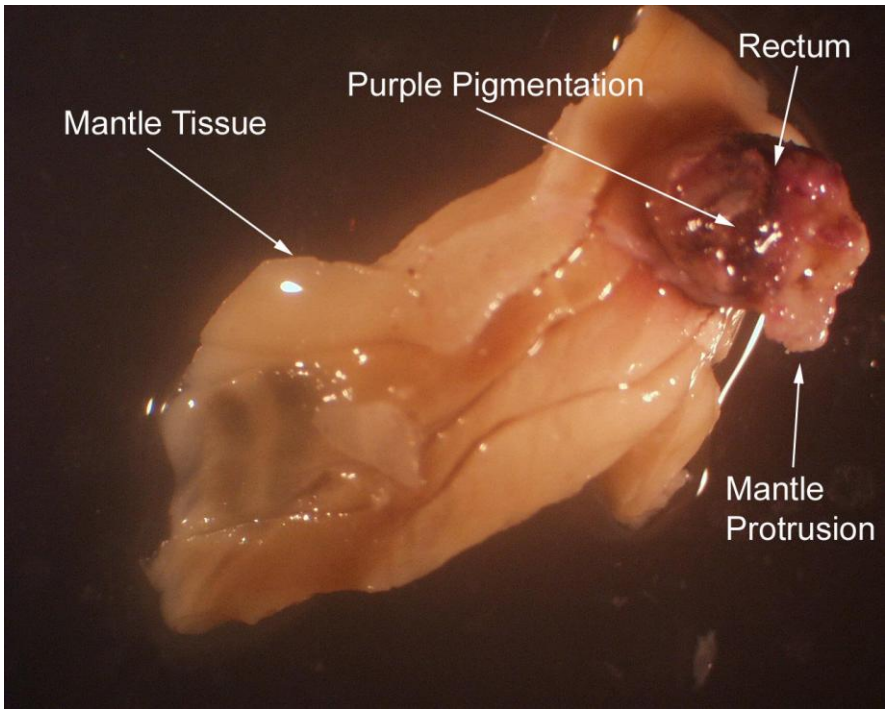

Hypobranchial Gland

c)

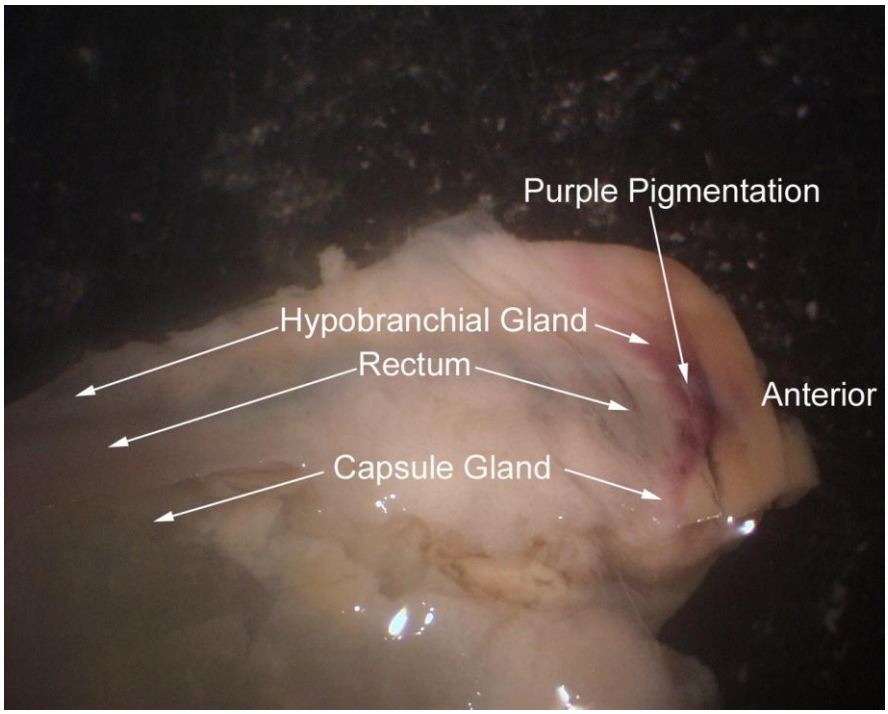

Prostate Gland
Hypobranchial Gland


Mantle Tissue
Hypobranchial Gland

a)


Rectum
Purple Pigmentation
Mantle Tissue
Mantle
Protrusion

b)


Purple Pigmentation
Hypobranchial Gland
Rectum
Anterior
Capsule Gland