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Application of anaesthetics for sex identification and bioactive compound recovery from wild *Dicathais orbita*

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ABSTRACT

Anaesthetics are used extensively on marine molluscs for non-destructive sampling and to manipulate specimens in ecological studies and aquaculture. *Dicathais orbita* is an edible southern Australian muricid (Neogastropoda) with potential for use as an indicator species for ecological monitoring and new species development in aquaculture. This species produces bioactive compounds that are currently under investigation for the development of a novel anticancer therapy. No previous studies have investigated the use of anaesthetics to collect bioactive compounds. Thus, a suite of anaesthetics were trialled for their efficacy in relaxing *D. orbita* out of the shell to

identify sex and for stimulating bioactive compound production. The recovery time significantly varied between the different anaesthetic applications ($p < 0.001$). Magnesium chloride proved most effective in relaxing specimens enough to identify sex and recovery time did not differ from the seawater control ($p > 0.05$). This method was successfully applied to six populations of *D. orbita* in order to establish a 1:1 sex ratio at 6 sites in South Australia. No evidence of imposex was observed at any location. Benzocaine and the carrier solvent ethanol were less effective for identifying sex, but stimulated expulsion of the bioactive precursors. This indicates that ethanol may be inducing a stress response in these gastropods rather than a standard anaesthetisation. Consequently, the most suitable anaesthetic for use on gastropods will depend on the specific use and requires testing for species specific responses.

Introduction

The individual disciplines of ecology, marine conservation, aquaculture and bioprospecting can be considered to be intrinsically linked. Aquaculture can be regarded as a conservation tool by ameliorating the strain on wild fisheries through making up the shortfall between wild fisheries production and consumer demand (Naylor et al., 2000) and providing seed stock for wild fisheries restocking (Seto and Doi, 2000). However, bioprospecting and aquaculture success depends on emulating aspects of ecology (Folke and Kautsky, 1989; Henderson et al., 2001). In turn, sustainable production of bioactive compounds discovered through bioprospecting can be managed through aquaculture (Sipkema et al., 2005; Benkendorff, 2009). Hence, research into species of

interest for aquaculture, bioprospecting, ecological monitoring or conservation can ultimately turn to one of the other disciplines at discrete stages of development.

A pertinent example of these multidisciplinary research interests is provided by marine molluscs in the family Muricidae (Neogastropoda). Severe population disruption of some commercially fished muricids (whelks) has provided impetus for research into aquaculture of this family to ensure sustainable supply (Nugranad et al., 1994; Xavier Ramesh et al. 1994; Gutiérrez and Gallardo, 1999; Woodcock and Benkendorff, 2008). Muricids are also highly valued for the production of their purple dye secretion, best known as Tyrian purple (Cooksey, 2001; Naegel, 2004). The bioactive precursors of Tyrian purple are of continuing interest for antibiotic and anticancer activities (Benkendorff et al., 2000; Westley et al., 2006; Meijer et al., 2003; Naegel and Murillo Alvarez 2005; Benkendorff et al., 2009). Ecologically speaking, the predatory and scavenging nature of muricid whelks also makes them important in structuring the biota within their habitat (Fairweather, 1988; Hunt and Scheibling, 1998; Stewart and Creese, 2004). Hence, the presence of healthy populations of these gastropods can indicate relative health and changes to ecosystems (Smith, 2005). Furthermore, imposex, development of a pseudo penis in female Muricidae, is used as an indicator for pollution from endocrine disruptors (Axiak et al., 2003; de Castro et al., 2004; Fujinaga et al., 2006).

The ability to accurately identify the sex of whelk specimens is therefore important for a wide range of ecological and aquaculture applications. Information on the sex ratio of populations and any incidence of imposex is required to determine effective population size for ecological monitoring. Non destructive sex determination is required for studying mating and breeding behaviours and facilitates selective breeding programs for aquaculture. Additionally, sex identification may prove useful for bioprospecting, as some species may produce sex specific secondary metabolites or chemical compositions (e.g. *Dicathais orbita* (Gmelin) Westley and Benkendorff, 2008). Sex identification is relatively simple in dioecious species, if the sex of specimens can be established by the presence or absence of an external penis (Hargis, 1957). However, whelks seal themselves inside the shell when disturbed, pulling their operculum closed. Methods of mass relaxation, that reduce individual handling, could greatly increase the efficiency of sex identification for large scale ecological surveys, breeding programs and bioprospecting purposes.

A range of anaesthetics are used extensively on marine molluscs for non-destructive sampling in ecological studies (Prince and Ford, 1985; McShane and Smith, 1988; Vasconcelos et al*.,* 2006) and in aquaculture to manipulate specimens (Heasman et al., 1995; White et al*.*, 1996; Butt et al., 2008; Acosta-Salmon and Davis, 2007). The most commonly used with gastropods are potassium chloride, sodium pentobarbital, magnesium chloride, ethanol, carbon dioxide gas and benzocaine (Culloty and Mulcahy, 1992; Aquilina and Roberts, 2000; Edwards et al., 2000; Butt et al., 2008).

However, no previous studies have specifically compared the use of different anaesthetics for sex determination in marine gastropods. There are also no available reports on the use of anaesthetics to collect bioactive compounds from marine organisms. However, on the coast of Central America, regular collection of secretions from the muricid *Plicopurpura pansa* is achieved by mechanical stimulation of each snail (Rios-Jara et al., 1994; Michel-Morfín and Chávez, 2000; Naegel, 2005). This non-destructive method for collecting valuable purple dyes was considered an exception among muricids (Naegel and Murillo-Alvarez, 2005), with all previous work being performed on the hypobranchial glands dissected from large numbers sacrificed snails (e.g. Baker and Sutherland, 1968; Michel et al., 1992; Westley and Benkendorff, 2008) or their egg masses (Benkendorff et al., 2000). However, purple dye has been occasionally observed in association with the feeding activities of Muricidae (Westley et al., 2006) and Verhecken (1989) reported that some muricids produce a mass of foamy mucus that colours purple when captured. This implies that it may be possible to induce the external secretion of bioactive precursors to Tyrian purple, either by relaxation or as part of a stress response.

In this study, a number of anaesthetics were trialled for their efficacy in relaxing specimens for sex determination and inducing the secretion of bioactive compounds in the muricid, *D. orbita*. Natural sex ratios and the incidence of imposex are currently unknown for South Australian populations of *D. orbita*. Phillips (1969) reported a 1:1 ratio from Rottnest Island in Western Australia and noted that it is not possible to sex these animals with certainty whilst they are still alive due to problems in getting them to

extrude themselves from the shell enough to observe the penis. Anaesthetics were trialled for their effectiveness in non-destructive sex determination and stimulating release of bioactive compounds. Detailed chemical analysis of secretions was also performed to confirm the presence of bioactive compounds within the secretions. The most effective anaesthetic was used to sample wild populations from six locations in South Australia to test the applicability of this method for large scale sex identification for the purpose of ecological monitoring.

Methods

Pilot study

Six anaesthetic treatments were tested at two concentrations for their efficacy in relaxing *D. orbita* out of the shell (Table 1). This allows the sex of the specimen to be identified via the presence or absence of a penis (Hargis, 1957). A control for this part of the study consisted of fresh seawater. For each treatment and control, six animals of varying shell length were placed in a bucket containing 2L of aerated fresh seawater. Animals were placed in the bucket upside down to check for righting response and allowed to acclimatize for 1h prior to addition of treatment. In all experiments, all animals righted themselves within the hour long acclimatization and moved from the position they were originally placed in. Righting response was used as the measure of recovery after treatment. Water temperature remained stable at 18^{o}C for each trial. Observations for signs of relaxation of the foot muscle were made continually throughout the experiment.

Animals were tested for relaxation by removing them from the treatment and gently but firmly pulling on the operculum to withdraw the animal enough to view the area where the penis would be present in males (Figure 1). All animals were tested for relaxation by this method at the conclusion of one hour, if no visible sign of relaxation was observed prior to this time. Animals were returned to buckets containing aerated fresh seawater in an upside down position as soon as they had been sexed and were continually monitored for recovery. Recovery was measured as the time taken for righting response to occur. After this time all animals were returned to their aquaria in the presence of ample food and shelter and monitored visually daily for 5 days.

Anaesthetic trial

Based on the success of this pilot trial (Table 1) a more detailed study was undertaken on the four most promising chemicals dissolved in seawater: 1mg/L sodium pentabarbital, 200 $mg.L^{-1}$ benzocaine, 5% ethanol, 0.5 M magnesium chloride and a control consisting of fresh seawater. This study involved selecting ten individuals from each of three shell length size classes; small (23-33 mm), medium (33.5-53 mm) and large (>53.5 mm). Whelks were tagged with numbered tags (Hallprint Pty Ltd, Victor Harbour, South Australia, Australia) for identification purposes. Each animal was tested once with one treatment only. Procedures were the same as for the pilot trial; each animal was placed on the dorsal surface of its shell in 2 L of aerated sea water and allowed to acclimatize for 1 h. The treatment was then slowly added to the bucket. Specimens were checked for relaxation after 1 h. Size class was recorded, as was success in sexing, and the sex, if identified. After anesthetization treatment, whelks

were transferred to tubs containing aerated seawater for recovery. Recovery time was recorded and animals were returned to aquaria in the presence of ample food and shelter and visually monitored daily for 5 days. On the sixth day, three male and three female whelks from each size class were sacrificed for dissection of the gonads. Observation of the ingesting and capsule glands in females and a prostate gland and penis for males confirmed that sex had been identified correctly.

Bioactive compounds

Observations were also made on whether each anaesthetic induced *D. orbita* to expel the bioactive precursors to Tyrian purple. Mucous strands and white and yellow 'flecks' were collected from around the aperture of treated whelks and were observed for the development of a purple colouration after exposure to sunlight. Triplicate samples of the collected mucus were also extracted in chloroform (AR grade Sigma), according to Benkendorff et al. (2000). The dried extract was redissolved in DCM (HPLC Grade Sigma) and analysed by liquid chromatography-mass spectrometry (LC-MS). This was achieved by separating the compounds on a high performance-liquid chromatographer (HPLC, Waters Alliance) coupled to a mass spectrometer (MS, Micromass, Quatro micro™) according to the protocol of Westley and Benkendorff (2008). HPLC was carried out using a Phenomenex, Synergi, Hydro-RP C_{18} column (250 x 4.6 mm x 4 µm). Parallel UV/Vis diode-array detection (DAD) at 300 and 600 nm was used. A flow rate of 1mL.min^{-1} of 0.1% formic acid in acetonitrile was used with an increasing gradient of acetonitrile in water from 30% for 1 min, then 60% acetonitrile for 3 min and then 100% acetonitrile for 15 min. This was then returned to 30% for 15 min.

Predominant compounds within the extracts were detected using electrospray ionisation-mass spectrometry (ESI-MS) and identified by known retention time and doublet (monobrominated) or triplet (dibrominated) mass ion clusters for Br^{79} Br^{81} (Westley and Benkendorff, 2008).

Sex identification in wild populations

A total of over 600 specimens (shell length 19-101.5 mm) were collected at random from six sites along the coast of South Australia (Figure 2). Three sites were selected on the metropolitan coast of the Fleurieu Peninsula: O'Sullivans Beach, Marino Rocks and Brighton jetty. The remaining three sites were located on the Eyre Peninsula: Lipson Cove, Boston Point, and an abalone sea ranch at Elliston. All sampling of wild populations occurred from October 2007 to March 2008.

All animals were placed in a 40 L tub with approx 5 L of continually aerated water and allowed to recover from handling. A solution of magnesium chloride (final concentration 0.5M) was added to the tub slowly to allow mixing with minimal disturbance. The specimens were left for 1 h and then assessed for relaxation. Sex was recorded and shell length was measured with vernier callipers. Each animal was then placed back in a recovery tub containing 30 L of aerated fresh seawater until recovery had occurred.

Analysis

Data was analysed using SPSS ver. 14. Mean recovery time was cube root transformed to satisfy the assumptions of normality and homogeneity of variance. A two-way ANOVA was used to test mean recovery time for each shell size class and treatment, followed by Tukeys HSD post hoc test.

Mean shell length of male and female *D. orbita* from wild populations was square root transformed to satisfy the assumptions of normality and homogeneity of variance. This data was then compared between sites and genders using a two-way fixed factor ANOVA followed by Tukeys HSD post hoc test. Sex ratio was analysed for deviation from 1:1 male:female using the chi squared test.

Results

Anaesthetic trial

Of the four anaesthetic treatments identified as promising for use in sex identification from the pilot study (Table 1), magnesium chloride proved most effective (96.6% sexed, Table 2). Ethanol ranged from 40-70% successful across the three size classes (Table 2). None of the other anaesthetics produced enough relaxation within the one hour time period to identify sex.

All whelks recovered within 1.5 h of anaesthetic treatment (Figure 3). Results of the two-way ANOVA for recovery time showed there was no interaction between shell size class and treatment ($F=0.579$, $P= 0.794$). However, recovery time for different shell size classes ($F=4.7$, $P=0.011$) and anaesthetics ($F=6.294$, $P<0.001$) was significantly

different (Figure 3). *D. orbita* in the large size class take significantly longer to recover than the medium size classes (P=0.008). However, there was no significant difference in recovery time between small and medium size classes (P=0.45) or between small and large size classes (P=0.162). With regard to recovery time from each treatment, magnesium chloride, benzocaine and ethanol did not differ significantly from the control (P>0.05). Recovery time from sodium pentobarbital was significantly longer than from magnesium chloride (P=0.001), benzocaine (P=0.000) and the control (P=0.033, Figure 3), but not from ethanol (P=0.338).

Dissections confirmed that sex identifications based on presence or absence of a penis was accurate in all specimens and no evidence of any imposex was detected i.e. all specimens identified as male had a fully formed penis and prostate gland, whereas only females had ingesting or capsule glands. Furthermore, imposex has not been observed in hundreds of additional *D. orbita* specimens dissected from South Australia in our laboratory over the last five years (pers. obs., Westley et al., 2009).

Bioactive compound production

Although less effective in identifying sex, ethanol did stimulate the expulsion of mucus that turned purple on exposure to light (Table 2). Benzocaine (dissolved in ethanol) also stimulated purple precursor expulsion. Analysis of the chloroform extracts of the mucus from *D. orbita* by LC-MS revealed six peaks corresponding to brominated indoles (Figure 4). The dominant compound present within this crude extract registered an HPLC peak detected at 11.23 min (D) with a molecular mass *m/z* 255,257

corresponding to tyrindoleninone (Figure 4). The second most dominant peak within this extract occurred at 12.02 min (E) with an isotopic cluster at *m/z* 511, 513, 515 corresponding to the molecular ion of tyriverdin [MH^+; $Br^{79}Br^{79}$, Br^{79} Br^{81}, $Br^{81}Br^{81}$] A slightly smaller proportion of 6-bromoisatin was detected at 6.39 min (B, Figure 4) with major ions at *m/z* 224, 226. A smaller peak registered at 9.48 min (C) with major ions at 255/257 and 256/258 corresponding to tyrindoxyl/tyrindoleninone. A minor HPLC peak registered at 5.5 min with major ions at *m/z* 336, 338 was identified as tyrindoxyl sulphate (A, Figure 4). An extremely small peak was also detected with the 300nm diode array at 14.02 min (F), with an isotopic cluster at *m/z* 417, 419, 421 and identified as 6,6'-dibromoindigo. In replicate extracts, the same compounds were detected but in varying relative concentrations.

Sex ratio and shell length of wild populations

The application of 0.5 M magnesium chloride to sex identification in field populations had a success rate of > 94% across all sites (Table 3). The sex ratio at each site did not differ significantly from 1:1 (χ^2=0.159, P=0.690, Figure 5). On a number of occasions the sites were observed the day following experiments to confirm recovery *in situ*. Selectively marked individuals from the trial were observed feeding or actively hunting live prey.

The mean shell length of male and females differed between sexes and sites (Table 3). There was no interaction between site and gender (F=0.506, P=0.772). However, male *D. orbita* were consistently larger than females (F=4.278, P=0.039). Mean shell length

was also significantly different between sites (F=32.031, P<0.001). The Brighton jetty population had the largest mean size (72.6 mm) and the largest individual (101.5 mm). *D*. *orbita* from this site were significantly larger than *D. orbita* from all other sites (P< 0.001). Specimens from Boston Point on the Eyre Peninsula were shown to be significantly different to specimens from all sites except Lipson Cove (P < 0.001), another natural reef in the Spencer Gulf. Specimens from the abalone sea ranch at Elliston (mean 48.4 mm) were significantly smaller than specimens from all other sites, except the two natural reefs at O'Sullivans Beach (P=0.356) and Marino (P=0.344, Table 3), south of Adelaide in the Gulf St Vincent. .

Discussion

Anaesthetics can be successfully applied to non-destructive sex identification and the collection of bioactive Tyrian purple precursors from the Muricidae *D. orbita*. No mortality occurred during or up to 5 days after experimentation with any treatment in this study, even after the expulsion of mucus containing bioactive precursors. Using mechanical stimulation on the Central American muricid *P. pansa*, Naegel (2005) reported that although a decline in expulsion of compounds occurred throughout 98 days of daily collection, mortality was less than 18%. However, previous work on *P. pansa* by Michel-Morfín and Chávez (2000) revealed that mortality from repeated collection of dyes was highest (25%) after the fifth collection at weekly intervals. This indicates that Muricidae can generally survive the stress of handling, anaesthetisation and some repeated "milking" of their secondary metabolites. Nevertheless, further

research will be required to establish whether there are any long-term effects of anaesthetisation on reproduction and biosynthetic processes in *D. orbita*.

The six anaesthetics originally trialled at the pilot study stage have been used on one or more species of mollusc for the purpose of anaesthetisation. The tremendous variability in success of these treatments for relaxation and sex identification indicates the species specificity of anaesthetics for particular uses within this phylum. The primary evaluation parameter for the effectiveness of anaesthetization treatments in this study was the ability to relax the animals enough to observe the presence or absence of a penis. From this perspective magnesium chloride was easily the most effective treatment (mean=99.6% sexed). The reliability of this method for sex identification in *D. orbita* was confirmed by dissection and observation of the gonadic organs. Other studies using magnesium chloride support its use for sex identification in a wide range of molluscs (Messenger et al*.,* 1985; Culloty and Mulcahy, 1992; Coney, 1993; Acosta-Salmón and Davis, 2007; Butt et al., 2008). Sodium pentobarbital has been used to great effect in the narcotisation of Haliotidae (White et al., 1996; Aquilina and Roberts, 2000; Sharma et al., 2003). However, this anaesthetic only produced minimal relaxation in 20% of oysters, *Ostrea edulis* (Linnaeus) (Culloty and Mulcahy, 1992). Similarly, in the case of *D. orbita,* the pilot study indicated a tendency towards relaxation. However, in more detailed experimentation on the various sizes classes, the overall effect was for specimens to withdraw into the shell with the operculum closed or nearly closed, preventing successful sex identification. Recovery from sodium pentobarbital also took

significantly longer than most other treatments. Importantly however, recovery from the two anaesthetic treatments that were found to be most useful in this study (magnesium chloride and ethanol) was not significantly different to the seawater controls. Animals were observed actively feeding in the days post-recovery, although the potential for long-term sublethal effects cannot be excluded.

In *D. orbita*, the recovery time after exposure to anaesthetics can depend on size. Recovery in snails greater than 53cm took up to twice as long, and was more variable when compared to the smaller animals. Metabolic rate in marine invertebrates varies inversely with increasing body mass (Von Bertalanffy, 1957). Hence, it is possible that increasing recovery times for larger specimens of *D. orbita* are due to the difference in metabolic rate. Edwards et al. (2000) found that larger mean sized specimens of *Haliotis rubra* (Leach) showed lower daily growth rates than the smaller mean sized *Haliotis laevigata* (Donovan) after treatment with benzocaine. This implies that the immediate recovery times could be indicative long-termer effects of anaesthetics, which appear to vary as a function of species and size. Delayed recovery was observed in larger animals across all treatments, as well as in the seawater control. Consequently, it is possible that larger animals are responding differently to the effects of handling than smaller animals due to previous experience. Although kept to a minimum in this study, handling specimens may have the same effect as predation attempts in the natural environment (Alexander and Kovich, 1991). Larger animals have presumably survived predation attempts by remaining hidden within the shell for longer, a key defence

against predation in marine gastropods (Bertness et al., 1981). Delgado et al. (2002) demonstrated this with queen conch (*Strombus gigas*, Linneaus), where defensive response time for specimens previously exposed to predators was more acute than those with no prior exposure.

The application of ethanol and benzocaine was effective for relaxing *D. orbita*, and ethanol was sometimes useful for sex identification, although benzocaine tended to cause greater retraction into the shell. In studies on Haliotidae, both ethanol and benzocaine proved effective in detaching animals from the substrate (Prince and Ford, 1985; Edwards et al. 2000). Aquilina and Roberts (2000) reported that in haliotids, benzocaine dissolved in ethanol had the effect of making the podial muscle hard and contracted. In a study on *S. gigas,* Acosta-Salmón and Davis (2007) report that benzocaine caused to animal to 'kick' before withdrawing into the shell with no resultant relaxation. Hence it seems that treatment with ethanol and benzocaine has the effect of disrupting the control of the podial muscles rather than an anaesthetic effect. This treatment also appears to also cause a stress response in *D. orbita*, resulting in the expulsion of mucus containing bioactive Tyrian purple precursors. Thus the application of ethanol appears to cause similar effects to the mechanical 'molestation" of the podial muscle and operculum of *P. pansa* (Rios-Jara et al., 1994; Michel-Morfín and Chávez, 2000; Naegel, 2005). As no purple mucus was produced from relaxed specimens treated with sodium pentobarbital or magnesium chloride, it appears that the expulsion

of this mucus is a stress response possibly caused by muscle contraction, rather than muscle relaxation.

Liquid chromatography mass spectrometry conclusively identified the bioactive brominated indole precursors to Tyrian purple within the mucus secreted by *D. orbita*. Previously these compounds have been only been obtained destructively from the hypobranchial glands, reproductive glands and egg masses of this species (Baker and Sutherland, 1968; Benkendorff et al., 2000; Westley and Benkendorff, 2008). The variation in relative proportions of the different brominated indoles in replicate extracts is most likely the result of varying exposure to oxygen and sunlight, which induces a series of oxidative and photolytic reactions in the precursor compounds (Benkendorff et al., 2000; Cooksey, 2001; Westley and Benkendorff, 2008). Importantly, a high proportion of the anticancer compound tyrindoleninone (Benkendorff et al., 2009) was detected in all of the mucus extracts analysed, making this mucus extract a viable source for future research and development.

Application of magnesium chloride in the field proved to be a highly efficient method for sex identification in natural populations. Over a hundred animals were sexed within two hours at each location. The sex ratio of *D. orbita* did not differ significantly from 1:1 across six locations in South Australia, encompassing natural reefs located on different Peninsulas in the two separate Gulfs, as well as an artificial jetty and an abalone sea ranch. This result is consistent with previous investigations of *D. orbita* (syn. *aegrota*) on Rottnest Island in Western Australia (Phillips, 1969) and indicates that the effective

population size is not different from the actual population size for this species. Furthermore, no evidence of imposex was observed in this study. If heavy metal pollution or some other factor was causing imposex at any of these sites, a greater proportion of male characteristics should have been observed. In muricid populations where imposex is known to occur, effective population size is limited by a skewed sex ratio (Cole, 1941; Ramon and Amor, 2002; Fujinaga et al*.,* 2006).

Significant spatial variation was found in the mean size of *D. orbita* within South Australia. Significant variation in size has been previously reported for different populations of *D. orbita* at a larger spatial scale, throughout this species geographic range (Phillips, 1969). It is common for local conditions to affect the relative size and condition of gastropods e.g. (Bayne and Widdows, 1978, Burrows and Hughes, 1990, McShane et al., 1994). Most notable from this study is the extreme size variation between the two sites most subject to anthropogenic influence; Brighton Jetty and Elliston. The Brighton Jetty population has larger individuals than other sites and is fed by regular bait lost from people fishing from the jetty. Whelks are often observed feeding on carrion at this site (pers. obs). It is common for a varied diet to increase growth of whelks, especially if a component of carrion is included that requires less energy than live prey (Nasution and Roberts, 2004; Woodcock and Benkendorff, 2008). Furthermore, regular observation at this site indicates that very little or no recreational harvest occurs here, as few people swim out to the deeper jetty pylons where the majority of whelks are clustered. Conversely, the abalone sea ranch at Elliston provides an artificial habitat that is regularly cleaned by divers and this population had

significantly smaller size whelks than most other sites. The whelks naturally recruit onto the nets of the sea ranch where there is diverse array of prey. However, as the abalone stock is also vulnerable to predation by the whelks (e.g. Woodcock and Benkendorff, 2008) divers are employed to regularly remove the whelks. The natural reefs in South Australia are protected by legislation that prohibits the taking of any benthic creature to a depth of 2 metres (Fisheries Act, 1982, revised 2007). Nevertheless, some people have been observed to ignore these laws and target *D. orbita* along with other organisms, particularly at Marino Rocks, which is close to the metropolitan city of Adelaide (pers. obs.). The relatively isolated nature of Lipson Cove and Boston Point may reduce the overall harvest of whelks from these sites thus accounting for a larger mean size.

Overall, this study provides important baseline data for future monitoring of *D. orbita* populations in the face of increasing anthropogenic pressures. Magnesium chloride has been identified as a suitable anaesthetic for sex identification in both laboratory and field based studies. Assuming there are no long-term adverse effects from the short-term anaesthetic exposure, magnesium chloride is likely to be the most useful anaesthetic for large scale sexing for future aquaculture or breeding programs in the Muricidae. Conversely, ethanol appears to cause muscle contraction indicating a stress response in molluscs. This has proved useful for enabling non-destructive collection of the bioactive brominated indoles produced by *D. orbita.*

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References

Acosta-Salmón, H., Davis, M., 2007. Inducing relaxation in the queen conch *Strombus gigas* (L.) for cultured pearl production. Aquaculture 262, 73–77.

Alexander, J.E., Kovich, A.P., 1991. Predation risk and avoidance behavior in two freshwater snails. Biol. Bull. 180, 387-393.

Aquilina, B., Roberts, R., 2000. A method for inducing muscle relaxation in abalone *Haliotis iris*. Aquaculture 190, 403-408.

Axiak,V., Micallef, D., Muscat, J., Vella, A., Mintoff, B., 2003. Imposex as a biomonitoring tool for marine pollution by tributyltin: some further observations. Environ. Int. 28(8), 743-749.

Baker, J. T., Sutherland, M. D., 1968. Pigments of marine animals VIII. Precursors of 6,6′- dibromoindigotin (Tyrian purple) from the marine mollusc *Dicathais orbita* Gmelin. Tetrahedron Lett. No. 1:43–46.

Bayne, B.L., Widdows, J., 1978. The physiological ecology of two populations of *Mytilus edulis* L. Oecologia 37, 137–62.

Benkendorff, K. 2009. Aquaculture and the Production of Pharmaceuticals and Nutraceuticals. In *New technologies in aquaculture: Improving production efficiency, quality and environmental management*. Ed. G. Burnell and G. Allen. Woodhead Publishing, Cambridge, 39pp.

Benkendorff, K., Bremner, J.B., Davis, A.R., 2000. Tyrian purple precursors in the egg masses of the Australian muricid, *Dicathais orbita*: a possible defensive role. J. Chem. Ecol. 26(4), 1037-1050.

Benkendorff, K., McIver, C. Abbott, C.A. 2009. Bioactivity of the *Murex* homeopathic remedy and of extracts from an Australian muricid mollusc against human cancer cells. Evidence-based Complementary and Alternative Medicines. Provisionally accepted, Feb, 2009.

Bertness, M.D., Garrity, S.D., Levings, S. C., 1981. Predation pressure and gastropod foraging: a tropical-temperate comparison. Evolution 35, 995-1001.

Burrows, M. T., Hughes, R. N., 1990. Variation in growth and consumption among individuals and populations of dogwhelks, *Nucella lapillus* : a link between foraging behaviour and fitness. J. Anim. Ecol. 59(2), 723-742.

Butt, D., O'Connor, S.J., Kuchel, R., O'Connor, W.A., Raftos D.A., 2008. Effects of the muscle relaxant, magnesium chloride, on the Sydney rock oyster (*Saccostrea glomerata*). Aquaculture 275, 342–346.

Cole, H.A., 1941.Sex-ratio in *Urosalpinx cinerea*, the American oyster drill. Nature 147, 116-117.

Coney, C.C., 1993. An empirical evaluation of various techniques for anesthetization and tissue fixation of freshwater Unionoida (Mollusca: Bivalvia), with a brief history of experimentation in molluscan anesthetization. Veliger 36, 413-424.

Cooksey, C., 2001Tyrian Purple: 6,6'-dibromoindigo and related compounds. Molecules 6, 736-769.

Culloty, S. C., Mulcahy, M. F., 1992. An evaluation for anesthetics for *Ostrea edulis*. Aquaculture 107, 249-252.

de Castro, I.B., Meirelles, C.A.O., Mathews-Cascon, H., Antonio, M., 2004. *Thais (Stramonita) rustica* (Lamarck, 1822) (MOLLUSCA: GASTROPODA: THAIDIDAE), a potential bioindicator of contamination by organotin, northeast Brazil. Braz. J. Oceanog. 52(2),135 –139.

Delgado G. A., Glazer R. A., Stewart N. J. 2002. Predator-induced behavioral and morphological plasticity in the tropical marine gastropod *Strombus gigas.* Biol. Bull. 203, 112–120.

Edwards, S., Burke, C., Hindrum, S., Johns, D., 2000. Recovery and growth effects of anaesthetic and mechanical removal on greenlip (*Haliotis laevigata*) and blacklip (*Haliotis rubra*) abalone. J. Shellfish Res. 19(1), 510.

Fairweather, P. G., 1988, Movements of intertidal whelks (*Morula marginalba* and *Thais orbita*) in relation to availability of prey and shelter. Mar. Biol. 100: 1, 63-68.

Fisheries Management Act, South Australia, Australia, revised 2007. http://www.legislation.sa.gov.au/LZ/C/A/FISHERIES%20MANAGEMENT%20ACT%202007/CURRENT/2007.4.UN.PDF. Accessed online 23 March 2009

Folke, C., Kautsky, N., 1989. The role of ecosystems for a sustainable development of aquaculture. Ambio. Stockholm 18(4), 234-243.

Fujinaga K., Ilano A. S., Hayakari C., Oyama Y., Matsuishi T., Nakao S.2006.The present state of imposex in several edible whelks in three coasts along Hokkaido, Japan. Aquaculture Sci. 54(2), 179 -185.

Gutiérrez, R.M., Gallardo, C.S., 1999. Prey attack, food preference and growth in juveniles of the edible muricid snail, *Chorus giganteus*. Aquaculture 174, 69-79.

Hargis, W.J., 1957. A rapid live-sexing technique for *Urosalpinx cinerea* and *Eupleura caudata*, with notes on previous methods. Limnol. Oceanogr. 2(1), 41-42.

Heasman, M.P., O'Connor, W.A., Frazer, A.W.J., 1995. Induction of anaesthesia in the commercial scallop, *Pecten fumatus* Reeve. Aquaculture 131(3-4), 231-238.

Henderson, A., Gamito, S., Karakassis, I., Pederson, P., Smaal, A., 2001.Use of hydrodynamic and benthic models for managing environmental impacts of marine aquaculture. J. Appl. Ichthyol. 17(4), 163-172.

Hunt, H.L., Scheibling, R.E., 1998. Effects of whelk (*Nucella lapillus* (L.)) predation on mussel (*Mytilus trossulus* (Gould), *M. edulis* (L.)) assemblages in tidepools and on emergent rock on a wave-exposed rocky shore in Nova Scotia, Canada. J. Exp. Mar. Biol. Ecol. 226(1), 87-113.

McShane, P.E., Smith, M.G., 1988. Measuring recruitment of abalone *Haliotis rubra* Leach (Gastropoda:Haliotidae) - comparison of a novel method with two other methods. Aust. J. Mar. Freshw. Res. 39, 331-336.

McShane, P.E., Schiel, D.R., Mercer, S.F., Murray, T., 1994. Morphometric variation in *Haliotis iris* (Mollusca:Gastropoda): analysis of 61 populations. N.Z. J. Mar. Freshwat. Res. 28, 357-364.

Meijer, L., Skaltsounis, A-K., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X. P., Vonica, C. A., Brivanlou, A., Dajani, R., Crovace, C., Tarricone, C., Musacchio, A., Roe, S. M., Pearl, L., Greengard, P., 2003. GSK-3-selective inhibitors derived from tyrian purple indirubins, Chem.Biol. 10, 1255-1266.

Messenger, J.B., Nixon, M., Ryan, K.P., 1985. Magnesium chloride as an anesthetic for cephalopods. Comp. Biochem. Physiol. 82C, 203-205.

Michel, R., Lazar, J., McGovern, P., 1992. The chemical composition of indigoid dyes from the hypobranchial glandular secretions of Murex molluscs. J. Soc. Dyers Colour. 108,150-154.

Michel-Morfin, J.E., Chávezo, E.A., 2000. Effect of repetitive dye extraction over yield and survival rate of the purple snail *Plicopurpura pansa* (Gould, 1853). J. Shellfish Res. 9, 913-917.

Naegel, L.C.A., 2004. *Plicopurpura pansa* (Gould, 1853) from the pacific coast of Mexico and Central America: a traditional source of Tyrian purple. J. Shellfish Res. 23(1), 211-214.

Naegel, L.C.A., 2005. The effect of periodically “milking” to obtain Tyrian purple from *Pliocpurpura pansa* (Gould, 1853) on the frequency of expulsion and mortality. J. Shellfish Res. 24(1), 85-90.

Naegel, L.C.A., Murillo Alvarez, J.I., 2005. Biological and chemical properties of the secretions from the hypobranchial gland of the purple snail *Plicopurpura pansa* (Gould, 1853). J. Shellfish Res. 24(2), 421-428.

Nasution, S., Roberts, D., 2004. Laboratory trials on the effects of different diets on growth and survival of the common whelk, *Buccinum undatum* L. 1758, as a candidate species for aquaculture. Aquacult. Int. 12, 509–521.

Naylor, R., Goldburg, R., Primavera, J., Kautsky, N., Beveridge, M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.

Nugranad, J., Poomtong, T., Promchinda, K., 1994. Mass culture of *Chicoreus ramosus* (L., 1758) (Gastropoda: Muricidae). Phuket Mar. Biol. Cent. Spec. Publ. 13, 67-70.

Phillips, B. F. (1969). The population ecology of the whelk *Dicathais aegrota* in Western Australia. Aust. J. Mar. Freshwat. Res. 20, 225-65.

Prince J. D., Ford W. B. 1985. Use of anaesthetic to standardize efficiency in sampling abalone populations (genus Haliotis; Mollusca : Gastropoda). Aust. J. Mar. Freshwat. Res. 36, 701-706.

Ramón, M., Amor, M.J., 2002. Reproductive cycle of *Bolinus brandaris* and penis and genital duct size variations in a population affected by imposex. J. Mar. Biol. Ass. UK, 82(3), 435-442.

Rios-Jara, E., León-Alvarez, H.G., Lizárraga-Chávez, L., Michel-Morfín, J.E., 1994. Producción y tiempo de recuperación del tinte de *Plicopurpura patula pansa* (Neogastropoda: Muricidae) en Jalisco, México. Rev. Biol. Trop. 42(3), 537-545.

Seto, Y., Doi, S., 2000. Seed production trial of the deep sea whelk *Buccinum bayani* using deep sea water. UJNR Technical report. 28, 85-88.

Sharma, P.D., Nollens, H.H., Keogh, J.A., Probert, P.K., 2003. Sodium pentobarbitone-induced relaxation in the abalone *Haliotis iris* (Gastropoda): effects of animal size and exposure time. Aquaculture 218, 589–599.

Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J., Wijffels, R.H., 2005. Large-Scale Production of Pharmaceuticals by Marine Sponges: Sea, Cell, or Synthesis? Biotech. Bioeng. 90(2), 201-222.

Smith, S.D.A., 2005. Rapid assessment of invertebrate biodiversity on rocky shores: where there's a whelk there's a way. Biodivers. Conserv. 14, 3565–3576.

Stewart, M.J., Creese, R.G., 2004. Feeding ecology of whelks on an intertidal sand flat in north-eastern New Zealand. N.Z. J. Mar. Freshwat. Res. 38(5), 819-831.

Vasconcelos, P., Gaspar, M.B., Pereira, A.M., Castro, M., 2006. Growth rate estimation of *Hexaplex (trunculariopsis) trunculus* (Gastropoda: Muricidae) based on mark/recapture experiments in the Ria Formosa lagoon (Algarve coast, southern Protugal). J. Shellfish Res. 25(1), 249-256.

Verhecken A, 1989. The indole pigments of Mollusca. Annales de la Societe Royale Zoologique de Belgique 119(2), 181-197

Von Bertalanffy, L., 1957. Quantitative laws in metabolism and growth. Quart. Rev. Biol., 32, 217-231.

Westley, C., Benkendorff, K., 2008. Sex-specific Tyrian purple genesis: precursor and pigment distribution in the reproductive system of the marine mollusc, *Dicathais orbita.* J. Chem. Ecol. 34(1), 44-56.

Westley, C. B., Lewis, M. C., and Benkendorff, K. 2009. Histomorphology of the female pallial gonoduct in *Dicathais orbita* (Neogastropoda: Muricidae): Sperm passage, fertilization and sperm storage potential, Invert. Biol. Accepted 18th Feb, 2009.

Westley, C., Vine, K., Benkendorff, K., 2006. A proposed functional role for indole derivatives in reproduction and defense of the Muricidae (Neogastropoda: Mollusca), Indirubin, the Red Shade of Indigo. pp. 31–44, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

White, H.I., Hecht ,T., Potgeiter, B., 1996. The effect of four anesthetics on *Haliotis midae* and their suitability for application in commercial abalone culture. Aquaculture 140, 145-151.

Woodcock, S.H., Benkendorff, K., 2008. The impact of diet on the growth and proximate composition of juvenile whelks, *Dicathais orbita* (Gastropoda: Mollusca). Aquaculture. 276(1-4), 162-170.

Xavier Ramesh, M., Patterson Edward, J.K., Ayyakkannu, K., 1994. Experimental culture of *Chicoreus virgineus* (Roding, 1798). Phuket Mar. Biol. Cent. Spec. Publ. 13, 99-102.

Table 1. Six anaesthetic treatments, each at two concentrations, were initially tested in a pilot study to determine the most effective treatments for relaxing and sex identification of *Dicathais orbita*. A control consisted of fresh seawater.

Treatment	Delivery Agent	Concentration	Signs of relaxation
KCl (Sigma-Aldrich)	seawater	0.1 M	No
		0.2 M	No
$MgCl_2$ (Chem supply)	seawater	0.3 M	No
		0.5 M	**yes**
Ethanol (Chem supply)	seawater	3% v/v	No
		5% v/v	**yes**
CO_2 (BOC gases)	bubbled through seawater	pH 5.2	No
		pH 5.7	No
Benzocaine (Merck Pty. Ltd)	Ethanol then mixed in seawater (EtOH final conc. = approx 4%)	100 $mg.L^{-1}$	No
		200 $mg.L^{-1}$	**yes**
Sodium pentobarbital (Sigma-Aldrich)	seawater	0.5 $mg.L^{-1}$	No
		1 $mg.L^{-1}$	**yes**
Fresh seawater control		NA	No

Table 2. Effectiveness of anaesthetic treatments for sex identification in *Dicathais orbita* and the expulsion of mucus that turned purple in sunlight. The mean recovery time (min) is determined as the time taken for the snails to right themselves after replacement in fresh seawater.

Treatment	**Shell length size class**	**mean recovery time (min)**	**% sex determined**	**Purple mucus**
Ethanol, 5%	small	33.4	40	Yes
	medium	24.7	70	Yes
	large	59.5	60	Yes
Benzocaine,200 mg.L^{-1}	small	25.9	0	Yes
	medium	27.1	0	Yes
	large	48.1	0	Yes
Sodium pentobarbital, 1 mg.L^{-1}	small	57.4	0	No
	medium	29.3	0	No
	large	54.1	0	No
Magnesium chloride, 0.5 M	small	25.8	90	No
	medium	8.3	100	No
	large	33.7	100	No
Seawater control	small	19.5	0	No
	medium	10.8	0	No
	large	36.3	0	No

Table 3. Success of sex identification in wild populations and mean shell length (mm ± standard error) of male and female *Dicathais orbita* from six locations across South Australia.

Location	n	Mean shell length (mm)		Proportion sexed at each location %
		Females	Males	
O'Sullivans Beach	107	49.8 ± 2	52.5 ± 1.7	95.3
Marino Rocks	110	48.5 ± 10.3	53.1 ± 13	98
Brighton Jetty	104	72.9 ± 1.9	71.8 ± 1.7	95.4
Lipson Cove	119	53 ± 1.9	57.7 ± 2.6	94.1
Boston Point	117	58.3 ± 2.6	60.2 ± 2.8	97.4
Elliston	109	47.8 ± 0.75	49.1± 0.8	96.3

Figure legends

Figure 1: Relaxed specimen of *Dicathais orbita* showing the location of the penis.

Figure 2: *Dicathais orbita* were collected from O'Sullivans Beach (OS), Marino Rocks (MR) and Brighton jetty (BJ) on the Fleurieu Peninsula and Lipson Cove (LC), Boston Point (BP) and Elliston (EL)on the Eyre Peninsula of South Australia.

Figure 3: Mean recovery time minutes (±S.E.) of three size classes of *D. orbita* post treatment with one of four anaesthetics. The different capital letters indicate significant differences between size classes, whereas the different small letters indicate significant differences between anaesthetic treatments from Tukeys pairwise comparisons on the cube-root transformed data.

Figure 4: Liquid chromatogram showing composition of brominated indoles in a representative chloroform extract from the mucus of *D. orbita*. The x axis represents the retention time (minutes) in the LC column before the compounds were detected by the diode array. Y axis represents the absorbance units (AU) from the diode array at 300nm. The resulting peaks are identified as: A) tyrindoxyl sulphate; B) 6-bromoisatin; C) tyrindoxyl/tyrindolinone; D) tyrindoleninone; E) tyriverdin and F) 6,6'-dibromoindigo.

Figure 5. Natural sex ratio distributions of *D. orbita* from various locations on the Fleurieu and Eyre peninsulas of South Australia.

Figure 1

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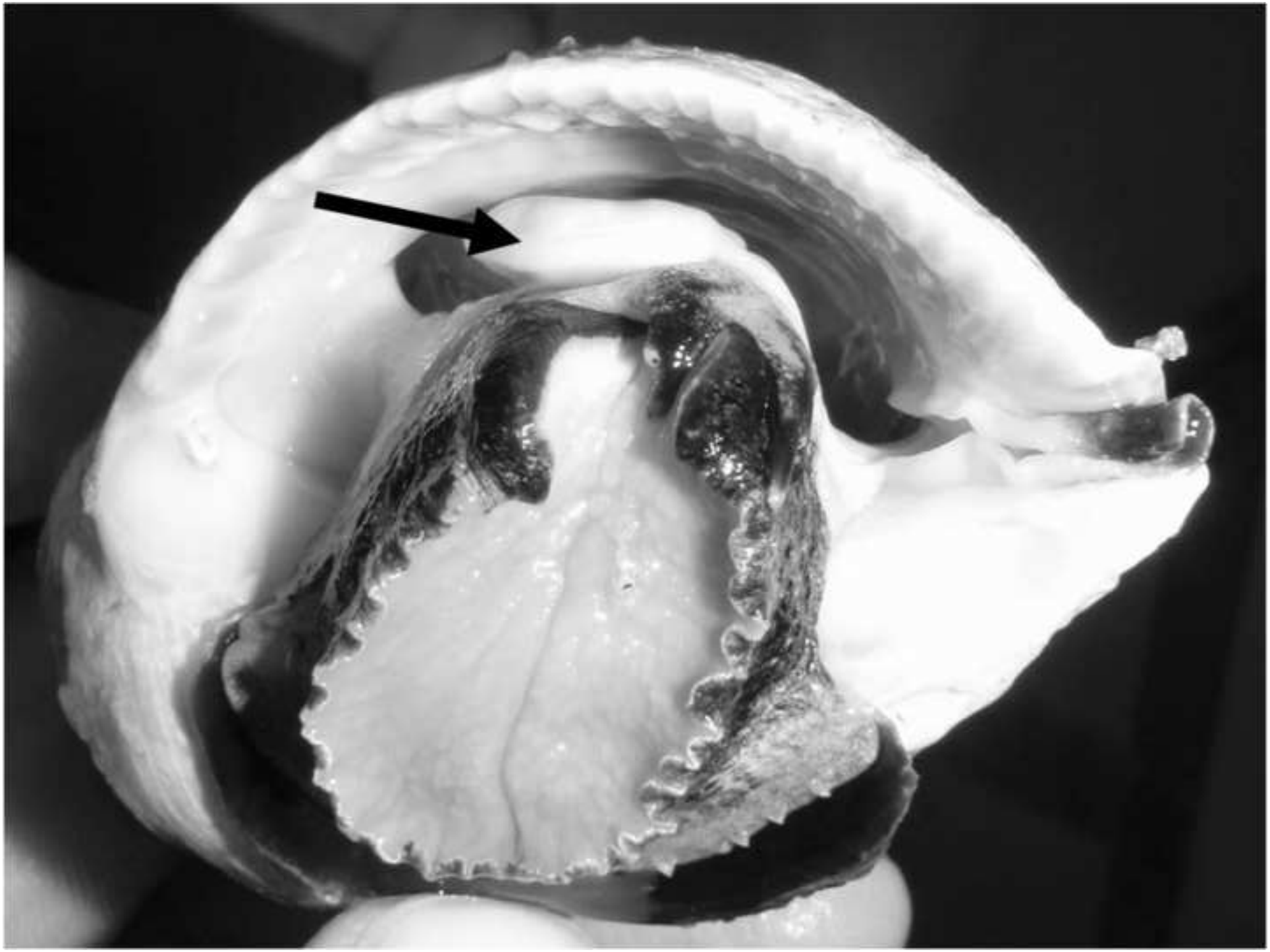

Figure 2

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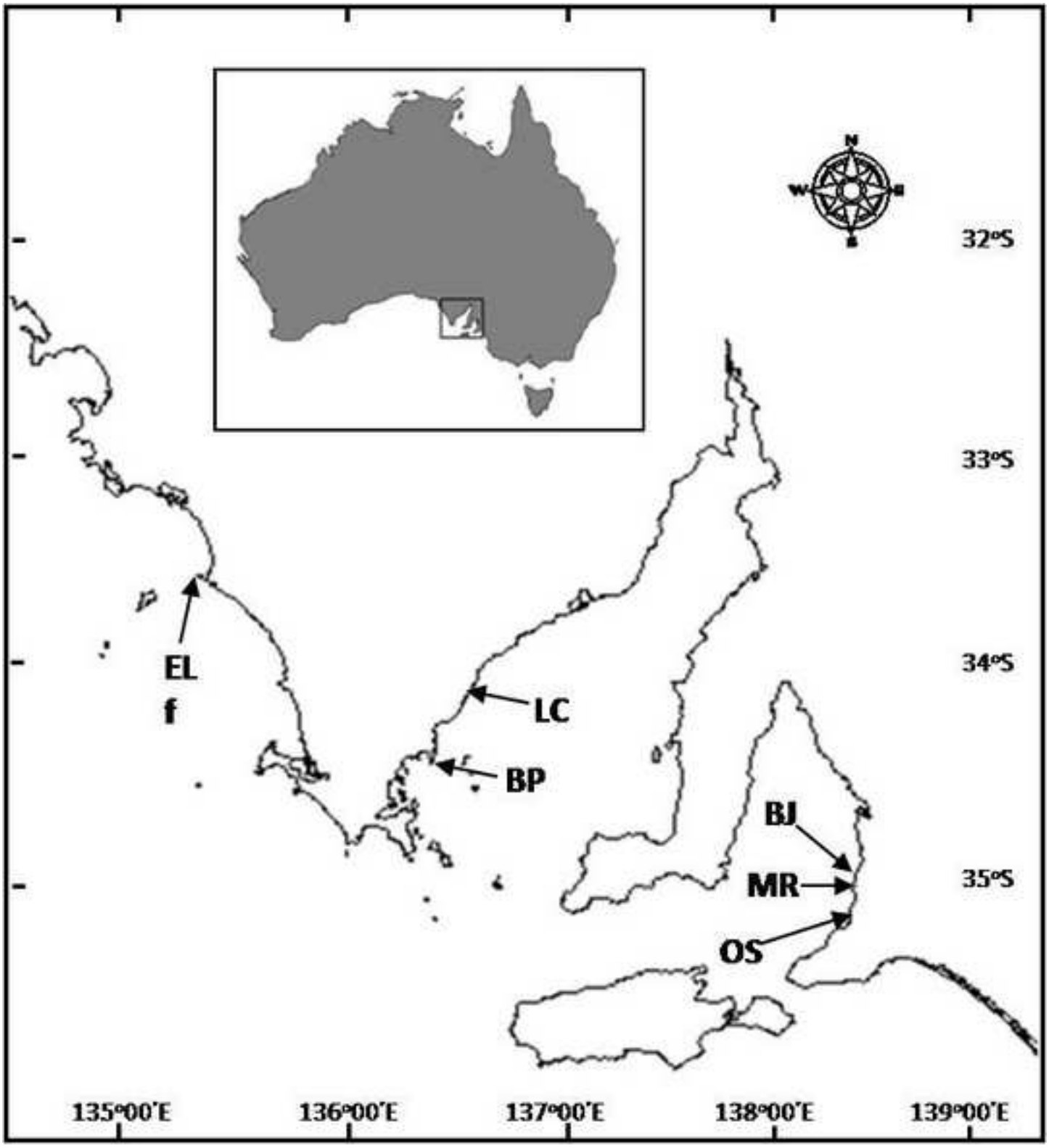

Figure 3

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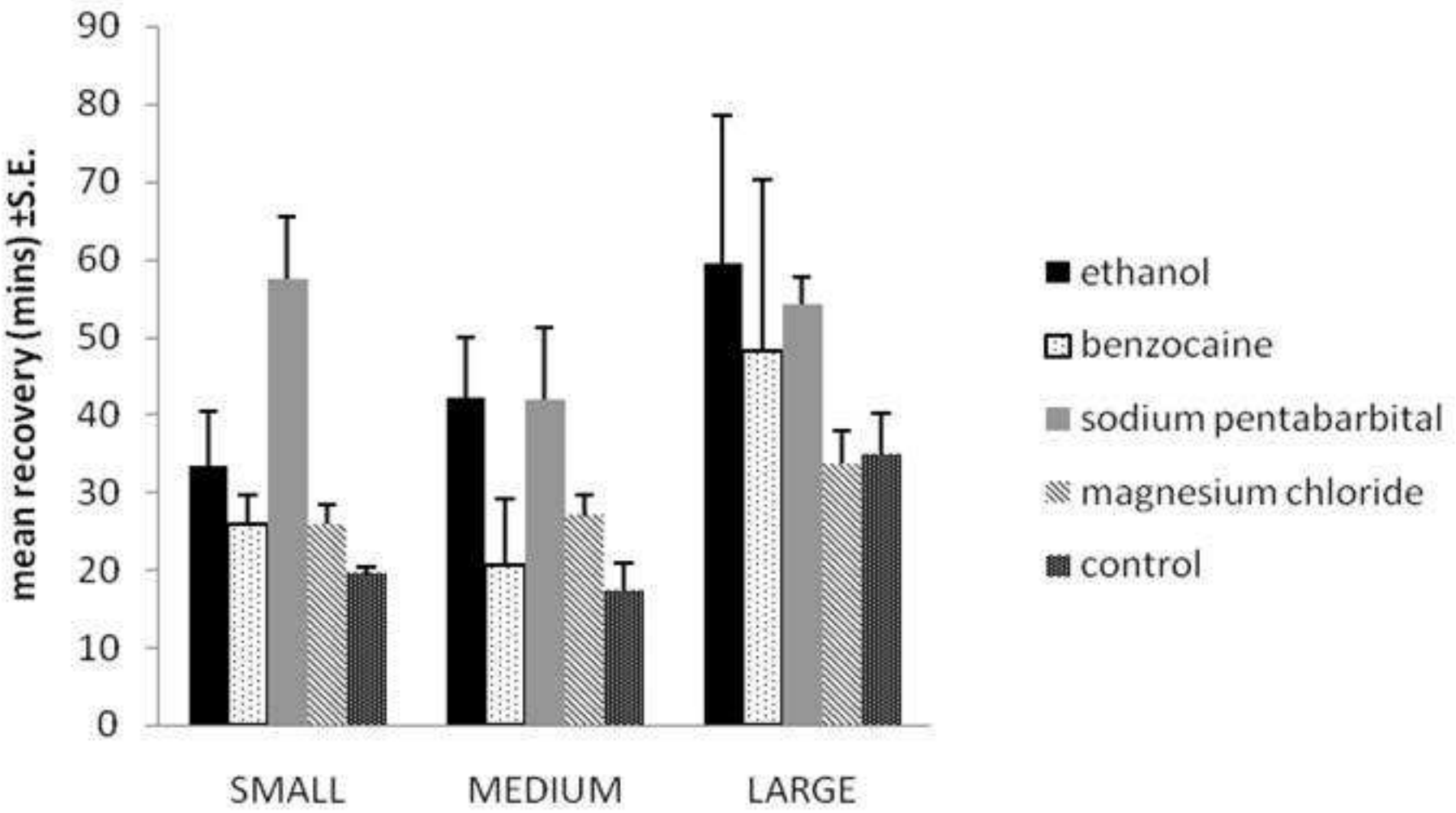

Figure 4

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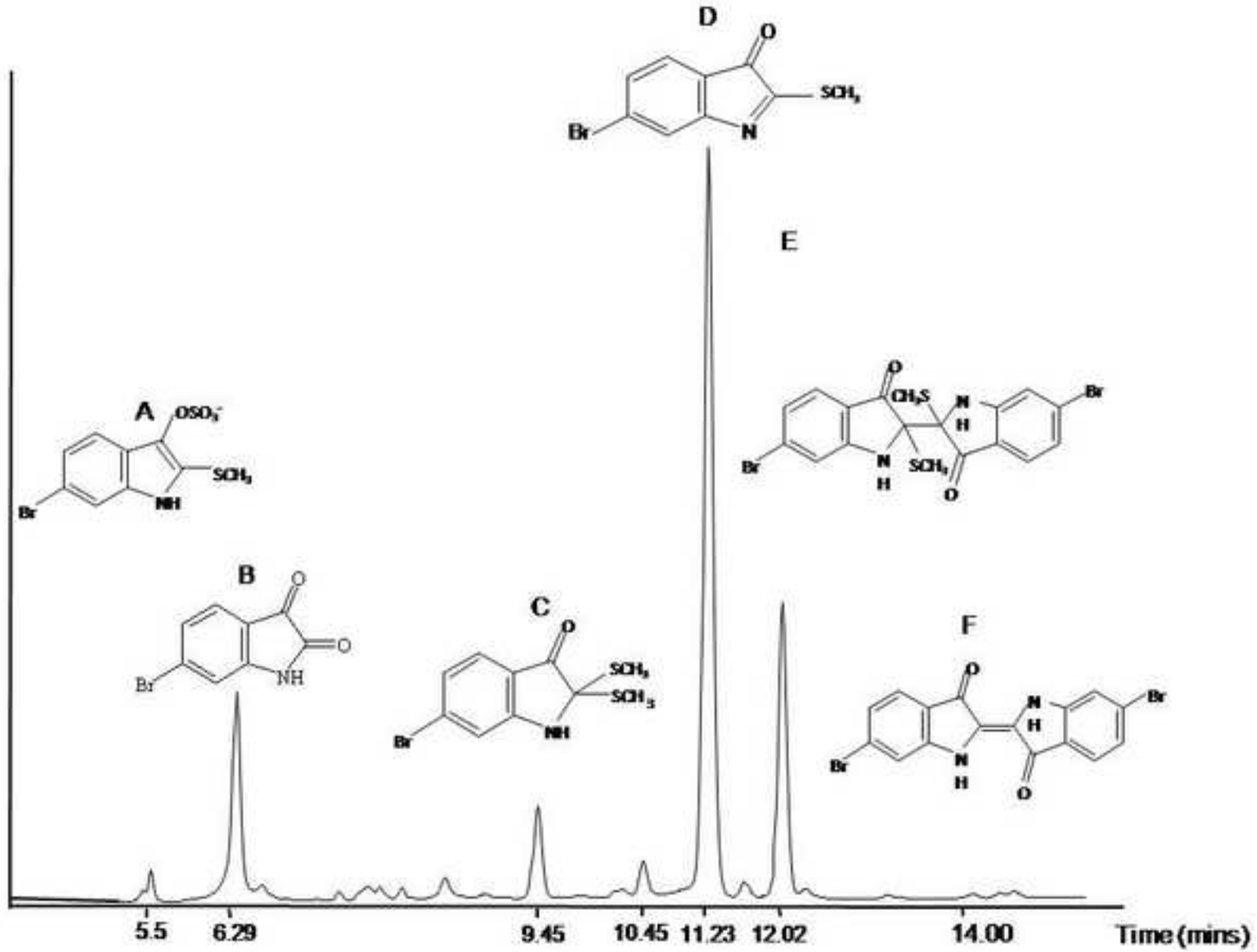

Figure 5

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