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SEX-SPECIFIC TYRIAN PURPLE GENESIS: PRECURSOR AND PIGMENT DISTRIBUTION IN THE REPRODUCTIVE SYSTEM OF THE MARINE MOLLUSC, *Dicathais orbita*.

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Abstract- Exploitation of Tyrian purple from muricid molluscs, since antiquity, has prompted much interest in its chemical composition. Nevertheless, there remains a paucity of information on the biosynthetic routes leading to observed sexual differences in pigmentation. A liquid chromatography-mass spectrometry method was developed to simultaneously quantify dye pigments and precursors in male and female *Dicathais orbita*. The prochromogen, tyrindoxyl sulfate, was detected for the first time using this method in hypobranchial gland extracts of both sexes. Intermediates tyrindoxyl, tyrindoleninone and tyriverdin were detected in female hypobranchial glands, along with 6,6'-dibromoindigo, while males contained 6-bromoisatin and 6,6'-dibromoindirubin. Multivariate analysis revealed statistically significant differences in the dye composition of male and female hypobranchial glands (ANOSIM, P = 0.002), providing evidence for sex-specific genesis of Tyrian purple in the Muricidae. Dye precursors were also present in male and female gonoduct extracts, providing a mechanism for the incorporation of bioactive intermediates into muricid egg masses. These findings provide a model for investigating sex-specific chemical divergences in marine invertebrates and support the involvement of Tyrian purple genesis in muricid reproduction.

Key Words- Brominated indoles, hypobranchial gland, Muricidae, reproduction, sexual dimorphism.

INTRODUCTION

Tyrian purple, also known as Shellfish purple and Royal purple, is an historically important dye, traditionally obtained from the hypobranchial glands of the Muricidae (Neogastropoda: Mollusca). Exploitation of this dye from as early as the 17th Century BC, has attracted the ongoing interest of natural and cultural historians, archeologists, dyers and colorists, artists, chemists, and biologists (Baker, 1974; Cooksey, 2001a, 2006; Haubrichs, 2004, 2006; Karapanagiotis and de Villemereuil, 2006; Westley et al., 2006). In Historia Naturalis, Pliny the Elder provided the first detailed description of colours obtained by dyeing with different techniques and muricid species in ancient Rome (Bailey, 1929). Much later, Cole (1685) described how the pigment develops in a series of colour reactions under the influence of sunlight. This process can now be explained by a series of oxidization, dimerization and photolytic cleavage reactions (Fig. 1). A major advance was made by Friedlander (1909), who resolved the structure of the principle pigment, 6,6'-dibromoindigo (**5**). Baker and Sutherland (1968) identified colourless tyrindoxyl sulfate (**1**), as the ultimate dye precursor in the Australian muricid, *Dicathais orbita* and subsequent studies revealed the intermediate precursors; tyrindoxyl (**2**), tyrindolinone, tyrindoleninone (**3**) (Baker and Duke, 1973, 1976), and tyriverdin (**4**) (Christophersen et al., 1978; Fujise et al., 1980). Further investigations of dyed artifacts and hypobranchial gland secretions from various muricids have uncovered additional precursors, artifacts and minor pigments (Michel et al., 1992; Wouters, 1992; Koren, 1995, 2006; Cooksey, 2001a, b, 2006; Cooksey and Withnall, 2001; Karapanagiotis and de Villemereuil, 2006). These include the yellow oxidation by-product, 6-bromoisatin (**7**), the red structural isomer of (**5**), 6,6'-dibromoindirubin (**6**) (Fig. 1), indigo (**8**), indirubin (**9**),

6-bromoindigo (**10**), 6-bromoindirubin (**11**) and 6'-bromoindirubin (**12**) (Appendix 1).

Figure 1

The first indication of a link between dye production and reproduction in the Muricidae was provided by Aristotle's comments on "purpuras" in Historia Animalium ~350 BC (Peck, 1970). He stated that, "When the purpuras have honeycombed (*sic* deposited egg capsules), their bloom (*sic* hypobranchial gland) is at its worst", a fact also reinforced by Pliny the Elder in 1st Century AD (Bailey, 1929). However, this association was overlooked until more recent investigations on egg masses of the Muricidae revealed the presence of indigoid compounds (Palma et al., 1991; Benkendorff et al., 2000, 2001, 2004). In the egg masses of *D. orbita,* Benkendorff et al. (2000) not only reported relatively high concentrations of tyriverdin (**4**) and tyrindoleninone (**3**), but also demonstrated that these intermediates have potent bacteriostatic and mild cytotoxic activity, respectively. This discovery prompted the proposal of a novel biochemical role for indigoid compounds in the Muricidae, whereby they are incorporated into egg masses as a form of maternal investment in the chemical defence of developing embryos (Benkendorff et al., 2000; Westley et al., 2006). The potential for precursor transfer from the adult hypobranchial gland to the adjacent reproductive glands has recently been supported by accounts of deep red pigmentation in capsule and prostate glands of *D. orbita* (Benkendorff et al., 2004). Furthermore, preliminary experiments involving the excision of hypobranchial glands from reproductive organs, suggests that the pallial gonoduct influences pigment

synthesis (Benkendorff et al., 2004). However, as this study was limited to visual accounts, quantitative analysis is required to confirm the composition of dye products and hence, the biochemical relationship between these glandular structures.

Another question that remains to be adequately resolved is whether dye composition and pigmentation differ between sexes of the Muricidae. Studies on hypobranchial gland secretions of *Murex trunculus* by Elsner and Spanier (1985), initially indicated that female glands produce predominantly purple dyes, while dyes of masculine origin gain blue pigmentation due to the presence of indigo (**8**). However, the method of specimen sexing was not reported and subsequently, Verhecken (1989) proposed the opposite. Verhecken's proposal is consistent with recent visual accounts of red dye pigmentation in male *M. trunculus*, and a more blue shade of purple in females (Fig. 2, Boesken Kanold, pers. comm.). Michel and colleagues (1992) failed to confirm any correlation between dye colour and sex; however a high incidence of pseudohermaphrodism may have influenced their results. Nevertheless, the final pigmentation of male samples after storage in the dark was described as predominantly purple, suggesting that male glands contain relatively less of the non-brominated indoxyl precursors and hence, less indigo (Michel et al., 1992). An alternative hypothesis for the prevalence of red purple dyes in males could be related to the formation of red indirubins, structural isomers of indigoids that evolve in oxygen-rich environments (Fig. 1). Sensitive chemical analysis of male and female dye is clearly required to objectively assess any sex-related differences in composition.

Figure 2

Over the last couple of decades, advances in chemical analysis by high performance liquid chromatography (HPLC) and mass spectrometry (MS) have facilitated the rapid and accurate detection of indigoid pigments (McGovern et al., 1990; Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1995, 2006; Szostek et al., 2003; Andreotti et al., 2004; Puchalaska et al., 2004; Karapanagiotis and de Villemereuil, 2006; Polec-Pawlak et al., 2006). To date however, no one has applied modern LC-MS techniques to simultaneously analyze the full suite of precursors and pigments that constitute Tyrian purple. In the current investigation, an effort was made to preserve glandular biochemistry in order to examine dye genesis in the presence of precursors. Extracts from the hypobranchial and reproductive glands of *D. orbita* were employed, as this species possesses a comparatively simple biosynthetic pathway to Tyrian purple from a single brominated prochromogen (**1**, Fig. 1) (Baker and Sutherland, 1968) representative of most Muricidae (Cooksey, 2006). Furthermore, previous studies on *D. orbita* provided the first evidence for dye precursors in egg capsules (Benkendorff et al., 2000), and observations of pigmentation in the reproductive organs (Benkendorff et al., 2004). This investigation aims to quantify precursor and pigment composition in the female pallial gonoduct, specifically, the capsule, albumen and ingesting glands, as a means for establishing maternal investment in embryonic chemical defence. Following from earlier observations (Benkendorff et al., 2004), analysis of dye composition in hypobranchial glands, attached and detached from male prostate and female capsule glands, was also undertaken to further establish the relationship between dye genesis, reproduction and sex.

METHODS AND MATERIALS

Six male and six female *D. orbita* specimens were sampled from subtidal rocky platforms along the metropolitan coastline of South Australia prior to the breeding season (July-August, 2005 and 2006). An additional three females were collected during breeding season (December, 2005) specifically for dissection and extraction of ingesting glands. Females were identified by the presence of an albumen and capsule gland and the absence of a penis, posterior to the right eye tentacle, and sperm ducts, which occur in pseudohermaphrodites after exposure to tributylin tin (Gibson and Wilson, 2003).

The shell of each 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 where the visceral mass was separated from the dorsal mantle by an incision along the lateral margins of the columnar muscle. The dorsal mantle was folded back, to reveal the pallial gonoduct, and pinned with the ventral surface facing up to expose the hypobranchial gland. In female specimens, care was taken to ensure that ingesting and albumen glands were dissected free of hypobranchial gland tissue. Prostate and capsule glands were dissected away from the medial and brachial regions of the hypobranchial gland in three male and three female specimens, respectively. It should be noted that removal of the rectal gland and rectal hypobranchial gland from these reproductive structures was not possible. For the “detachment experiment”, excised medial and branchial hypobranchial gland regions from both sexes were reserved for inclusion as “detached”

replicates. In another three specimens of each sex, the pallial gonoduct and hypobranchial gland were left intact to give comparative “attached” replicates. To produce extracts, which represent the true biochemical composition of dye products from *D. orbita*, all dissected glands were left in their natural posture and exposed to ambient laboratory oxygen and lighting for 12h post dissection.

After dye development, each gland (24 in total) was transferred to an amber vial containing enough dimethyl formamide (DMF) to submerge the tissue. Glands were then macerated and extracted for 48hrs, before being gravity filtered through glass wool. Prior to compositional analysis, all extracts were sonicated and centrifuged to precipitate tissue residues. Extracts were analyzed using high performance-liquid chromatography (HPLC, Waters Alliance) couple to a mass spectrometer (MS, Micromass, Quatro micro™). HPLC separation was performed on a Phenomenex, Synergi, Hydro-RP C_{18} column (250 x 4.6mm x 4μm) with parallel UV/Vis diode-array detection (DAD) at 300 and 600nm. The elution scheme was modified from Szostek et al. (2003) and Puchalska et al. (2004) using 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. Compounds were identified using electrospray ionization-mass spectrometry (ESI-MS) with a flow rate of 300μl/min. Relative proportions of each compound were calculated from integrated absorption data in diode-array using MassLynx 4.0 software. Proportions were expressed as a percentage of the total dye composition, including all detected precursors and end-products. To facilitate identification of the dye constituents, synthetic standards were analyzed by identical procedures.

Synthetic standards for all possible indole and indirubin end-products (Appendix 1, Karapanagiotis and de Villemereuil, 2006) were prepared in DMF to a concentration of 40μM. Standards included indigo (Sigma, 229296), 6-bromoindigo (MDPI, 19393), 6,6'-dibromoindigo (courtesy of Prof. P. Imming), indirubin (Apin Chemicals LTD, 20338I), and 6-bromoindirubin, 6'-bromoindirubin and 6,6'-dibromoindirubin (courtesy of Prof. A. L. Skaltsounis). Apart from retention time (Table 1), discrimination between structural isomers was achieved by differences in visible absorption spectra at λ_{max} 600nm for indigoids and λ_{max} 550nm for indirubins. Indigo (**8**) and indirubin (**9**) were further discriminated by the registration of a doubly charged quasi-molecular ion $[M+2H]^{2+}$ at *m/z* 132, which allowed unequivocal identification of **8** (Puchalaska et al., 2004). Major ions in ESI-MS obtained at the apex of HPLC peaks for monobrominated compounds (**10**-**12**) produced duplet ion clusters at *m/z* 339, 341 $[M-H]^{+}$. Similarly, 6,6'-dibromoindigo (**5**) and 6,6'-dibromindirubin (**7**) were identified by triplet ion clusters at *m/z* 417, 419, 421 for Br^{79} Br^{79}, Br^{79} Br^{81}, Br^{81} Br^{81}.

Synthetic standards were not available for precursors, however mass spectrums have previously been used to identify intermediate precursors (Michel et al., 1992; Benkendorff et al., 2000; Cooksey and Withnall, 2001, Andreotti et al., 2004), based on expected mass and isotopic clusters for the mono- and dibrominated compounds. As the mass spectrum of tyrindoxyl sulfate has not yet been published, the presence of this compound in extracts was confirmed by thin layer chromatography (TLC). TLC was conducted on aluminum-backed silica gel plates (Merck), employing an n-butanol-EtOH-acetic acid-water (8:2:1:3) solvent

system. Development in 1M HCl results in the formation of a purple spot, characteristic of tyrindoxyl sulfate (Baker and Sutherland, 1968). Presence of murexine and senecioylcholine was also investigated by TLC, as these choline esters are known to be associated with the prochromogen (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 and rose pigmentation in UV-active spots indicates the presence of senecioylcholine and murexine, respectively (Roseghini et al., 1996).

Statistical analysis of differences in male and female dye composition (n =6) were undertaken using Primer Version 5 Software. Multivariate analyses were undertaken on square root transformed data to increase the weighting of minor constituents. Non-metric multidimensional scaling (nMDS) was performed on a Bray-Curtis similarity matrix (Clarke and Gorley, 2001) and portrayed in a two-dimensional plot. Significant differences in dye composition between the sexes were then explored using ANOSIM. SIMPER was undertaken to identify which indigoids contributed to compositional differences.

RESULTS

Dyes and precursors in the hypobranchial and reproductive glands. LC-MS analysis revealed the presence of brominated indole derivatives in dimethyl formamide (DMF) extracts from all hypobranchial and reproductive glands (Table

1). The dominant compound present in all samples registered an HPLC peak at 5.1min (e.g. Fig. 3). Major ions in ESI-MS, obtained at the apex of this peak, were at *m/z* 338, 336, which correspond to the molecular ions of tyrindoxyl sulfate (**1**) (Br^{79}, Br^{81}, Fig. 3). Fragment ions at *m/z* 240, 242 and *m/z* 224, 226 correlate with the loss of a sulfate ion [$MH-SO_4$], and a methyl group [$M^-H-SO_4-CH_3$] respectively, from the tyrindoxyl sulfate molecule. Since this compound has not been previously characterized using mass spectrometry, we used thin layer chromatography (TLC) confirm the presence of **1**. A single spot (R_f 1.0), that turned purple after exposure to HCl was detected in all extracts except one ingesting gland, which also failed to produce a peak corresponding to tyrindoxyl sulfate in LC-MS (Table 1). TLC analysis also revealed a colourless UV-active spot (R_f 0.12) in all glandular extracts containing tyrindoxyl sulfate. Application of the Dragendorff Reagent resulted in rose pigmentation, indicative of murexine (Roseghini et al., 1996).

Table 1

Figure 3

Intermediate dye precursors were detected as minor components in LC-MS analyses (e.g. Fig. 3) of male and female hypobranchial and capsule gland DMF extracts (Table 1). Co-eluting peaks at 10.1min with major ions in ESI-MS at *m/z* 255, 257 and 256, 258 correspond to the molecular mass of tyrindoleninone (**3**) and tyrindoxyl (**2**). Fragment ions at *m/z* 240, 242 formed by the elimination of a methyl group $[M-CH_3]^+$ are consistent with the mass spectrum data for **3**. The second duplet ion cluster at *m/z* 256, 258 and fragment ions at *m/z* 241, 243,

correspond to those of **2** $[M-H]^+$ and the loss of a methyl group $[M-H-CH_3]^+$ during electron bombardment. The HPLC peak detected at 6.5min with *m/z* 224, 226, in both male and female hypobranchial glands (Table 1), can be attributed to the pseuomolecular ion $[M-H]^+$ of 6-bromoisatin (**6**), which has a molecular mass of 225, 227 (Br^{79}, Br^{81}).

An additional dye precursor, identified exclusively in female hypobranchial and capsule gland extracts, registered a chromatographic peak at 12.0min (Table 1). Although major ions in ESI-MS at *m/z* 417, 419, 421 correspond to the mass of dibrominated standards **5** and **7**, the retention times disagree (Appendix 1). Minor peak isotopic clusters detected at 513, 515, 517 correspond to the quasi-molecular ion of tyriverdin [MH^+; Br^{79} Br^{79}, Br^{79} Br^{81}, $Br^{81}Br^{81}$]. Additional ion clusters at *m/z* 465 (Br^{79} Br^{81} $[M-SCH_3]^+$ from the elimination of a single methane thiol group) and *m/z* 419 (Br^{79} Br^{81} $[MH-2SCH_3]^+$ formed by the elimination of dimethyl disulphide) further confirm this peak as tyriverdin (**4**).

The dye pigments appear as relatively minor constituents in *D. orbita* extracts (e.g. Fig. 3, Table 1). The two dibrominated dye pigments **5** and **7** were detected in some of our extracts, with retention times and mass spectra that were consistent with synthetic standards (Appendix 1). No peaks corresponding to the mono- or non-brominated indigo or indirubin standards (Appendix 1) were detected in any *D. orbita* extracts.

Sex-specific pigment genesis. During the exposure period, hypobranchial glands sequentially developed yellow, red and green pigmentation before gaining various

shades of purple and blue, irrespective of sex. The major purple dye pigment extracted from female hypobranchial and capsules glands was identified as 6,6'-dibromoindigo (**5**) (Table 1). One male hypobranchial gland extract was also found to contain trace amounts of 6,6' dibromoindigo (**5**, Table 2). However, in contrast to the females, the major dye pigment of male hypobranchial and prostate glands corresponded to 6,6'-dibromoindirubin (**7**) (Table 1, 2). Within each sex, the dye and precursor composition was identical in all replicate hypobranchial glands whether "attached" or "detached" from the reproductive system; with male extracts dominated by 6,6'-dibromoindirubin and females by 6,6'-dibromoindigo (Table 2). Tyriverdin (**4**), was only detected in the female extracts (Table 2)

Multidimensional scaling (MDS) ordination on the dye compositions revealed a distinct separation between male and female hypobranchial gland extracts (Fig. 4). Multivariate analysis of similarities (ANOSIM) confirmed that these differences were statistically significant (Global R = 0.757, p = 0.002). Similarity of percentage (SIMPER) analysis revealed an average dissimilarity of 21% in the dye composition between male and females. Intermediates and final pigments contributed substantially to the sexual differences, whereas the ultimate precursor, tyrindoxyl sulfate (**1**), occurred in similar abundance in both sexes. Tyriverdin (**4**) and 6,6'-dibromoindigo (**5**) were consistently more abundant in females samples, whereas 6-bromoisatin (**6**) and 6,6'-dibromoindirubin (**7**) characterized male samples. Blind examination of LC-MS chromatograms confirmed that we could reliably determine the sexual origin of *D. orbita* extracts based on these chemical compositions.

Table 2

Figure 4

DISCUSSION

Analysis of hypobranchial gland extracts by LC-MS, in conjunction with synthetic standards, confirmed the presence of dibrominated dyes generated from a single brominated prochromogen in *Dicathais orbita*. Tyrindoxyl sulfate (**1**) was detected in hypobranchial gland extracts and also occurred throughout female reproductive glands and male prostate glands (Table 1). Comparison of male and female extracts provided clear evidence for sex-specific Tyrian purple genesis (Table 2, Fig. 4). Females dyes were composed of 6,6'-dibromoindigo (**5**), while male hypobranchial glands contained the red isomer (**7**). This is the first study in which the full suite of Tyrian purple precursors have been analyzed simultaneously alongside the final dye pigments. As suggested by Michel and colleagues (1992), precursors in the final dye product are most likely due to reduced light exposure resulting from the screening affect of mucoid glandular secretions or already formed dye. Consequently, this method of dye development may be viewed as somewhat incomplete compared to the majority of studies where muricid hypobranchial gland secretions were developed on filter paper. However, preservation of the complete glandular biochemistry can prove useful in deciphering the genesis of specific dye products under natural physiological conditions. This approach has enabled a significant advance in understanding the chemistry behind sex specific colour differences in Muricidae dyes (see Fig. 2).

Our findings of only one prochromogen corresponding to tyrindoxyl sulfate (Fig. 3) in *D. orbita* hypobranchial gland extracts are consistent with previous reports that **1** is the sole dye precursor in this species (Baker, 1974). Using TLC developed in Dragendorff Reagent, we also detected only one choline ester corresponding to murexine, which is known to be associated with tyrindoxyl sulfate in *D. orbita* (Baker and Duke, 1976). Whilst murexine was not detected in our LC-MS analyses, the ability to detect the prochromogen **1** in muricid hypobranchial gland extracts should allow for identification of prochromogens in more complex extracts (e.g. *M. trunculus*), where substituted and un-substituted sulfate esters of indoxyl and 6-bromoindoxyl co-exist (McGovern and Michel, 1990). The absence of any peaks corresponding to the mono- or non-brominated indigo or indirubin standards (Appendix 1) in our *D. orbita* extracts (Fig. 3, Table 1), further confirms **1** as the sole ultimate dye precursor (Fig. 1) in this Australian species. Nevertheless, this study does provide new evidence for 6,6'-dibromoindirubin in glandular extracts of *D. orbita* (Table 1), as 6,6'-dibromoindigo was previously thought to be the sole dye component in secretions from this species (Baker, 1974). It now appears that genesis of the structural isomer is also possible, similar to that reported for other Muricidae (Clark and Cooksey, 1997; Cooksey, 2001a, b, 2006; Cooksey and Withnall, 2001; Naegel and Cooksey, 2002; Withnall et al., 2003; Koren, 2006).

The molecular weight (Table 1) and mass spectrum fragment ion data obtained for the intermediate precursors tyrindoxyl (**2**) and tyrindoleninone (**3**) is consistent with previous studies on Muricidae extracts. Both of these indole precursors have been detected using mass spectrometry from the hypobranchial gland extracts of

Nucella lapillus (Cooksey and Withnall, 2001), with **2** also detected in *Murex brandaris* (Michel et al., 1992), and *M. trunculus* (Andreotti et al., 2004), whereas **3** has been reported from egg mass extracts from *D. orbita* (Benkendorff et al., 2000). Also expected from previous studies was the HPLC peak corresponding to 6-bromoisatin (**6**), which is known to evolve from other dye precursors under oxidative conditions (Cooksey, 2001a). This compound was detected in both male and female hypobranchial glands, as well as the female capsule glands (Table 1) and has been previously detected in the egg masses of *D. orbita* (Benkendorff et al., 2000). The immediate precursor tyriverdin, however, was detected for the first time using ESI-MS. Field desorption/ field ionization MS of tyriverdin has been previously successful in identifying this intermediate (Christophersen et al., 1978), while chemical ionization and electrospray (positive ion) MS failed to produce a molecular ion (Benkendorff et al., 2000). It appears that a change in ionization mode to negative in the ESI-MS facilitates detection of this compound.

Detection of Tyrindoxyl sulfate (**1**) in male prostate and all female capsule, albumen and the majority of ingesting gland extracts (Table 1) strongly supports a role for indole derivatives in muricid reproduction. Tyrian purple precursors are thought to be synthesized in the branchial and rectal regions of the hypobranchial gland, before being transported by muco-cilliary action to the medial region for storage (Roller et al., 1995). Similar to other muricids (Middlefart, 1992a, b; Roller et al., 1995) the rectal region of the hypobranchial gland in *D. orbita* surrounds the ventral surface of the rectum, which is embedded in the prostate or capsule gland (Benkendorff et al., 2004). This apparent association could facilitate the transfer of dye precursors and pigments from their site of synthesis in the

hypobranchial gland to the adjacent prostate and capsule glands. Detection of the prochromogen (**1**) in more posterior female reproductive glands could be explained by residual biosynthetic activity, as muricid genital ducts are thought to arise from an ancestral right hypobranchial gland (Kay et al., 1998). Failure to detect intermediates or pigments (**2**-**7**) in the albumen and ingesting glands of females (Table 1) implies the absence of arylsulfatase, which is required for the hydrolysis of **1** (Fig. 1) (Dubois, 1909; Baker and Sutherland, 1968). By comparison, pigment production in the capsule and prostate glands (Table 1) suggests either the presence of arylsulfatase, in addition to **1**, or diffusion of hydrolyzed intermediates from the hypobranchial gland.

Detection of Tyrian purple precursors in the reproductive glands of *D. orbita* could provide a mechanism for incorporating bioactive intermediates into the egg masses of this species (see Benkendorff et al., 2001). Although the exact location of fertilization remains unclear, the capsule gland has been proposed in *N. lapillus* (Fretter, 1941). This poses a practical site for precursor incorporation into egg capsules as tyrindoxyl sulfate and arylsulfatase (Dubois, 1909; Baker and Sutherland, 1968) could be acquired from the adjacent hypobranchial gland. An alternative fertilization site in muricids is the albumen gland, where sperm are thought to pass from the duct of the ingesting gland into the albumen gland where eggs are received from the oviduct (Fretter, 1941). If so, it may be possible that the prochromogen is incorporated into albuminous secretions before being passed into the capsule gland along with fertilized eggs. Detection of **1** in male prostate gland extracts (Table 1) could provide further means for transferring high concentrations of bioactive dye precursors to the egg masses of *D. orbita*

(Benkendorff et al., 2001). As the prostate gland adds prostatic fluid to sperm within the pallial vas deferens during passage to the penis (Middlefart, 1992a, b), the prochromogen could also be incorporated into seminal secretions. During copulation, semen is released into the bursa copulatorix or ventral channel of the female uterus (Fretter, 1941), where it ultimately combines with albuminous secretions. Consequently, males could feasibly contribute tyrindoxyl sulfate to the intracapsular fluid constituent during capsule manufacture as an additional paternal investment. Previously it was suspected that precursors in the egg masses of *D. orbita* were of adult origin (Benkendorff et al., 2004; Westley et al., 2006), however the question remained as to how these compounds arrive in the egg capsules of this species. This investigation provides chemical evidence for Tyrian purple precursors and pigments in the reproductive system of a Muricidae.

Statistically significant sexual dimorphism was found in the chemical composition of Tyrian purple from the hypobranchial glands of *Dicathais orbita* (Fig. 4; P = 0.002). Our results support previous observations of a more blue-purple pigmentation in female hypobranchial muricid secretions, compared to the red-purple dyes gained from males (Fig. 2, Boesken Kanold pers. comm.; Verhecken, 1989; Michel et al., 1992). In the past, the blue hue of Tyrian purple secretions has been attributed to the presence of indigo (8) and/or 6-bromoindigo (10) (Verhecken, 1989; Michel et al., 1992; Benkendorff et al., 2004). However, neither of these blue compounds were detected during quantitative analysis of *D. orbita* extracts in this study (Table 1, 2), including glands in which blue pigmentation was clearly observed. This demonstrates the unreliability of simple colour observations for drawing conclusions about pigment composition in natural

samples. The sex-specific dye pigmentation in *D. orbita* is clearly not due to variations in purple dibrominated vs. blue non- or mono-brominated indigoids, but is rather due to the presence of precursors and structural isomers of Tyrian purple. The blue-purple colour of female secretions can be accounted for by the presence of blue-purple (5) and green (4), whereas the red hue of male dyes can be attributed to red-purple (7) and yellow (6) (Table 1, 2). Previous observations have also suggested that dye pigmentation depends on whether mantle integrity is maintained between the pallial gonoduct and the hypobranchial gland (Benkendorff et al. 2004). Blue secretions in “detached” glands were speculated to result from the evolution of indigo (8) in the absence of bromoperoxidase or bromine in the rectal hypobranchial gland or pallial gonoduct. However, in this investigation both “attached” and “detached” glands displayed consistent blue and purple pigment mixtures (Table 2), confirming that the sexual differences occur directly in the hypobranchial glands and are not dependant on the presence of reproductive glands.

The blue colouration observed in some of our *D. orbita* glands and extracts is most likely due to the molecular behavior of dibromoindigo. In solution or at low concentration, dibromoindigo can appear blue, while at high concentrations or as a textile dye, it develops a purple hue (Cooksey, 2001b). The blue pigmentation arises from monomers of 6,6’-dibromoindigo, while the formation of dimers or higher polymers gives a purple colour due to the van der Waals attraction between bromine atoms. This results in closer molecular stacking and shifts the absorption maximum towards the red (Cooksey, 2001b). Furthermore, as dibromoindigo displays a strong anisotropy of light absorption in the solid state

(Susse and Krampe, 1979), the absorption maximum (λ_{max} 540nm and 640nm) greatly depends on molecular orientation (Cooksey and Withnall, 2001). Consequently, the blue pigmentation in *D. orbita* secretions could result from the molecular arrangement and/or concentration of 6,6'-dibromoindigo, but is clearly not due to isolation from bromoperoxidase or bromine.

Examination of the dye genesis in male and female samples under natural conditions has interesting implications for differences in glandular physiology. For example, detection of the Tyrian purple isomer **7** and higher concentrations of 6-bromoisatin (**6**) in male hypobranchial glands suggests more aerobic conditions in comparison to female glands. It is hypothesized that **6** arises from the photochemical oxidation of the tyrindoxyl sulfate, tyrindoxyl or tyrindoleninone, among other intermediates (Cooksey, 2001a, 2006; Cooksey and Withnall, 2001). 6-bromoisatin (**6**) is considered to be a precursor to brominated indirubins (Clark and Cooksey, 1997; Cooksey, 2001a, 2006), which supports the evolution of **7** in male hypobranchial glands, where the highest concentrations of **6** were detected (Table 1). Surprisingly, **6** was not detected in conjunction with **7** in male prostate glands, thus suggesting a more complete reaction series in this gland. Conversely, in female extracts the presence of sulfur compounds, such as dimethyl disulfide and the intermediate dye precursors tyrindoleninone (**3**) and tyriverdin (**4**), suggests the female glandular environment may be more reducing than oxidizing. The presence of **4**, in conjunction with comparatively low percentages of **6** explains the evolution of 6,6'-dibromoindigo (**5**) instead of **7**. In the presence of sunlight, photoliable tyriverdin undergoes cleavage to yield dimethyl disulphide (McGovern and Michel, 1990; Cooksey, 2001a; Cooksey and

Withnall, 2001) and **5**. The liberation of dimethyl disulphide would help maintain a reducing environment (McGovern and Michel, 1990) resulting in increased yields of **5** and a relative decrease in the oxidation by-products **6** and **7**. Although oxygen availability appears to explain differences in male and female dye composition, the reason for this divergence in glandular chemistry requires further investigation.

Our LC-MS analyses of hypobranchial gland secretions from *D. orbita* provide the first chemical evidence for sex-specific genesis of Tyrian purple (Tables 1 & 2, Fig. 4) in the Muricidae. Sex-specific pigments that result in visual colouration occur in many species, including marine invertebrates (Bandaranayake, 2006). These visual colour differences generally aid in mate selection. However, in the case of the Muricidae, the pigmentation from hypobranchial gland metabolites does not exist as an external visual cue and thus is more likely to occur as a by-product of their biologically active precursor compounds (Benkendorff et al., 2000; Westley et al., 2006). Sex is a known determinant in the synthesis of antibacterial ceratotoxins in the Mediterranean fruit fly, *Ceratitis capitata* (Marchini et al., 1993; Rosetto et al., 1996), the venom of spiders (Rash and Hodgson 2002) and the allelochemical, sarcophytoxin, in the soft coral, *Sarcophyton glaucum* (Fleury et al., 2006). However, this is the first account of sex-specific secondary metabolite synthesis in the Mollusca and provides a good model for exploring the driving forces for such biosynthetic divergences in marine invertebrates. Indole derivatives have been documented in a huge range of marine invertebrates (Christophersen, 1983; Alvares and Salas, 1991), including one report of dibromoindigo in the marine acorn worm, *Ptychodera flava laysanica* (Higa and

Scheuer, 1976). But unlike most other species, we now have a good understanding of the biosynthetic origin and anatomical distribution of these indole derivatives in *D. orbita*. This will facilitate future physiological and ecological investigations without confounding from inappropriately pooling between sexes or biosynthetic organs.

Understanding the sexual differences in muricid brominated indoles could also have useful implications for future development of natural medicines from these molluscs. The purple secretion from muricids is currently listed on the Homeopathic Materia Medica (Westley et al., 2006), although chemical and pharmacological research to substantiate the bioactivity of this remedy is currently lacking. Nevertheless, recent studies indicate that brominated indirubins in muricid Tyrian purple inhibit cell proliferation with selectivity towards GSK-3α/β receptors (Meijer et al., 2003; Magiatis and Skaltsounis, 2006). Our results suggest that extracts from male muricids will yield the highest concentrations of these bioactive bromoindirubins. However, extracts from the females contain the intermediate precursors, tyrindoleninone and tyriverdin, with reported anticancer and bacteriostatic activity, respectively (Benkendorff et al., 2000; Westley et al., 2006; Vine et al., 2007). Consequently, there is much scope for future comparative studies to optimize extraction procedures for the development of novel natural remedies from these marine molluscs.

In conclusion, preservation of the glandular biochemistry, followed by quantification using HPLC-DAD coupled to ESI-MS, enables comparative investigations into the natural genesis of Tyrian purple in Muricidae molluscs.

Tyrindoxyl sulfate (**1**) and the immediate precursor tyriverdin (**4**) were detected for the first time by LC-MS, thus providing a suitable procedure for the simultaneous analysis of all brominated precursors and pigments. The ultimate precursor **1** was detected throughout the female reproductive system, thus presenting a means for maternal investment in the chemical defence of *D. orbita* egg masses. A significant difference in the chemical composition of extracts from male and female hypobranchial glands provides evidence for sex-specific biosynthetic routes to Tyrian purple production. This sexual dimorphism is likely to be governed by glandular physiology, giving rise to an oxidizing and reducing environment in males and females, respectively. Together these findings have useful implications for future investigations into the selective pressures influencing sex-specific metabolite biosynthesis in marine invertebrates and the development of bioactive muricid extracts.

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FIGURE LEDGENDS

FIGURE 1 Tyrian purple genesis from tyrindoxyl sulfate in the hypobranchial gland of *Dicathais orbita*.

FIGURE 2 Extract preparations (a) and dried pigments (b) from *Hexaplex (Murex) trunculus* (photos provided by Inge Boesken Kanold, France). Male hypobranchial gland extracts, complete with prostate glands (left), display purple/red pigmentation in comparison to the purple/blue hue of female hypobranchial and capsule gland extracts (right).

FIGURE 3 Liquid chromatography-mass spectrometry analysis of a typical extract from the hypobranchial gland of a female *Dicathais orbita*. The chromatogram obtained from the diode array at 300,600nm shows the relative composition of the dye precursors and pigments. Inset is the electrospray ionization mass spectrum obtained from the apex of the major chromatographic peak obtained at 5.1min showing dominant signals, which agree with the molecular mass (*m/z* 336, 338) and stable fragment ions of tyrindoxyl sulfate (**1**). Other peaks in the chromatogram correspond to tyrindoxyl/tyrindoleninone (**2/3**), tyriverdin (**4**), 6,6'-dibromoindigo (**5**) and 6-bromoisatin (**6**).

FIGURE 4: Two-dimensional nMDS plot for the Tyrian purple composition found in extracts from the hypobranchial glands of male and female *Dicathais orbita*. Data based on the percent composition of each precursor (**1-4**) and pigment (**5-7**) after square root transformation.

TABLE 1 High performance liquid chromatography (HPLC) retention times (t_R) and electrospray ionization mass spectrum (*m/z*) values for the various indole derivatives[a] in dimethyl formamide extracts of male and female glandular extracts from *Dicathais orbita*.

Dye Component	t_R (min)	*Major Ions* (*m/z*)	Males		Females			
			Hypobranchial	Prostate	Hypobranchial	Capsule	Albumen	Ingesting
Tyrindoxyl sulfate **1**	5.1-5.5 [b]	336, 338	89.66 ± 2.42	82.06 ± 14.86	92.21 ± 3.37	94.18 ± 0.55	100.00 ± 0.00	66.67 ± 57.74 [c]
Tyrindoxyl **2**/ Tyrindoleninone **3**	9.5-10.1	256, 258/ 255, 257	3.20 ± 0.92	0.00	3.72 ± 0.82	1.93 ± 0.91	0.00	0.00
Tyriverdin **4**	12.0-12.1	417, 419, 421 463, 465, 467 511, 513, 515	0.00	0.00	2.57 ± 1.24	2.20 ± 0.49	0.00	0.00
6,6'-dibromo-indigo **5**	14.4-15.4 [d]	417, 419, 421	0.12 ± 0.00	0.00	0.50 ± 0.35	0.96 ± 0.02	0.00	0.00
6-bromoisatin **6**	6.4-6.6	224, 226	6.40 ± 2.84	0.00	0.98 ± 0.96	0.72 ± 0.08	0.00	0.00
6,6'-dibromo-indirubin **7**	16.7-16.9	417, 419, 421	0.70 ± 0.35	17.94 ± 14.86	0.00	0.00	0.00	0.00

[a] The relative proportions of each compound were calculated from integration data taken at 300 and 600nm using a diode array in the HPLC, and expressed as the mean percent of total dye composition ± SD, where N= 3. It should be noted that extinction coefficients are not available for all these compounds, thus prohibiting adjustment of integrated peak values for absolute quantification. Therefore percentages of total dye composition should be viewed as relative for comparison between samples rather than actual compound proportions.

[b] Molecular ions corresponding to **1** in ingesting glands registered for peaks at 7.0 and 7.9min. Despite shifts in t_R, TLC analysis confirmed the presence of **1** in these extracts at R_f 1.0.

[c] **1** was absent from one ingesting gland extract, but represented 100% dye composition in the two remaining replicates.

[d] The large t_R range for **5** is due to a shift downfield in some extracts after HPLC column replacement (Appendix 1).

TABLE 2 Liquid chromatography-mass spectrometry analyses of Tyrian purple precursors and pigments in dimethyl formamide extracts of attached and detached male and female hypobranchial glands.

Dye Component	Male		Female	
	Attached [a]	Detached [b]	Attached [a]	Detached [b]
Tyrindoxyl sulfate **1**	+++	+++	+++	+++
Tyrindoxyl **2**/ Tyrindoleninone **3**	+++	+++	+++	+++
Tyriverdin **4**	-	-	+++	+++
6,6'-dibromoindigo **5**	+	-	+++	+++
6-bromoisatin **6**	+++	+++	+++	+++
6,6'-dibromoindirubin **7**	+++	+++	-	-

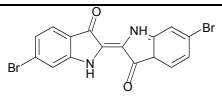
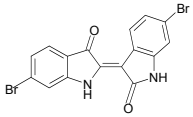
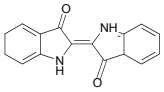
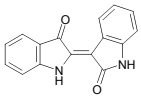
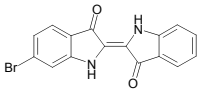
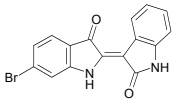
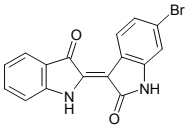
[a] Attached extracts represent glands in which connection with the pallial gonoduct was maintained (N=3).

[b] Detached extracts comprise hypobranchial glands excised from the prostate or capsule gland (N=3).

"+++" indicates presence in all three replicate extracts; "+" indicates presence in one of three replicate extracts; "-" indicates absence in all three replicate extracts.

APPENDIX 1

Characteristics of indigoid and indirubin standards and diagnostic parameters obtained by liquid chromatography-mass spectrometry.

Compound Number	Standard	Structure	$[M-H]^+$	t_R
5	6,6'-dibromoindigo[1]		417, 419, 421	15.4
7	6,6'-dibromoindirubin		417, 419, 421	16.5
8	Indigo		261	10.7
9	Indirubin		261	10.5
10	6-bromoindigo		339, 341	12.7
11	6-bromoindirubin		339, 341	13.5
12	6'-bromoindirubin		339, 341	13.2

$[M-H]^+$ = the pseuomolecular ion (Br^{79}, Br^{81}) registered as the dominant signal in ESI mass spectrums in the negative ionization mode.
t_R = the retention time in minutes.
[1] Female extracts displayed a shift in retention time compared to the synthetic standard (table 1). This was attributed to HPLC column replacement (despite identical specifications). To confirm the identity of dye components, a female hypobranchial extract was spiked with the dibromoindirubin standard, which also contained trace amounts of the dibromoindigo isomer. In comparison to un-spiked extracts, an increase in relative peak intensity in the spiked extract at 14.5min and an additional peak at 16.0min confirmed the dominance of dibromoindigo in female extracts. Subsequent re-analysis of male extracts confirmed that the retention time shift was due to column replacement rather than any specific properties of the female extracts.

Figure
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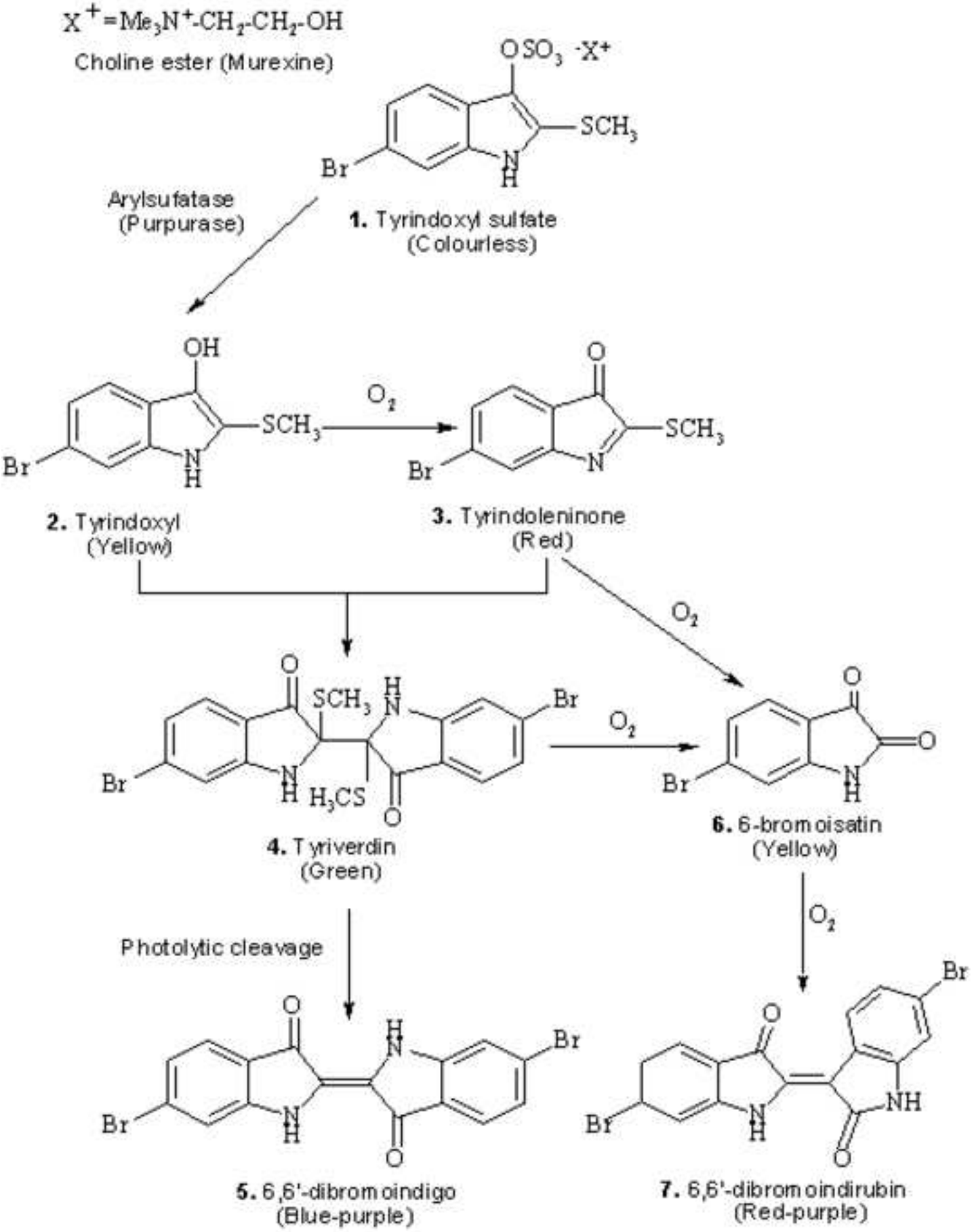

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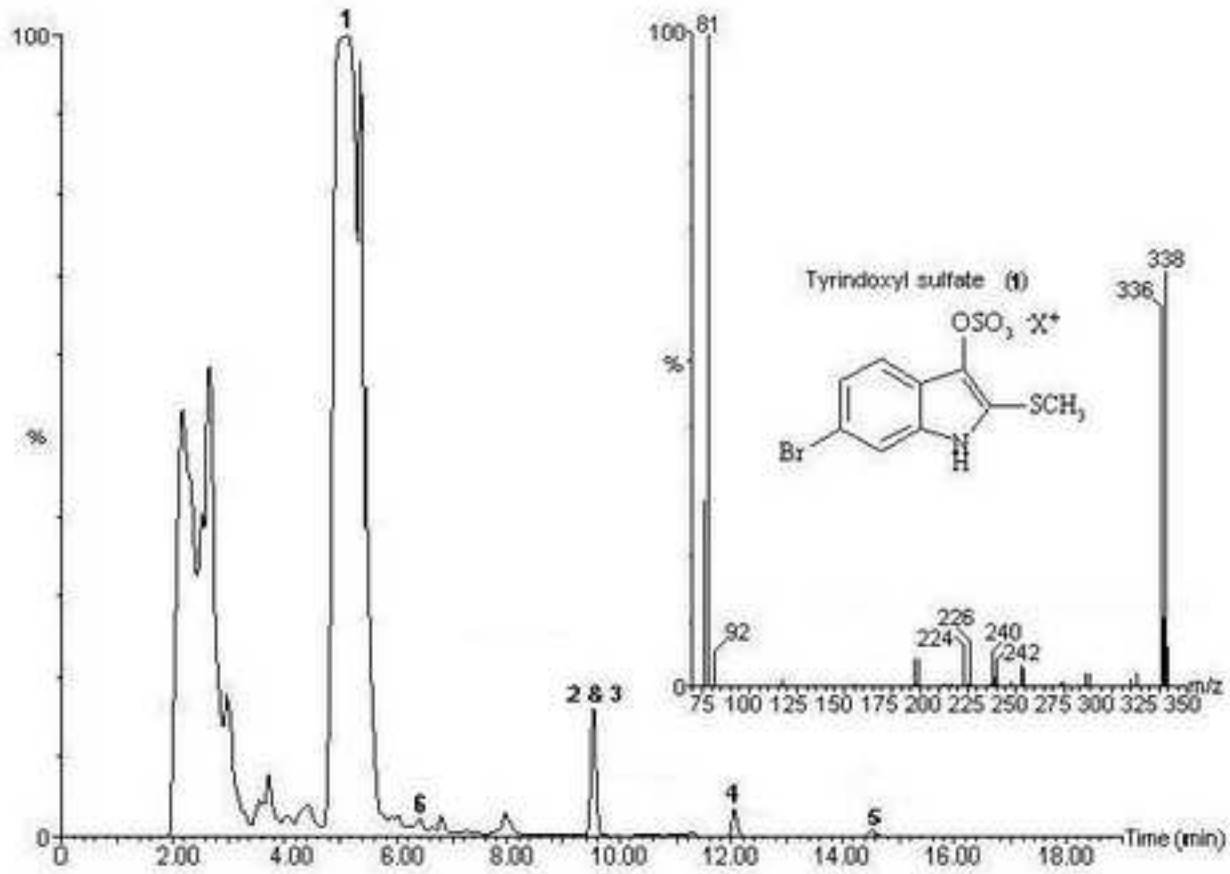
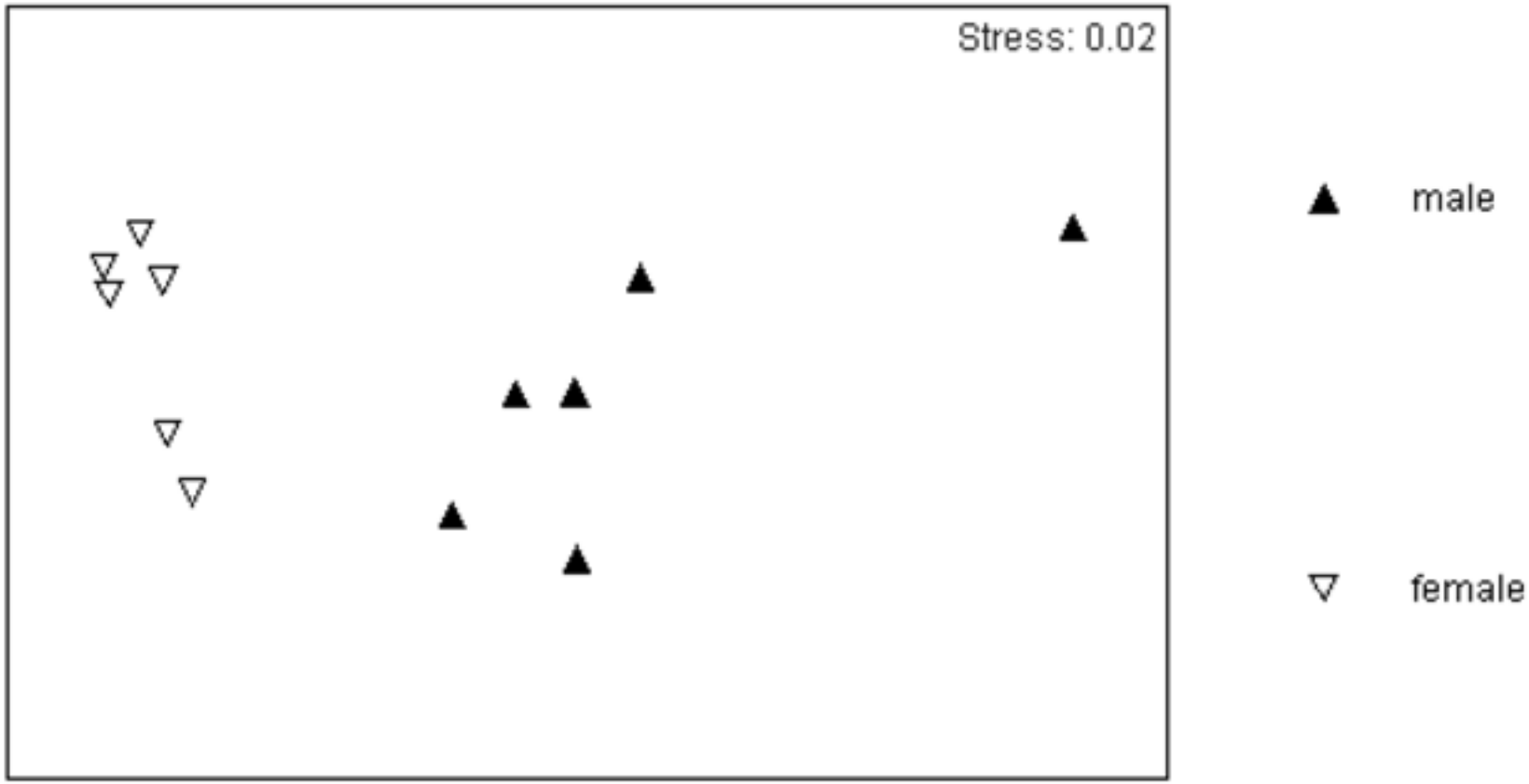
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