Aspects of life history and ecology of *Dicathais orbita* Gmelin, 1781 related to potential aquaculture for bioactive compound recovery

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By

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Abbreviations

FSW	Fresh seawater
GSI	Gonadosomatic index
DAD	Parallel UV/Vis diode-array
ESI-MS	electrospray ionisation-mass
RAS	Recirculating aquaculture system
P	Pigment
Bf	Bifurcation
H	Hypobranchial gland
DG	Digestive gland
G	Gonad
SBwt	Soft body weight
SL	Shell length
to	Tuberculate ornementation
V	Velum
Ft	Foot
Sc	Siphonal canal
Op	Operculum
Es	Eye spots
SGR	Specific growth rate
dph	days post hatching
KCl	Pottasium chloride
HPLC	High performance liquid
AUD	Australian dollars

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

21/5/2014

Warwick Noble

Date

Abstract

World aquaculture production, including the production of pharmaceuticals and nutraceuticals, is increasing to supplement fisheries harvest from wild stocks. Muricids (Neogastropoda) are widely fished around the globe and produce a range of interesting bioactive compounds (Chapter 1). Several species of muricids have been successfully cultured to supply seafood markets. The southern Australian muricid, *Dicathais orbita*, is recreationally harvested for food and more recently has been shown to produce potent bioactive compounds of interest for development as pharmaceutical leads. Successful aquaculture of *D. orbita* would provide a sustainable supply for ongoing development of pharmaceutical leads, as well as for seafood markets. Information on aspects of the life history of *D. orbita* will underpin successful aquaculture production.

Neogastropods are dioecious, but morphologically identical when in their shell. In order to non-destructively assess effective population size of wild stocks of *D. orbita* and manipulate lab held stock, it was necessary to develop a means to identify the sex of the species. A suite of anaesthetics were trialled for their efficacy in relaxing *D. orbita* out of the shell to identify sex organs and for stimulating bioactive compound production through a stress response (Chapter 2). Magnesium chloride proved most effective in relaxing *D. orbita* specimens enough to identify sex. Benzocaine and the carrier solvent ethanol were less effective for identifying sex, but stimulated expulsion of the bioactive precursors. The presence of bioactive brominated indoles in the expelled mucus of *D. orbita* was confirmed by liquid chromatography/mass spectrometry and provides a novel, sustainable means for obtaining these compounds without killing the snails.

The reproductive cycle of *D. orbita* was studied using a gonadosomatic index in wild and captive populations, along with spawning and post hatching larval development (Chapter 3). *D. orbita* follows an annual reproductive cycle, peaking in early summer (December) in South Australia. Female *D. orbita* spawn ~ 40 egg capsules in a session and each capsule contains an average of 5542 eggs with an average diameter of 105.2 µm. Post-hatching larval development proceeded through 5 stages over 41 days with average shell length increasing from 253 to 974.3 µm and shell width increasing from 203.8 to 980.5 µm. Information on the reproductive cycle and larval development patterns of *D. orbita* will allow for enhancement of reproductive condition and larval production.

Larval rearing experiments, to determine the effects of temperature and diet on the growth and survival of *D. orbita* larvae under laboratory conditions, used five different unicellular algal diets for larvae maintained at 16°C and 22°C (Chapter 4). Larvae reared at 22°C on a mixed diet, or diatoms alone, performed significantly better than those reared on green microalgal diets alone. Trials with settlement cues were undertaken on newly hatched to 38 day old *D. orbita* larvae to determine when larvae become competent. An array of natural cues (carrion, *Xenostrobus pulex*, adult mucus and *Ulvella lens*), as well as concentrations of KCl were tested. 20 mM KCl induced the greatest settlement, however no larvae metamorphosed under the conditions provided.

In conclusion, *D. orbita* can be grown under laboratory conditions and are highly fecund. Bioactive compounds can be extracted non-destructively from *D. orbita*, providing an extra resource that can be collected to value-add to seafood aquaculture. Larvae are planktotrophic with higher development rates at the higher water temperatures within their local range and grow to greater size on a diet comprised of

both green microalgae and diatoms. *D. orbita* is iteroparous with an annual reproductive cycle. High fecundity indicates that larval survival and recruitment is the limiting phase of the life cycle for *D. orbita*. Further studies will optimise culture conditions and cues for settlement and metamorphosis in order to close the life cycle of *D. orbita* for aquaculture production.

Acknowledgements

First and foremost I thank my supervisors, who patiently and unswervingly guided me along my post-graduate journey. Dr Kirsten Benkendorff, who will forever be an inspiration to me for her capacity to rapidly review all aspects of my work from justification and experimental design to manuscript preparation and submission, supported my development through this project. I gratefully acknowledge the extra opportunities she afforded me during my studies, which along with fond memories of conferences and international research placements have advanced my professional development immeasurably. Dr James Harris encouraged me to see that biological science alone will not provide the solutions to feasibility and technical problems encountered when in pursuit of new aquaculture activities. Although it was my enjoyment of open water that drew me to marine science, James' guidance in constructing experimental recirculating aquaculture systems showed me that closed water can be fun and informative also. Without Dr Benkendorff and Dr Harris's encouragement, discussion and personal examples I doubt I would have learned and gained as much on this journey as I have.

I also like to acknowledge and thank Jintana Nugranad and Tanes Poomtong, of Prachuap Khiri Khan Fisheries and Aquaculture, Thailand. I was fortunate to be able to spend time with them at their home research institution where Jintana and her staff taught me the skills to handle, grow and study molluscan larvae. Although my stomach was not prepared for the spiciness of Thai food, the lessons I learned while working with a wonderful group have helped me prepare this thesis.

My parents, Cliff and Peggy who provided me with patient ears to ramble about the ups and downs of my post graduate experience, and friends too many to list, for encouragement, and tolerance…I'll see you all soon.

Thesis structure

This thesis is presented in manuscript format. Thus some repetition of background and methods will be evident between chapters. To reduce repetition between chapters, a single reference list is contained at the end of the thesis. The introductory chapter (Chapter 1) provides context for the thesis through a broad overview of aquaculture, molluscan resources and neogastropod life history and concludes with overall aims of the thesis. Chapters 2-4 have independent hypotheses and aims which support the overall aims of the thesis. Chapter 2; Application of anaesthetics for sex identification and bioactive compound recovery from wild *Dicathais orbita.* and Chapter 4; Growth, settlement and survival of *Dicathais orbita* (Neogastropoda, Mollusca) larvae in response to temperature, diet and settlement cues, have been published, and Chapter 3; Reproductive cycle, spawning and post hatching larval development of *Dicathais orbita* (Neogastropoda: Muricidae), has been provisionally accepted for publication. The full details of publication are provided at the start of each chapter. Within each manuscript, the "study" refers to the particular set of experiments associated only with that chapter. Chapter 5 provides an overall, concluding discussion of all previous chapters. Appendix A and B consist of large tables referred to in the introductory and discussion chapters, and appendix C provides information on initial experiments on reproductive conditioning o *D. orbita*.

To maintain continuity in presentation, all chapters have been formatted in a consistent manner. Although I am first author on all chapters and personally responsible for the experimental design, conducting the research and preparing manuscripts, Kirsten Benkendorff and James Harris were supervisors who provided intellectual input for experiments and editorial input to facilitate publication. The contributions of an additional author on one published manuscript is outlined in the description of chapter objectives.

CHAPTER 1: General introduction

1.1 Summary

Dicathais orbita is a marine gastropod that is distributed across rocky shores and reefs of southern Australia and New Zealand (Gowlett-Holmes, 2008). Natural products research on this species over more than 45 years has led to the discovery of antibacterial and anticancer compounds (Benkendorff, 2013). Creating a sustainable supply of *D. orbita* for continued research and larger scale production has provided the impetus for the work described in this thesis. This introduction provides an overview of molluscan resources in general as well as previous research on Muricidae resources, life history and aquaculture. More specifically the introduction provides context for the new research undertaken for this thesis.

1.2 Molluscan resources

Exploration and exploitation of marine biological resources have shaped human history and continue to support our present and ongoing existence. Among the diversity of marine resources, marine molluscs have played a significant role in human development. The earliest evidence of molluscan resource use by humans are shell middens dating back ~ 164 thousand years (Marean *et al.,* 2007) and have been found to litter coasts around the world (de Boer *et al.,* 2000, Álvarez *et al.,* 2011). Coincident with the inclusion of marine molluscs in the human diet appears to be the first use of non-dietary molluscan resources, in the form of tools (Callomon & Snyder, 2009) and personal adornment (Mohanraj *et al.,* 2011). More recently the oceans have been the source of a considerable number of drug candidates and natural products. Although fisheries remains the dominant resource from the oceans, the search for natural products from the marine environment has expanded and the number of compounds that are investigated for pharmaceutical and biotechnological

development now lists in the thousands (Blunt *et al.,* 2012). However, the path from basic research to identification of interesting bioactive compounds, to trials of these pharmaceutical leads, can take 12-15 years (Hughes *et al.,* 2011), exceeding the time it takes to exploit fisheries stocks.

The human population is now above 7 billion and demand for marine resources is greater than ever. Since the 1950s, world fisheries production nearly quadrupled reaching its peak in the 1990s (Figure 1.1a). Finfish are the dominant catch, but increasing production is clear across all taxonomic groups. Mollusc production increased from 2.8 million tonnes between 1952-1957, to over 130 million tonnes between 1992-1996 (Figure 1.1a). The decreased production experienced since the 1990s, across all fisheries sectors, was largely due to widespread overexploitation, whilst increase in production from early in the new millennium reflects increased effort and advances in fishing technology (Leiva & Castilla, 2002, Rosenberg, 2003, Pauly *et al.,* 2005) (Figure 1.1a) .

The cultivation and fishing of marine molluscs has a long history in many countries and has become widely established as a coastal livelihood. Bivalves dominate both fisheries and aquaculture production, whilst abalone (*Haliotis* spp.) and the Chilean loco, *Concholepas concholepas* (Muricidae), represent the largest fishery and aquaculture production within gastropods (FAO, 2012). Since the 1990s, however, there has been significant upscaling and the introduction of specialized equipment to increase take from both fisheries resources and aquaculture production (Ram *et al.,* 1993, Leiva & Castilla, 2002, Defeo & Castilla, 2005, FAO, 2012). In order to avoid supply disruptions previously experienced from traditional wild molluscan fisheries (Leiva & Castilla, 2002, Romero *et al.,* 2004), more focus has been dedicated to assessing commercial harvesting of previously artisanal or recreational

fisheries (Martín *et al.*, 1995, Morel & Bossy, 2004, Narvarte *et al.*, 2007, de Vooys & van der Meer, 2010, Mohan *et al.*, 2012).

Aquaculture production, including the production of pharmaceuticals and nutraceuticals (Hunt & Vincent, 2006, Benkendorff, 2009), has increased to supplement wild fish stocks (Ram *et al.*, 1993) and now is responsible for over a third of all seafood (Figure 1.1b). Within aquaculture, mollusc production is approximately equal by weight to finfish, which in turn are only exceeded by marine plant production (Figure 1.1b).

Along with the promise of increased production are counter arguments that aquaculture of carnivorous species requires large inputs of wild fish as feed (Naylor *et al.*, 2005) and that all aquaculture has widespread ecological impacts, such as habitat modification or loss, release of organic and inorganic waste and introductions and transfers of invasive species (Barnes *et al.*, 1998). Some of these concerns can be addressed through co-culturing or polyculturing fed aquaculture species (e.g. finfish) with inorganic extractive species (e.g. macroalgae) that convert inorganic waste such as ammonia from the water column so that one species will help mitigate the waste impacts of the other species. Integrated multitrophic aquaculture (IMTA) is a form of co-culture that goes one step further in seeking balanced management of the ecosystem by adding organic extractive aquaculture species (e.g. bivalves) that remove organic waste such as faeces to the combination of fed and inorganic extractive species (Troell *et al.*, 2009). Numerous combinations of species, using the range of habitats from coastal (Ridler *et al.*, 2007) to open ocean (Troell *et al.*, 2009), offer the potential to deliver abundant and diverse resources beyond present monoculture capacity, while minimising disturbance to the surrounding ecosystem. The opportunity to use local species adds considerably to the benefits of IMTA

through addressing concerns about suitability to local conditions and the potential for escapes from introduced species (Ross *et al.*, 2008).

IMTA may increase product quality for some or all of the species used by feeding lower trophic levels to higher ones. For instance, the use of natural diets such as cultivated macroalgae can increase health and condition of abalone in comparison to formulated, pellet diets (Stone *et al.*, 2014). By feeding runts or weaker abalone stock to predatory or scavenging molluscs, IMTA could be extended to three or more species (see Benkendorff, 2009) thus minimising waste further.

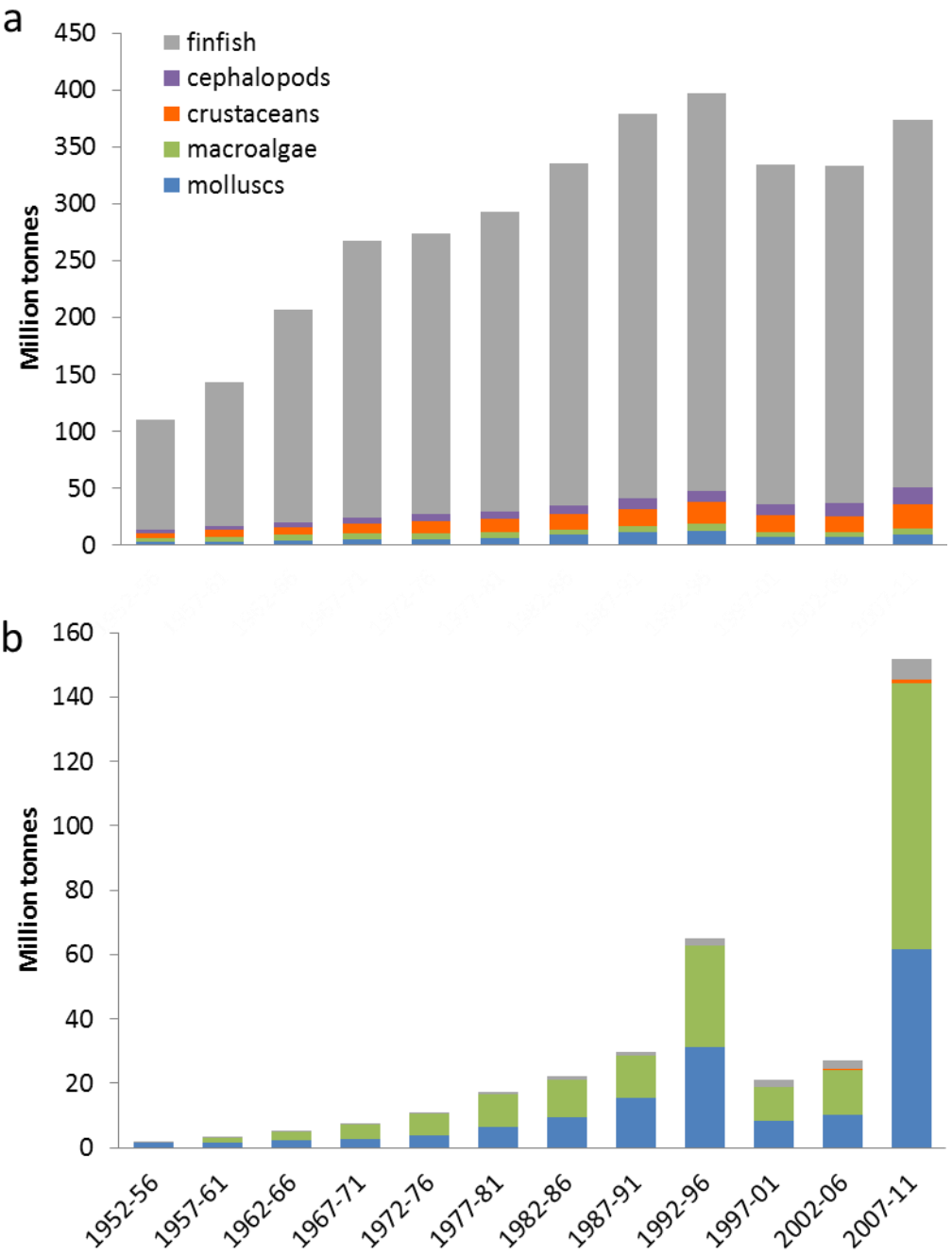

Figure 1.1. Estimation of a) global marine fisheries production and b) marine aquaculture production from 1952-2011 (FAO, 2012).

1.3 Neogastropoda – Whelks

One highly diverse clade of molluscs, the Neogastropoda (Poppe & Tagaro, 2006, Colgan *et al.*, 2007) are relatively under represented globally in fisheries and

aquaculture (FAO, 2012), although harvest as a food resource is widespread (Appendix A). There are about 16,000 species of neogastropods classified into six superfamilies: Buccinoidea, Muricoidea, Olivoidea, Pseudolivoidea, Conoidea, and Cancellarioidea (Colgan *et al.,* 2007). Within these, a number of families from the Buccinidae and the Muricoidea have been grouped together and are known commonly known as whelks. Whelks have been selected for harvest on both small and large scale across the world (Kingsford *et al.,* 1991, de Vooys & van der Meer, 2010). Despite some inconsistency, worldwide whelk fisheries production has doubled since 1991 (Figure 1.2). Much of the commercial demand for whelks comes from Asia, but domestic markets in Europe and the Americas consume most of the catch for locally significant species (Castilla & Gelcich, 2008, Leiva & Castilla, 2002, Vasconcelos *et al.,* 2009).

Trawling is used in offshore whelk fisheries (Anderson & Eversole, 1993, de Vooys & van der Meer, 2010), whereas collection by hand of more accessible inshore species is prevalent in smaller fisheries exploited by poor coastal communities (King *et al.,* 1990, Ramesh & Ayyakkannu, 1992, Shanthini & Patterson, 2005). Although considered predatory, studies on a range of species show that whelks have a preference for carrion (Nasution & Roberts, 2005, Woodcock & Benkendorff, 2008, Sangsawangchote *et al.,* 2010), and baited traps are a widespread fishing method (Nashimoto *et al.,* 1995, Sasikumar *et al.,* 2006, Park *et al.,* 2007).

The most important families of whelks from a fishery resource perspective are the Muricidae, Buccinidae and Fasciolariidae (Appendix A). Buccinids arguably represent the largest commercial whelk fisheries in the world, while species of fasciolarids are primarily fished by coastal populations in poor or developing countries, and muricid fisheries are dominated by commercial demand in South

America (Appendix A). The most heavily exploited species is *Buccinum undatum*, which is fished widely by countries surrounding the North Atlantic (Appendix A).

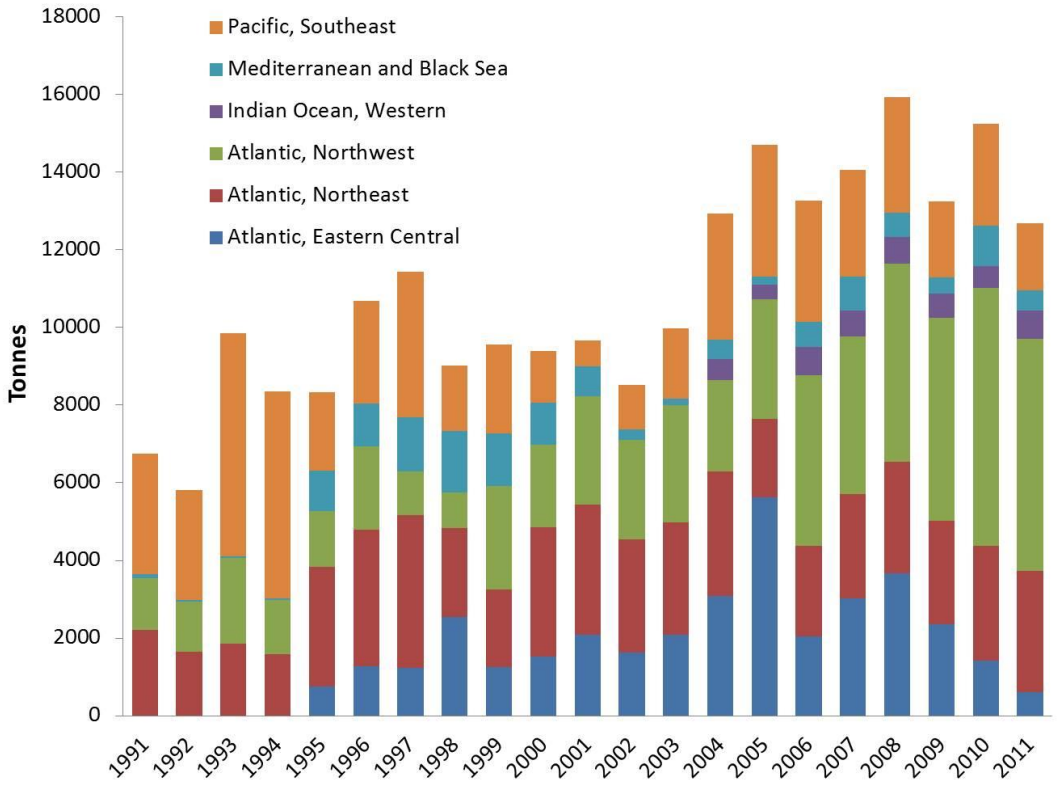

Figure 1.2. Whelk fisheries production, excluding fishing for ornate shells, from six geographic fishing areas between 1991 and 2011 (FishStatJ).

Pressure on commercially fished whelk populations of *B. undatum* and *C. concholepas*, have led to declines in fishery landings encouraging improved management through regular appraisals of these fishery resources (Kenchington & Lundy, 1996, Leiva & Castilla, 2002, Fahy *et al.*, 2005). Fisheries assessments have now been widely adopted and applied to well-established, large scale as well as emerging and artisanal fisheries (Muhando & Jiddawi, 1998, Morel & Bossy, 2004, Hermosilia & Narido, 2007, Mohan *et al.*, 2012, Peemoeler & Stevens, 2013).

1.4 Muricidae resources

Muricids are fished mostly through the Mediterranean and Asia, although there is substantial production of *C. concholepas* in Chile (Appendix A). Muricids are distinguished among whelks because in some areas they are harvested for the pigment, Tyrian purple (Naegel & Lopez-Rocha, 2006) along with meat and ornate shells (Radwin & d'Attilio, 1976, Castilla & Gelcich, 2008). The pigment is extracted from the animal and used as a dye for cloth. Tyrian purple has a history of use dating back at least 4000 years (Cooksey, 2013), and is so culturally important that, at times, control of production has inspired uprisings among indigenous people (Naegel & Lopez-Rocha, 2006). The popularity of this commodity has varied since it was first used, but was widely produced in the Mediterranean from the first century BC through to the end of the Middle Ages (Cooksey, 2013). Although demand has not since been as high due to the production of cheaper synthetic purple pigments, Tyrian purple is still readily available and can be purchased for 2439.50 Euros (3535.72 AUD) per gram (Kremer-Pigmente, 2013). This is close to seventy times the present value of 35 euros (51 AUD) per gram, for gold (Reserve Bank of Australia, 2013).

The main pigment in Tyrian purple is a brominated derivative of indole, 6,6 dibromoindigo, that occurs from a series of enzymatic, oxidative and photolytic reactions when brominated precursors from the hypobranchial gland are exposed to light (Cooksey, 2001). Natural products research on the precursors and intermediate compounds have identified bioactive compounds (Benkendorff, 2013), including antibacterial compounds (Benkendorff & Davis, 2000, Benkendorff *et al.,* 2001b, Ramasamy & Murugan, 2005), muscle relaxants (Roseghini *et al.,* 1996) and anticancer compounds (Benkendorff *et al.,* 2001a, Meijer *et al.,* 2003, Benkendorff *et al.,* 2011, Edwards *et al.,* 2012, Esmaeelian *et al*., 2013, Esmaeelian *et al.*, 2014).

The precursors are also suggested to contribute spiciness to the flavour of the meat when the hypobranchial gland is left attached (Cooksey, 2013) and this has the potential to confer the beneficial therapeutic effects of bioactive compounds when consumed.

In order to collect Tyrian purple, or compounds for natural product research, the individual animal is often destroyed for relatively low yield (Westley *et al.,* 2013, Cooksey, 2013). On the coast of Central America, regular collection of secretions from the muricid *Plicopurpura pansa* is achieved by mechanical stimulation of each snail (Ríos-Jara *et al.,* 2001). Mechanical stimulation as a sustainable extraction method appears to avoid the issues of overexploitation that could occur from destructive collection, since dye can be "milked" repetitively from the same animal with minimal mortality (Michel-MorfÍn & Chávez, 2000). However, milking the snails still appears to be causing declines in populations (Naegel & Lopez-Rocha, 2006). Tyrian purple 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. Observations of Tyrian purple production imply 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 if a sustainable stock of animals could be maintained and milked en masse.

1.5 Whelk aquaculture

Aquaculture of whelks is largely concentrated around those countries with the greatest demand or involved in fishery export. The documented severe population disruption due to fisheries overexploitation is a commonly encountered problem in whelk fisheries (Di Salvo & Carriker, 1994, Martín *et al.,* 1995, Gajardoa *et al.,*

2002, Leiva & Castilla, 2002). Inconsistent supply due to overexploitation has provided a major research impetus for the potential aquaculture of whelks, for the purpose of restocking wild populations (Naegel & García-Domínguez, 2006) and more generally for providing a sustainable supply to seafood markets (Nugranad *et al.,* 1994, Nugranad & Kerdpoom, 1995). Nevertheless, the aquaculture of whelks is relatively small scale in comparison to aquaculture of abalone, which dominates aquaculture to a similar degree as fisheries (FAO, 2012). However, despite the comparative small scale of whelk aquaculture, production may be highly significant to local markets where each species is caught. For instance, demand for *C. concholepas* in South America has driven a range of research aimed at enhancing aquaculture production of this species (Di Salvo, 1988, Manríquez *et al.,* 2008), and has produced as much as 12 tonnes per annum of this species (FAO, 2012). Similarly, in Thailand research on the muricid, *Chicoreus ramosus* and the buccinid, *Babylonia areolata* have led to successful aquaculture of these locally important species (Nugranad *et al.,* 1994, Nugranad & Kerdpoom, 1995, Chaitanawisuti & Kritsanapuntu, 1997, Chaitanawisuti *et al.,* 2002). While in the south of India, aquaculture of *Babylonia spirata* is recognised as having potential to provide significant nutritional and economic benefit to locals of region (Edward *et al.,* 2006). The success of aquaculture in general depends on understanding the biology and emulating aspects of the ecology of the target species (Folke & Kautsky, 1989).

1.6 Neogastropoda biology

Neogastropods are dioecious; although females sometimes attain a greater size than males, there are no other obvious morphological differences between males and females apart from the reproductive organs (Hughes, 1986, Power & Keegan, 2001, Elhasni *et al.,* 2013). Identifying sex in target species is necessary to manage broodstock, and for wild populations, knowing the sex ratio can inform the effective

population size and associated fisheries management strategies. From an ecological perspective, muricid whelks are important for structuring the biota within their habitat through their predatory and scavenging nature (Fairweather, 1988, Hunt & Scheibling, 1998, Stewart & Creese, 2004). Hence, the presence of healthy populations of these gastropods can indicate relative health and changes to ecosystems (Smith, 2005).

Neogastropods have been used as indicators for pollution from endocrine disrupting compounds released into waterways (Rees *et al.,* 2001, Pavoni *et al.,* 2007). Exposure to low levels of the heavy metal tributyltin (TBT), once widely used in antifouling paints, causes imposex. Imposex refers to the development of a pseudo penis in females, often rendering the affected females reproductively inviable (Gibbs & Bryan, 1986). The potential for this condition to negatively impact populations of a species that relies on internal fertilization for reproduction has been elucidated in numerous studies (Gibson & Wilson, 2003, Fujinaga *et al.,* 2006, Castro & Fillmann, 2012) and could significantly affect both whelk fisheries (Solé *et al.,* 1998) and aquaculture (Gooding *et al.,* 1999).

Reproduction of neogastropods follows an iteroparous strategy and fecundity increases asymptotically with size and age (Hughes, 1986). The reproductive cycle for neogastropods ranges from annual cycles with reproductive condition and spawning increasing with increasing water temperatures (Martel *et al.,* 1986, Vasconcelos *et al.,* 2012), to numerous spawnings with no relationship to water temperature (Ramón & Amor, 2002, Naegel & García-Domínguez, 2006). The same species may exhibit different cycles depending on geographic location. For example, the muricid *Bolinus brandaris* has an annual reproductive cycle in the north Atlantic and central Mediterranean (Vasconcelos *et al.,* 2012, Elhasni *et al.,* 2013),

while two reproductive periods have been observed in the western Mediterranean (Ramón & Amor, 2002).

Females neogastropods are often gregarious when spawning and a number of individuals may contribute groups of egg capsules to a communal mass that is attached to the substrate, with each egg capsule containing a few to thousands of eggs (Phillips, 1969, Romero *et al.*, 2004, Sağlam & Düzgüneş, 2007). Once spawned, females provide no further maternal investment to their offspring. However, the capsules and contents represent a significant investment and can account for over 80% of the weight lost through spawning (Stickle, 1975). The capsules are complex proteinaceous structures that are produced by the capsule gland and are made up of multiple layers (Hughes, 1986, Westley & Benkendorff, 2008). As well as providing physical protection from the elements (Przeslawski, 2004), capsules have been shown to contain active compounds that discourage microbial fouling (Przeslawski & Benkendorff, 2005, Lim *et al.*, 2007).

The number of eggs in each capsule, time for development of embryos and the number and stage of development that hatchlings emerge as are grouped into three main patterns: lecithotrophic, planktotrophic and direct development (Figure 1.3). Common to all three developmental modes; eggs or blastulae begin dividing and progress to gastrulae and trochophore stages where the first sign of the shell becomes evident, before becoming veligers within benthic egg capsules (Hughes, 1986). The nutritional resources for intracapsular development are provided by several means, including nurse eggs that disintegrate soon after egg deposition and are consumed by one or more siblings (adelphophagy)(Cumplido *et al.*, 2011), individual yolk reserves in each embryo, and/or the provision of nutrients in the intracapsular fluid, and (Hughes, 1986). Generally, larger eggs containing more yolk hatch into large

juveniles which are expected to survive better than smaller ones (Spight, 1976, Romero *et al.*, 2004).

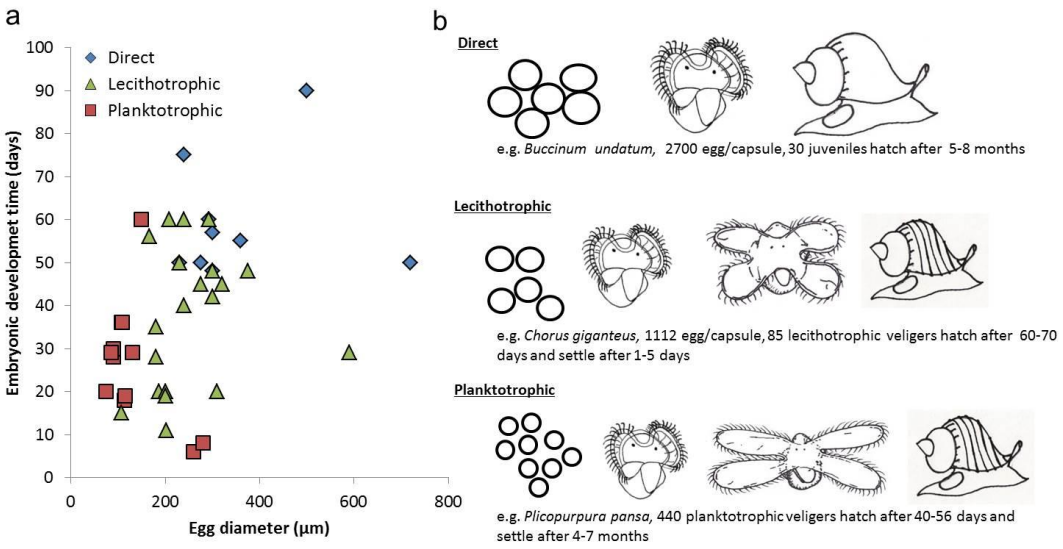

Figure 1.3. Different larval development modes in Neogastropoda: a) Egg diameter and embryonic development time for neogastropods showing three types of larval development b) conceptual illustrations of larval development types. Data compiled from Phillips (1969), Castilla & Cancino (1976), Spight (1976), Martel *et al.* (1986a), Di Salvo (1988), D'Asaro (1991), Campos *et al.* (1994), Di Salvo & Carriker (1994), Gonzáles & Gallardo (1999), Power & Keegan (2001), Naegel *et al.* (2003), Romero *et al.* (2004), Sreejaya *et al.* (2004), Meirelles &Matthews-Cascon (2005), Harding (2006), Sağlam & Düzgüneş (2007), Manríquez *et al.* (2008), Martínez *et al.* (2008), Gallardo & Cancino (2009) and Jagadis *et al.* (2013).

As a rule, small embryos without substantial yolk reserves hatch as planktotrophic veligers that depend on planktonic food to supply the energy needed for continued development and survival. Those with intermediate yolk reserves hatch as lecithotrophic veligers that do not require planktonic food but metamorphose within a few days or even hours after hatching. Direct developing species, those with large yolk reserves, complete metamorphosis within the capsule and emerge as crawling juveniles (Hughes, 1986), have lower dispersive potential and are likely to be more vulnerable to over collection.

Competency for invertebrate larvae can be considered as the point of development at which larvae are sufficiently developed to settle and metamorphose (Hadfield, 1984). The process of metamorphosis is energetically expensive and juveniles are subject to latent effects of maternal condition and larval development conditions (Pechenik, 2006). Larval mortality is typically high for lecithotrophic and planktotrophic species and may be linked to lack of maternal provision (Pechenik, 2006, Bertram and Strathmann, 1998), predation (Pechenik *et al.*, 2004), injury (Di Salvo & Carriker, 1994), temperature (Hoegh-Guldberg & Pearse, 1995) and advection from suitable habitats (Kempf, 1981, Moreno *et al.,* 1998). In the case of planktotrophic larvae, the quality and quantity of food can also lead to substantial mortality (Phillips, 1969, Zheng *et al.*, 2001, Zheng *et al.,* 2005, Edward *et al.*, 2006). Many of these factors must be managed when culturing larvae under laboratory conditions, but additional factors of larval density and pathogen infestation must also be managed (Di Salvo *et al.*, 1978, Pillay & Kutty, 2005). Managing so many factors leads to relatively low success especially in early attempts at closing the life cycle of a species. For instance, although there have been dozens of species of whelk larvae with planktotrophic development that have been cultured under laboratory conditions, only seven have been successfully cultured through metamorphosis (Table 1.1).

Table 1.1. Whelks that produce planktotrophic larvae and have been successfully metamorphosed under laboratory conditions. na = not available.

Muricidae	Initial culture density (larvae mL^{-1})	Mean time (days) for competence	Culture temperature (°C)	Settlement cue	Reference
Concholepas concholepas	0.1	118	20	25 mM K^+	Di Salvo, 1988, Campos *et al.,* 1994
Plicopurpura pansa	0.125	120	21-23	15 mM K^+	Naegel, 2003
Rapana venosa	0.7	33	26.6	spontaneous	Harding, 2006
Thais chocolata	1	120	22	Bio-encrusted rock	Romero *et al.,* 2004
Buccindae					
Babylonia formosae habei	1.5	11.5	24	spontaneous	Zheng *et al.,* 2001, 2010
B. spirata	na	na	27	12 mM K^+	Ke *et al.,* 2000,
B. areolata	10	na	28.5	spontaneous	Chaitanawisuti & Kritsanapuntu, 1999, Ke *et al.,*2000

Even if larvae are sufficiently developed to have reached competency, settlement out of the water column to metamorphose will be delayed unless larvae are provided with a suitable settlement cue (Jackson *et al.*, 2002, Pechenik *et al.*, 2002, Strathmann & Strathmann, 2007). Settlement cues may indicate suitable substrate because they provide habitat (Campos *et al.*, 1994, Kingsley-Smith *et al.*, 2005) or food for post-metamorphic juveniles (Avila, 1998, Manríquez *et al.*, 2004). Although natural settlement cues are species specific, excess potassium ions can induce metamorphosis in a range of marine invertebrates (Yool *et al.*, 1986) and proved useful to induce settlement and metamorphosis in a number of whelks under aquaculture conditions (Campos *et al.*, 1994, Ke *et al.*, 2000, Gallardo & Sánchez, 2001).

1.7 Dicathais orbita

Within a more local Australian context, the muricid whelk, *Dicathais orbita* inhabits rocky shores and reefs across southern Australia and New Zealand (Gowlett-Holmes, 2008). Small-scale, recreational harvesting is the extent of the fishery (Kingsford *et al.,* 1991), but *D. orbita* has been the subject of a great deal of study over the last 50 years encompassing ecology to bioprospecting (see Benkendorff, 2013 for a comprehensive review). Perhaps some of the most exciting findings in this considerable body of research are the recent discovery of bioactive compounds produced in the hypobranchial gland which show antitumour activity (Westley *et al.,* 2010a, Edwards *et al.,* 2012, Benkendorff, 2013) with nutraceutical potential. Whelks have a nutritional profile similar to other species (King *et al.,* 1990, Woodcock & Benkendorff, 2008, Vasconcelos *et al.,* 2009) and ingesting the hypobranchial gland along with the meat, as is done in some countries (Cooksey, 2013), could make *D. orbita* attractive as a functional food. Functional foods are similar in appearance to conventional foods, are consumed as part of a usual diet, and are known to improve health status beyond basic nutritional function expected of conventional foods (Shahidi, 2004). Functional foods and natural health products encompass a wide range of food and ingredients, with a variety of bioactives responsible for their efficacy in health promotion and disease prevention (Shahidi, 2004). It is logical to assume that consumer demand for health-promoting foods and the bioactive compounds they contain will continue to grow in the foreseeable future (Milner, 1999, Lordan *et al.,* 2011).

Harvesting previously unharvested Muricidae species in commercial quantities should be avoided based on experiences of overexploitation and subsequent population collapse in other whelk fisheries (Leiva & Castilla, 2002). Therefore, in order to ensure a sustainable supply for bioactive research and potential commercial

sale, a better understanding of the life history and ecology of *D. orbita,* as they relate to aquaculture production is needed. There is already keen interest from existing aquaculture farmers (pers. comm. Australian Bight Abalone, South Australian Seafood, Southern Australian Mariculture) and *D. orbita* could be added to existing farming practices as part of an IMTA approach.

Successful aquaculture of any species depends on the biological, technical and economic feasibility of the venture (Gutiérrez & Gallardo, 1999; Jeffs & Hooker, 2000; Le Francois *et al.*, 2002). Le Francois *et al.* (2002) proposed a framework in order to assess the suitability of a species to aquaculture development for stock enhancement, on-growing or production through the complete life cycle. It is beyond the scope of this study to discuss in detail the economic feasibility of aquaculturing *D. orbita,* however the premier point in the selection process described by Le Francois *et al.* (2002) is marketability. The high value of Tyrian purple along with the potential to market this species as a functional food suggests good marketability. Hence, the focus of this study is on the biological aspects of *D. orbita*. The experience and knowledge obtained provides a basis for future economic feasibility assessments.

1.8 Thesis aims, significance and objectives

The aims for this thesis are to provide further information on the reproductive cycle and post hatching larval development of *D. orbita* relevant to progress towards sustainable aquaculture of this species. Prior to these investigations, a reliable, non-destructive method of identifying sex and broad assessment of population sex ratio across South Australia needs to be undertaken. Studies on the reproductive cycle and sex identification will provide a means to manage broodstock populations in

captivity, as well as providing ecologically relevant information on the status of wild population structure.

1.9 Chapter aims and objectives

Chapter 2: Application of anaesthetics for sex identification and bioactive compound recovery from wild *Dicathais orbita. Journal of Experimental Marine Biology and Ecology*, 380, 1-2, 53-60.

Aims: The aims of Chapter 2 were to investigate non-destructive methods of sex identification for stock assessment and a method of sustainable "milking" of adult *D. orbita* for bioactive compounds. The aims are achieved through addressing the following objectives.

Objectives: 1) Test a range of different anaesthetics for efficacy in relaxing *D. orbita* out of the shell and allowing sex identification via observation of the penis.

2) Examine the recovery rate and any side effects associated with application of anaesthetics.

3) Test for the presence of Tyrian purple precursors in mucus secreted (milked) from anaesthetic application.

4) Establish the sex ratio of wild populations in the field by applying the optimal anaesthetic at a concentration determined in the laboratory experiments.

5) Undertake timed search surveys to estimate the population size of *D. orbita* at various sites on the South Australian coastline.

An additional author, Rebecca Cocks, was an honours student who undertook the chemical analysis of mucus from the milking experiment.

Chapter 3: Reproductive cycle, spawning and post hatching larval development of *Dicathais orbita* (Neogastropoda: Muricidae). *Molluscan Research* Provisionally accepted.

Aims: The aims of Chapter 3 were to investigate the reproductive cycle and the larval cycle of *D. orbita* for stock management. The aims are achieved by addressing the following objectives.

Objectives: 1) Use a gonadosomatic index to study the reproductive cycle of laboratory held and wild *D. orbita* through to spawning.

2) Provide information on the fecundity of wild and laboratory held *D. orbita* through the number of egg capsules laid by a female and the number of eggs in each capsule.

3) Provide a description and analysis of the morphological changes during larval development.

Chapter 4 Growth, settlement and survival of *Dicathais orbita* (Neogastropoda, Mollusca) larvae in response to temperature, diet and settlement cues. *Aquaculture Research* (2013). In Press, DOI: 10.1111/are.12298.

Aims: The aims of this chapter were to investigate the effect of temperature and diet on *D. orbita* larvae, and the effect of settlement cues on different aged larvae. The aims are achieved through addressing the following objectives.

Objectives: 1) Through growing *D. orbita* larvae at different temperatures and providing different diets, determine the optimal temperature and diet combination for larval growth.

2) Determine the optimal concentration of KCl and larval age combination for inducing settlement of *D. orbita* larvae grown at the optimal temperature and diet combination.

3) Test the efficacy of the optimal KCl concentration and larval age combination in comparison to natural settlement cues.

Chapter 5 provides a general discussion of the outcomes from this thesis and suggests future directions for research.

Some additional research not included in the main thesis was also undertaken and this has been included in Appendix C.

Appendix A provides a table summarizing the global production and value of whelk fisheries and aquaculture.

Appendix B provides a table summarizing the embryonic and larval development, diet and settlement cues for a range of whelk species.

Appendix C describes a lab experiment designed to manipulate and induce spawning of adult broodstock by altering temperature and light:dark regime. This experiment was not deemed publishable due to a substantial number of samples being unusable due to inadequate fixation. However, the experimental design necessitated a specialised recirculating aquaculture system (RAS) of considerable technical complexity. The task of designing the experiment, writing the methodology and researching, designing and constructing the RAS took a number of months and represents a system that could be used for future studies on broodstock conditioning of gastropods and is therefore included in this appendix.

CHAPTER 2: Application of anaesthetics for sex identification

Noble, W. J., Cocks, R. J., Harris, J. O., Benkendorff, K. 2009. *Journal of Experimental Marine Biology and Ecology*, 380, 1-2, 53-60.

2.1 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. *D. orbita* 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. 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 will require testing for species specific responses.

2.2 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.,* 2005), providing seed stock for wild fisheries restocking (Seto & Doi, 2000) and it has been reported to enhance wild fish stocks (Brehmer *et al.,* 2003; Dempster *et al.,* 2006). However, success of aquaculture depends on emulating aspects of ecology (Folke & Kautsky, 1989; Hawkins *et al.,* 1999; Henderson *et al.,* 2001). Similarly, bioprospecting often seeks leads for novel bioactive compounds through studies in chemical ecology (Faulkner, 1996; Paul *et al.,* 2001). The sustainable production of bioactive compounds discovered through bioprospecting can be managed successfully through aquaculture (Page *et al.,* 2005; 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 good example of these multidisciplinary research interests is provided by marine molluscs in the family Muricidae (Neogastropoda). Currently, muricids are fished throughout the world and sold in seafood markets as 'Whelks'. Overexploitation of whelks in some countries due to commercial wild harvest has led to severe population disruption (Di Salvo & Carriker, 1994; Martin *et al.,* 1995; Castilla, 1997; Leiva & Castilla, 2002; Uyan & Aral, 2003). This has provided a major research impetus for the potential aquaculture of Muricidae species, for the purpose of restocking wild populations (Di Salvo & Carriker 1994; Naegel & López-Rocha, 2007) and more generally for providing a sustainable supply (Nugranad *et al.,* 1994; Ramesh *et al.,* 1994; Nugranad & Kerdpoom, 1995; Gutiérrez & Gallardo, 1999;

Woodcock & Benkendorff, 2008). Muricids are also highly valued for the production of their purple dye secretion, best known as Tyrian purple (Baker, 1974; Cooksey, 2001; Naegel, 2004). Recent research into the precursors and minor dye constituents of Tyrian purple secretions has revealed antibiotic and anticancer activities (Benkendorff & Davis, 2000; 2001; Meijer *et al.,* 2003; Naegel & Alvarez 2005; Westley *et al.,* 2006). Consequently, these dye producing molluscs are under investigation for development as a new potential source of nutraceuticals (or natural medicines) (Benkendorff, 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 & Scheibling, 1998; Stewart & Creese, 2004). Hence, the presence of healthy populations of these gastropods can indicate relative health and changes to ecosystems (Smith, 2005). Muricids are also used as indicators of pollution from endocrine disrupting compounds released into waterways (Axiak *et al.,* 2003; Castro *et al.,* 2004; Fujinaga *et al.,* 2006b). Exposure to low levels of the heavy metal tributyltin, once widely used in antifouling paints, causes imposex. This condition leads to the development of a pseudo penis in females effectively rendering the affected females reproductively inviable (Gibbs & Bryan, 1986). The potential for this condition to negatively impact populations of a species that relies on internal fertilization for reproduction has been elucidated in numerous studies (Gibbs & Bryan, 1986; Ramón & Amor, 2002; Shi *et al.,* 2005; Fujinaga *et al.,* 2006a).

Whelks (Neogastropods) are dioecious and use internal fertilisation (Ponder, 1998), as opposed to external fertilisation in gastropod broadcast spawners. The ability to accurately identify the sex of whelk specimens is 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 and restocking purposes. Additionally, sex identification may prove useful for bioprospecting, as some species may produce sex specific secondary metabolites or chemical compositions (e.g. *Dicathais orbita* Westley & Benkendorff, 2008). In many dioecious species, sex identification can be relatively simple, if the sex of a specimen 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. Whilst some species of whelks will readily extend outside the shell after a period of time undisturbed, they will not necessarily extend far enough to easily observe the penis inside and this method also necessitates that each snail is dealt with individually. Methods of mass relaxation could greatly increase the efficiency of sex identification for large scale ecological surveys, breeding programs and bioprospecting purposes.

Anaesthetics are used extensively on marine molluscs for sampling non-destructively in ecological studies (Prince & Ford, 1985; McShane & Smith, 1988; Vasconcelos *et al.*, 2006) and in aquaculture to manipulate specimens (Sagara & Ninomiya, 1970; Heasman *et al.*, 1995; White *et al.*, 1996; Acosta-Salmón & Davis, 2007; Butt *et al.*, 2008). A range of different anaesthetics have been used on gastropods, with the most commonly used being potassium chloride, sodium pentobarbital, magnesium chloride, ethanol, carbon dioxide gas and benzocaine (Culloty & Mulcahy, 1992; Norton *et al.*, 1996; Aquilina & Roberts, 2000; Edwards *et al.*, 2000; Acosta-Salmón *et al.*, 2005; 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 (Michel-Morfín & Chávez, 2000, Naegel, 2004, 2005). This non-destructive method for collecting valuable purple dyes was considered an exception among muricids (Naegel & Alvarez, 2005), with all previous work being performed on the hypobranchial glands dissected from large numbers of sacrificed snails (e.g. Baker & Sutherland, 1968, Baker & Duke, 1973, Naegel & Cooksey, 2002, Westley & Benkendorff, 2008) or their egg masses (Benkendorff & Davis., 2000; Benkendorff *et al*., 2001). However, purple dye has been occasionally observed in association with the feeding activities of Muricidae (Roller *et al.,* 1995; 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*. *D. orbita* is endemic to southern Australian and New Zealand coastal waters (Gowlett-Holmes, 2008). Presently no commercial fishery of *D. orbita* exists, although small recreational harvests of *D. orbita* occur in New South Wales (Kingsford *et al.,* 1991) and South Australia (pers obs). Natural sex ratios and the incidence of imposex are currently unknown for S.A. 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. Consequently, there is a need to develop suitable non-

destructive methods for sampling this species in order to facilitate sustainable development. To better facilitate studies on the bioactivity of compounds from this species, anaesthetics were trialled for their effectiveness in stimulating release of these compounds. Detailed chemical analysis of secretions was also performed to confirm the presence of bioactive components 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.

2.3 Methods

2.3.1 Pilot study

Six anaesthetic treatments were tested at two concentrations for their efficacy in relaxing *D. orbita* out of the shell (Table 2.1). 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 2 L of aerated fresh seawater. Animals were placed in the bucket upside down to check for righting response and allowed to acclimatize for 1 h 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 2.1). 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.

Table 2.1. 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
		0.5M	**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

2.3.2 Anaesthetic trial

Based on the success of this pilot trial (Table 2.1) a more detailed study was undertaken on the four most promising chemicals dissolved in seawater: 1 mg L^{-1} 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 one hour. The treatment was then slowly added to the bucket. Specimens were checked for relaxation after one hour. 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.

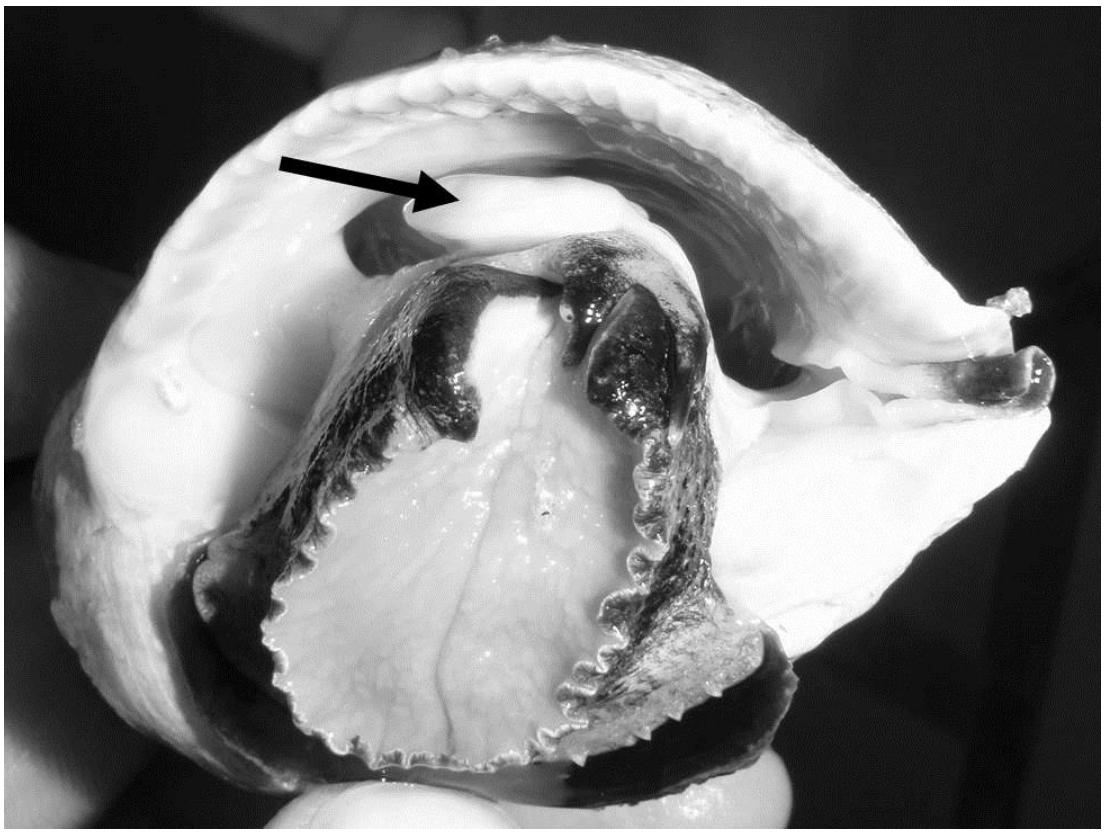

Figure 2.1. Relaxed specimen of *Dicathais orbita* showing the location of the penis.

2.3.3 Bioactive compound production

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 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).

Table 2.2. 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) (±S.E.) 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) (± S.E.)	% sex determined	Purple mucus
Ethanol, 5%	Small	33.4 ± 7	40	Yes
	Medium	24.7 ± 7.8	70	Yes
	Large	59.5 ± 19	60	Yes
Benzocaine, 200 mg L^{-1}	Small	25.9 ± 3.7	0	Yes
	Medium	27.1 ± 8.6	0	Yes
	Large	48.1 ± 22.1	0	Yes
Sodium pentobarbital, 1 mg L^{-1}	Small	57.4 ± 8	0	No
	Medium	29.3 ± 9.3	0	No
	Large	54.1 ± 3.7	0	No
Magnesium chloride, 0.5 M	Small	25.8 ± 2.6	90	No
	Medium	8.3 ±2.6	100	No
	Large	33.7 ± 4.2	100	No
Seawater control	Small	19.5 ± 0.8	0	No
	Medium	10.8 ± 3.4	0	No
	Large	36.3 ± 5.2	0	No

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 & Benkendorff, 2008).

2.3.4 Sex ratio and shell length of 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 2.2). 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 0.5 M) was added to the tub slowly to allow mixing with minimal disturbance. The specimens were left for an hour 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.

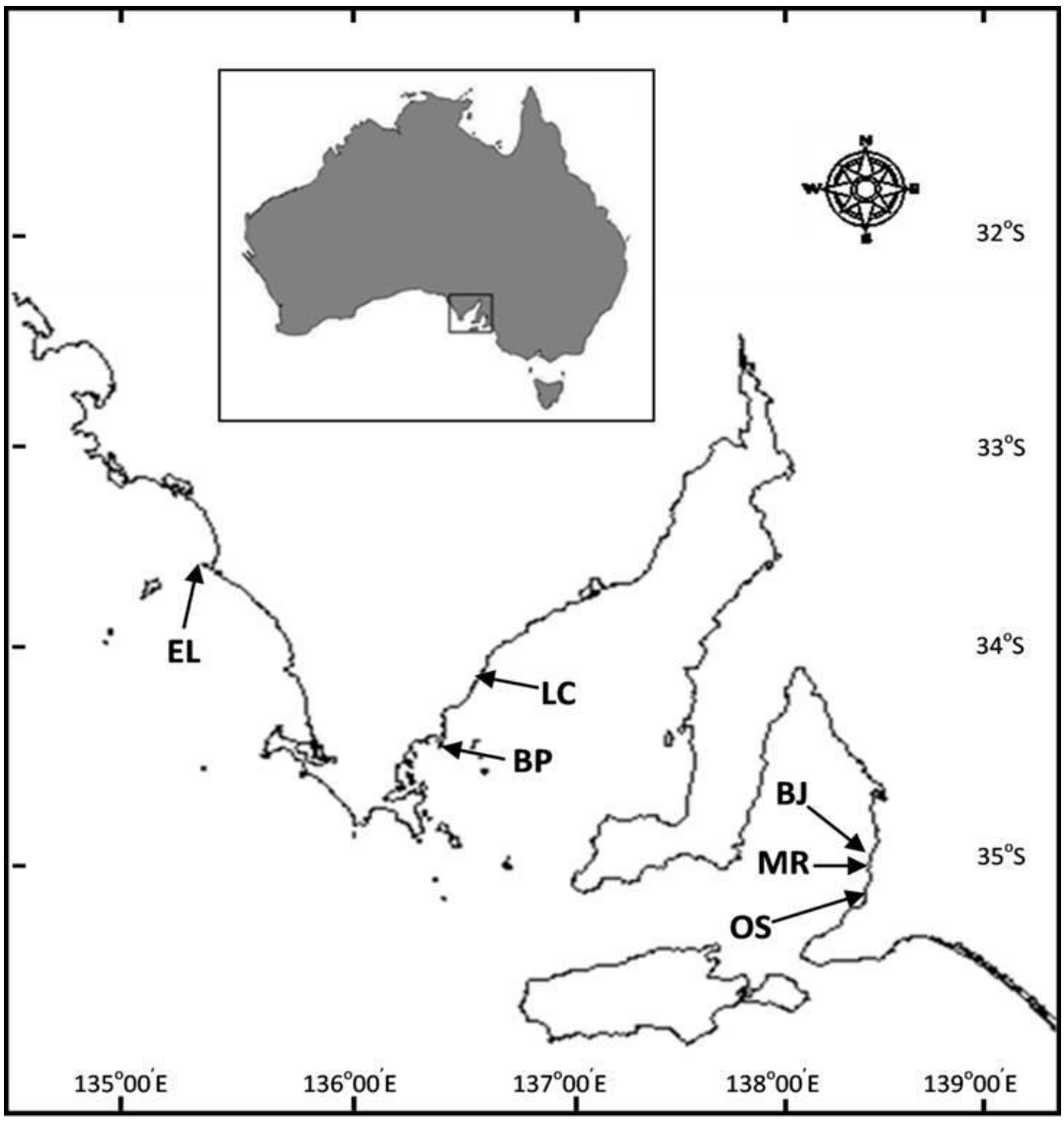

Figure 2.2. *Dicathais orbita* were collected from O'Sullivans Beach (OS), Marino Rocks (MR) and Brighton jetty (BJ) on the Fleurieu Peninsula and Lipson Cove (LC), Boston Point (BP) and Elliston (EL) on the Eyre Peninsula of South Australia.

2.3.5 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 sex 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.

2.4 Results

2.4.1 Anaesthetic trial

Of the four anaesthetic treatments identified as promising for use in sex identification from the pilot study (Table 2.2), magnesium chloride proved most effective (96.6 % sexed, Table 2.2). Ethanol ranged from 40-70 % successful across the three size classes (Table 2.2). 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 2.3). Results of the two-way ANOVA for recovery time (cube root transformed) 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 2.3). Tukeys post hoc analysis revealed that *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 2.3), but not from ethanol ($P = 0.338$).

Results from the dissections confirmed sex identifications based on presence or absence of a penis as accurate in all specimens and no evidence of any imposex 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, pers. com).

2.4.2 Bioactive compound recovery

Although less effective for identifying sex, ethanol did stimulate the expulsion of mucus that turned purple on exposure to light (Tabele 2.2). Benzocaine (dissolved in ethanol) did not show any effectiveness relaxing whelks enough to identify sex, but did stimulate 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 2.4). 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 2.4). 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 2.4) 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 2.4). 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.

2.4.3 Sex ratio and 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 (Figure 2.5). The sex ratio at each site did not differ significantly from 1:1 ($\chi^2 = 0.159$, P = 0.690, Figure 2.5).

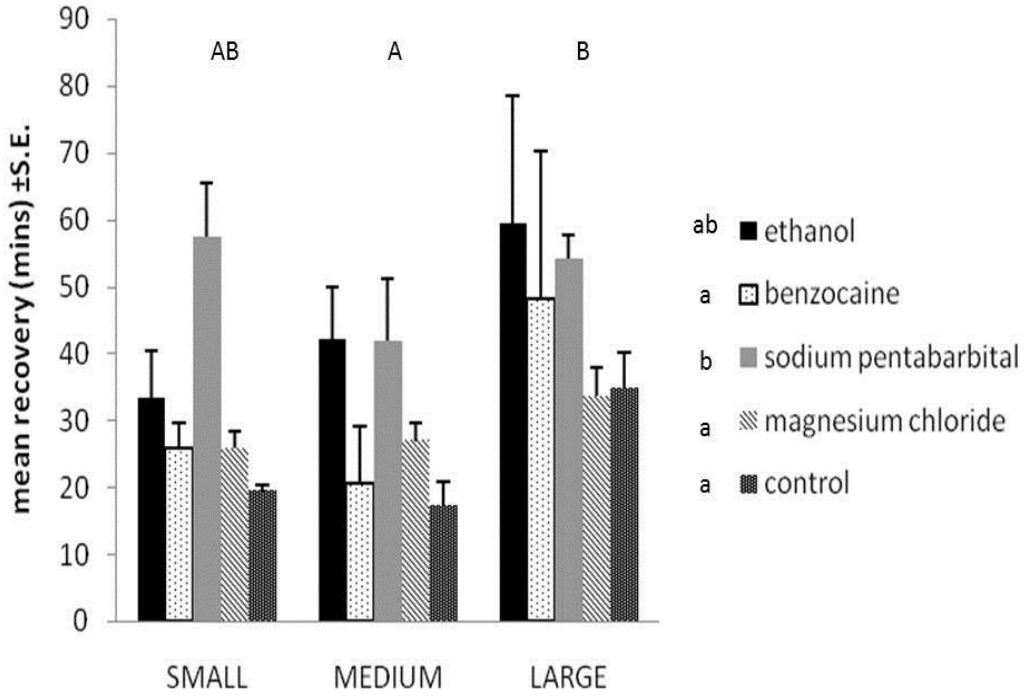

Figure 2.3. Mean recovery time minutes (± S.E.) of three size classes of *Dicathais orbita* post treatment with one of four anaesthetics. The different capital letters indicate significant differences between size classes, whereas the different small letters indicate significant differences between anaesthetic treatments from Tukeys pairwise comparisons on the cube-root transformed data.

The mean shell length of male and females differed between sexes and sites (Table 2.3). There was no interaction between site and sex (F = 0.506, P = 0.772). However, male *D. orbita* were consistently larger than females and overall, these differences were found to be significant (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 different to *D. orbita* from all other sites ($P < 0.001$).

Table 2.3. Success of sex identification in wild populations and mean shell length (mm ± S.E) 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

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 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 2.3), south of Adelaide in the Gulf St Vincent. The smallest mean shell length was recorded from Elliston (48.4 mm). 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.

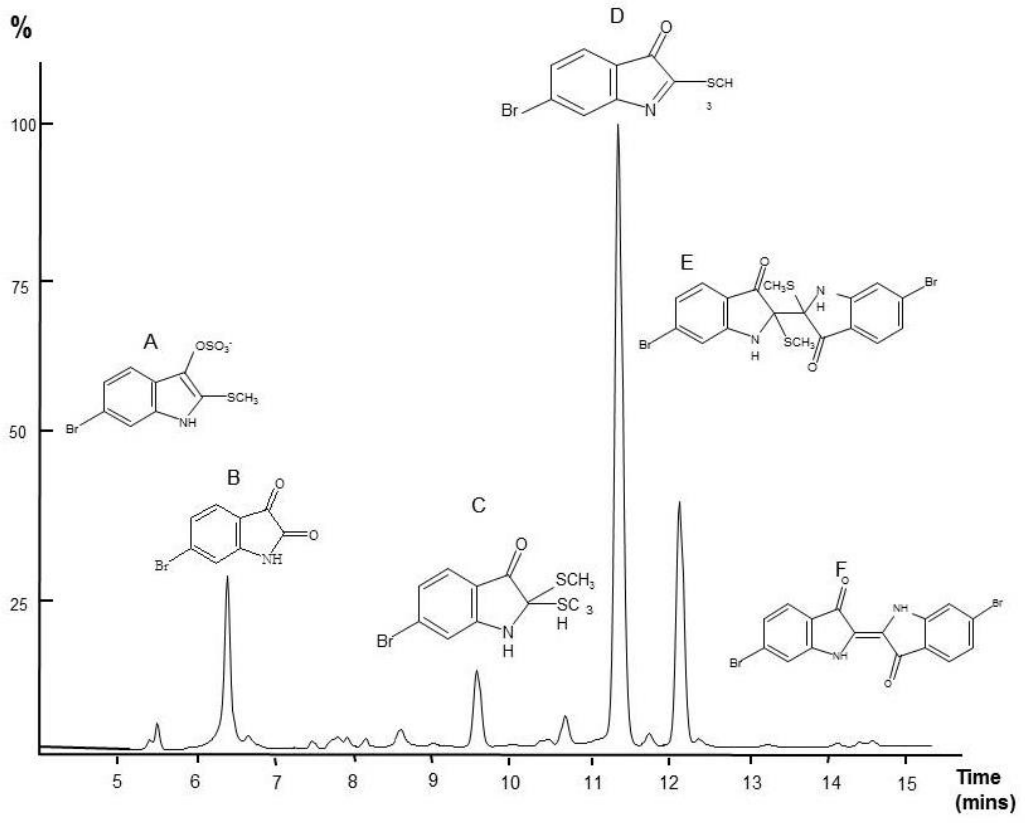

Figure 2.4. Liquid chromatogram showing composition of brominated indoles in a representative chloroform extract from the mucus of *D. orbita*. The x axis represents the retention time (minutes) in the LC column before the compounds were detected by the diode array. Y axis represents the absorbance units (AU) from the diode array at 300 nm. The resulting peaks are identified as: A) tyrindoxyl sulphate; B) 6-bromoisatin; C) tyrindoxyl/tyrindolinone; D) tyrindoleninone; E) tyriverdin and F) 6,6'-dibromoindigo.

2.5 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 South American muricid *Plicopurpura 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*.

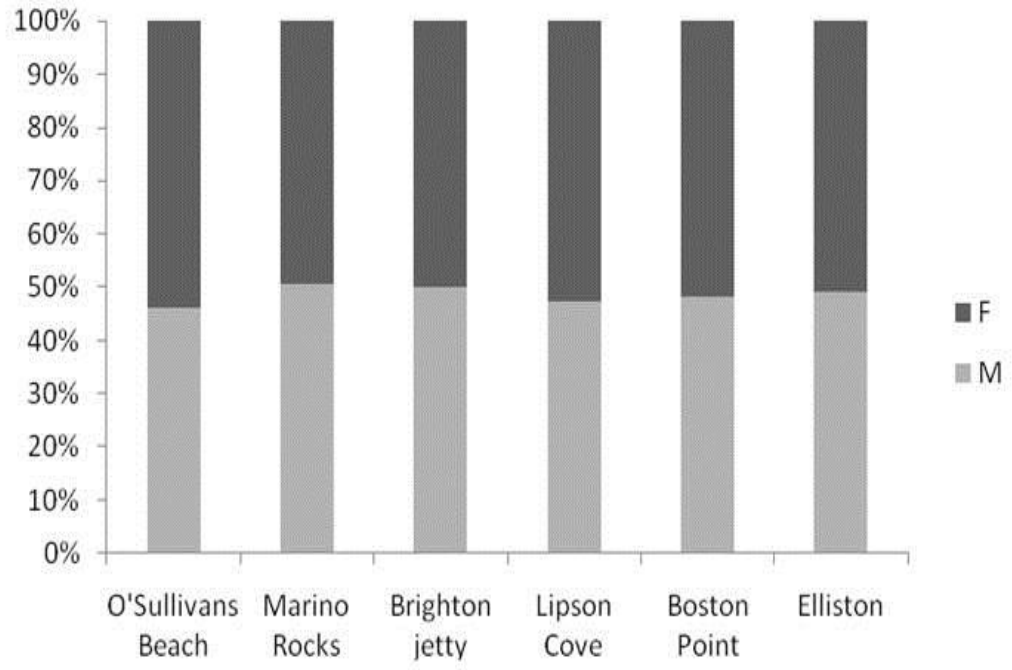

Figure 2.5. Natural sex ratio distributions of *D. orbita* from various locations on the Fleurieu and Eyre peninsulas of South Australia.

All of the six anaesthetics originally trialled at the pilot study stage have been used on one or more species of mollusc by other researchers for the purpose of anaesthetisation (e.g. Culloty & Mulcahy, 1992; Norton *et al.*, 1996; Aquilina & Roberts, 2000; Edwards *et al.*, 2000; Sharma *et al.*, 2003; Acosta-Salmón *et al.*, 2005; Acosta-Salmón & Davis, 2007; Butt *et al.*, 2008). 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 & Mulcahy, 1992; Coney, 1993; Huet *et al.,* 1995; Acosta-Salmón & Davis, 2007; Butt *et al.,* 2008). Sodium pentobarbital has been used to great effect in the narcotisation of Haliotidae (White *et al.,* 1996; Aquilina & Roberts, 2000; Sharma *et al.,* 2003). However, this anaesthetic only produced minimal relaxation in 20% of the oyster *Ostrea edulis* (Culloty & 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, thus preventing successful sex identification.

In *D. orbita*, the recovery time after exposure to anaesthetics can depend on size. Snails that were larger than 53 mm took up to twice as long to recover and were more variable in their recovery time when compared to the smaller animals. Metabolic rate in marine invertebrates varies inversely with increasing body mass (Von Bertalanffy, 1957). Ganapati and Ramasastry (1972) determined that although smaller specimens of the herbivorous intertidal gastropod, *Turbo intercostalis* expended less energy on respiration than did larger specimens, total metabolic rate decreased with increasing body size. Similarly, in the sand flat dwelling predatory gastropod *Polinices duplicates*, total respiration increased with increasing body size,

but growth efficiency and overall metabolic rate appeared to decrease with increasing body size (Huebner & Edwards, 1981). Hence, it is possible that increasing recovery times for larger specimens of *D. orbita* are due to the difference in metabolic rate, but this will need to be confirmed through experimentation.

A delayed recovery was observed in larger animals across all treatments and in the seawater control. Consequently, it is also 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 & Covich, 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; Lowell, 1986). Delgado *et al.* (2002) demonstrated this with queen conch (*Strombus gigas*), where defensive response time for specimens that had been previously exposed to predators was more acute than those with no prior exposure. Hence larger specimens that have lived longer may have experienced more predation attempts and been more reticent in extruding and righting themselves after an adverse handling event.

Irrespective of the size of *D. orbita*, significant differences were found in the recovery time from application of the different anaesthetics. Recovery from sodium pentobarbital 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. In this study recovery was primarily based on responsiveness and mobility. Animals were observed actively feeding in the days post-recovery, but the potential for long-term sublethal effects cannot be excluded.

Edwards *et al.* (2000) found that larger mean sized specimens of *Haliotis rubra* showed lower daily growth rates than the smaller mean sized *Haliotis laevigata* after treatment with benzocaine. This implies that the immediate recovery and long-term effects of anaesthetics could vary as a function of species and size, in addition to the specific anaesthetic applied.

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 & Ford, 1985; Edwards *et al.*, 2000). Aquilina & 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 & 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 & Chávez, 2000; Naegel, 2004; 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 & Sutherland, 1968; Baker & Duke, 1973; Benkendorff *et al.,* 2001; Westley & Benkendorff, 2008). The relative proportion of the different brominated indoles did vary somewhat in replicate extracts. This 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.,* 2001; Cooksey, 2001; Westley & Benkendorff, 2008). Importantly, a high proportion of the anticancer compound tyrindoleninone (Benkendorff, 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, there was no evidence of imposex 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; Ramón & Amor, 2002; Fujinaga *et al.,* 2006a, b; Mann *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 animals (Wolcott, 1973, Bayne & Widdows, 1978, Burrows & Hughes, 1990). 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) and *D. orbita* is known to scavenge fresh carrion (Phillips, 1969; Woodcock & Benkendorff, 2008). 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 (Chen *et al.,* 2005; Nasution & Roberts, 2005; Woodcock & 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.

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 & Benkendorff, 2008) divers are employed to regularly remove the whelks. These whelks are a current source of supply for preclinical trials using *D. orbita* extracts (Westley *et al.,* 2010a; Benkendorff *et al.,* 2011; Esmaeelian *et al.,* 2013) with potential for future polyculture. Conversely, 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 (WN 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 exposure, magnesium chloride is likely to be the most useful anaesthetic for any 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.*

Acknowledgements

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CHAPTER 3: Reproductive cycle, spawning and post hatching larval development of *Dicathais orbita* (Gmelin, 1791)(Neogastropoda, Mollusca)

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3.1 Abstract

Aquaculture production, including the production of pharmaceuticals and nutraceuticals, is increasing to supplement wild fish stocks. Understanding the life history of species of interest as biological resources will provide valuable information for sustainable production. The southern Australian muricid, *Dicathais orbita*, is harvested for food and produces potent bioactive compounds. The reproductive cycle of this species was studied in wild and captive populations using a gonadosomatic index, along with spawning and post hatching larval development. *D. orbita* follows an annual reproductive cycle, peaking in early summer (December) in South Australia. Females spawn ~ 40 egg capsules in a session and each capsule contains 5477-5607 eggs with a diameter between 49-150 µm. Post-hatching larval development proceeded through 5 stages over 41 days with shell length increasing from 314 (± St. Dev.) 46 to 1011 ± 143 µm and shell width increasing from 2262 ± 42 to 1013 ± 143 µm. The relatively high fecundity of adults and readiness to spawn in captivity with minimal difference to wild populations, along with the suitability of post hatching larvae for growth under laboratory conditions, makes this species promising for aquaculture.

3.2 Introduction

Aquaculture is a rapidly expanding and diversifying industry throughout the world (FAO, 2012). Aquaculture production, including the production of pharmaceuticals and nutraceuticals (Benkendorff, 2009) has increased to supplement wild stocks (Ram *et al.*, 1993). Muricid gastropods have long been harvested for shells, meat and Tyrian purple (Radwin & d'Attilio, 1976; Kingsford *et al.*, 1991; Naegel & Lopez-Rocha, 2006; Cooksey, 2013). Of particular note is the harvest of muricids to collect Tyrian purple which has been highly valued as a dye since ancient Roman times (Cooksey, 2013). Discovery of novel compounds with anti-tumour activity produced in the hypobranchial gland of the Australian muricid, *Dicathais orbita* (Benkendorff, 2013), has led to interest in this species beyond a small recreational harvest (Kingsford *et al.*, 1991) and the occasional beachcomber. Chemical synthesis of the anticancer agents is not yet possible as biosynthetic enzymes used by muricids to attach a methyl sulphide component to tyrindoleninone have not been identified (Benkendorff, 2013). Additionally, extracts contain several bioactive compounds (Chapter 2) that may act synergistically, and/or contribute different bioactivities useful in nutraceutical formulation. Hence, a primary focus is on the development of a potential nutraceutical or functional food (Benkendorff, 2009; Benkendorff, 2013) where bioactive components are present as natural constituents (Lordan *et al.*, 2011). The dye precursors can be collected by non-destructive anesthetisation in 5% ethanol (Chapter 2) offering the potential for sustainable supply. However the low yields relative to the amounts that would be required for a nutraceutical industry necessitate the development of sustainable production of the mollusc through aquaculture. The molluscan aquaculture industry is well developed throughout the geographic range of *D. orbita* in southern Australia and New Zealand and there is potential to value add to existing aquaculture in this region through polyculture or integrated multitrophic

aquaculture, as is already being done with other neogastropod species in other areas of the world (Kritsanapuntu *et al.,* 2006). An understanding of both the reproductive cycle and larval development are integral to achieving this goal.

Gurney and Mundy (2004) reviewed the published methods used to assess reproductive condition in the gastropod genus *Haliotis* and noted that a reliable gonad index could be determined by making a transverse cut through the gonad/digestive gland complex and comparing the gonad area to the total area of the cross section. This cross section, referred to as the gonadosomatic index (GSI), can be used regardless of the size of the animal. Hahn (1988) found that this index correlated with histology resulting in a clear representation of the reproductive cycle. The GSI has been successfully used to monitor gonad maturation and reproductive condition in the muricid, *Hexaplex trunculus* (Vasconcelos *et al.,* 2008b). These insights into the reproductive cycle of neogastopods can be supplemented with observations of spawning in aquaria and benthic egg capsules in the field, as has been done for the commercially and locally important species *C. concholepas* (Castilla & Cancino 1976) and *Chorus giganteus* (Gonzáles & Gallardo 1999) from South America, *P. pansa* from Mexico (Naegel *et al.,* 2003) and *Chicoreus virgineus*, from India (Jagadis *et al.,* 2013).

The Muricidae family display a complex range of larval development modes. All species deposit benthic egg capsules (D'Asaro, 1991), however, the development time varies considerably between species. Embryonic development of most muricids proceeds via a relatively short lecithotrophic phase, followed by a pelagic dispersive phase, which often involves planktotrophy. However, in some direct developing species with longer periods of intracapsular development, the larvae are cannibalistic or adelphophagic, supplemented by nurse eggs (Gonzáles & Gallardo, 1999), and less than 8% of eggs may be fertile (Gallardo, 1979). A better understanding of larval

development stages will lead to development of more suitable conditions and success in culture. For instance, the velum of planktonic larvae is used for motility and feeding (Strathmann *et al.,* 1993, Page 2007) and therefore velum size in these species may indicate insufficient food (Strathmann *et al.,* 1993; Klinzing & Pechenik 2000) or competence to metamorphose (Naegel *et al.,* 2003, Kulikova *et al.,* 2007).

The peak of the reproductive cycle, when female *D. orbita* lay rafts of egg capsules containing thousands of embryos on rocky shores and reefs, occurs in response to increasing water temperatures after winter (Phillips 1969). Planktotrophic larvae hatch within weeks and likely take advantage of high phytoplankton abundance that occurs due to the warm, summer water temperatures. Hatching success is close to 100% for *D. orbita* (Phillips 1969) and thousands of small planktotrophic larvae are released from the egg capsules, which can immediately start to feed and grow in the plankton (Phillips 1969, Chapter 4). However, the larval stages post-hatching are yet to be described for this species of Muricidae. Therefore, the aims of this study were to investigate the reproductive cycle of *D. orbita* as determined through gonadosomatic index, to document spawning for a South Australian population and to provide a description of the post-hatching larval development stages.

3.3 Methods

3.3.1 Reproductive cycle breeding and egg capsules

Dicathais orbita were collected monthly from jetty pilings at Brighton (-35.059772°, 138.502457°) and a rocky intertidal reef at Marino Rocks (-35.04385°, 138.50810°) along the metropolitan coast of Adelaide between August 2007-July 2008. Larger individuals were selected from each location to ensure that sexually mature specimens were used to assess reproductive cycle. Sex was identified after anaesthetisation using 0.5 M MgCl solution following the methods detailed in

Chapter 2. Five male and 5 female *D. orbita* were marked by etching the shell, frozen (-18°C) and retained for the study, while the others were returned to their habitat. Vernier callipers were used to measure shell length (SL) to the nearest 0.1 mm. A vice was used to crack the shells and the shell was removed without damaging the gonad/digestive gland complex (GDGc). Individuals were blotted dry to remove surface fluid and the GDGc was separated from the rest of the somatic tissues using a scalpel. The weights of the gonad and digestive gland (GDGwt) and of the remaining soft body (SBwt) were measured to the nearest 0.1 g. A transverse section (2-3 mm) of the GDGc was removed from behind the gastric caecum (Figure 3.1), placed flat on graph paper and photographed (Olympus, 1040SW) from above. Images were downloaded and the area of the cross section of the gonad/digestive gland complex (GDGca), the gonad area (Ga) and the digestive gland area (DGa) traced (Figure 3.1b) and measured (ImageJ) to the nearest 0.001 mm^2 after calibration with a stage micrometer.

The gonadosomatic index was determined as:

$$GSI = (Ga/GDGca) \times 100$$

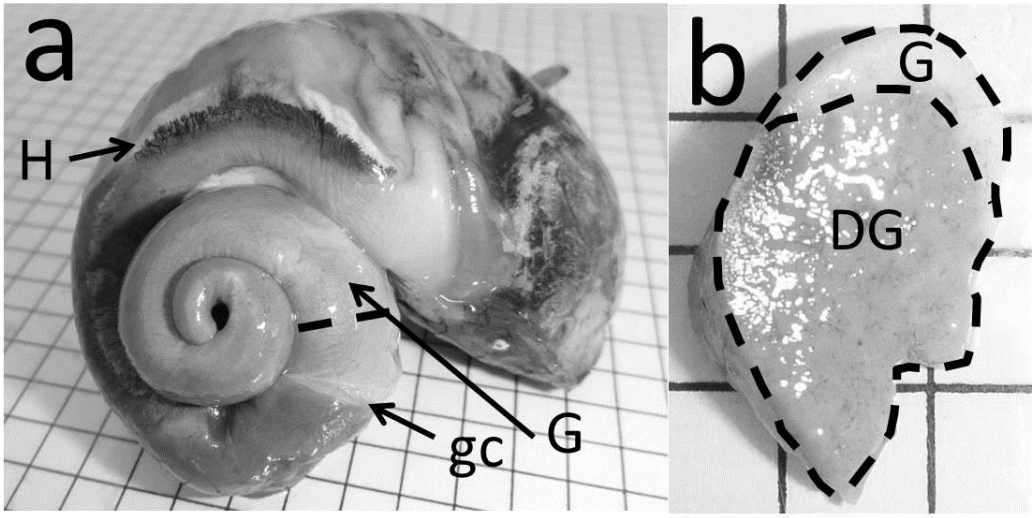

Figure 3.1. Assessment of reproductive activity using gonadosomatic index (GSI) in *Dicathais orbita*: a) specimen with shell removed showing transverse cut (dotted line) made in the digestive gland (DG) and gonad (G) complex behind the gastric caecum (gc) and in relation to the hypobranchial gland (H); b) outlines traced on the digitized image for measurement of the gonad and digestive gland (DG) areas to estimate the GSI.

3.3.2 Breeding and egg capsules

Each month throughout the year, observations were made within half an hour of low tide of the number of copulating pairs and the number of female *D. orbita* spawning egg capsules in the field. Experimental broodstock were maintained in recirculating aquaria at Flinders University and fed a diet of fresh oysters (*Crassostrea gigas* (Thunberg 1793)), mussels (*Mytilus galloprovincialis* (Lamarck 1891)) and frozen squid (*Sepioteuthis australis* Quoy & Gaimard 1832) and any spawning activity in the broodstock population was also recorded. The numbers of egg capsules in a group were counted for a selection of egg masses from wild populations (n = 29) and broodstock populations (n = 17). Egg capsules were collected from the broodstock tank (n = 15) and field sites (n = 15) from September through to January and used to study capsule morphometrics. Digital callipers and a dissecting microscope (10 x magnification) were used to measure the height and width of capsules, as well as the

length and width of aperture to the nearest 0.01 mm (Figure 3.2). The size of eggs (n = 150) from newly deposited capsules were measured using a micrometer. The average number of eggs per capsule from wild (n = 15) and broodstock (n = 15) egg masses was determined as was the number of larvae hatching from wild (n = 5) and broodstock (n = 5) capsules.

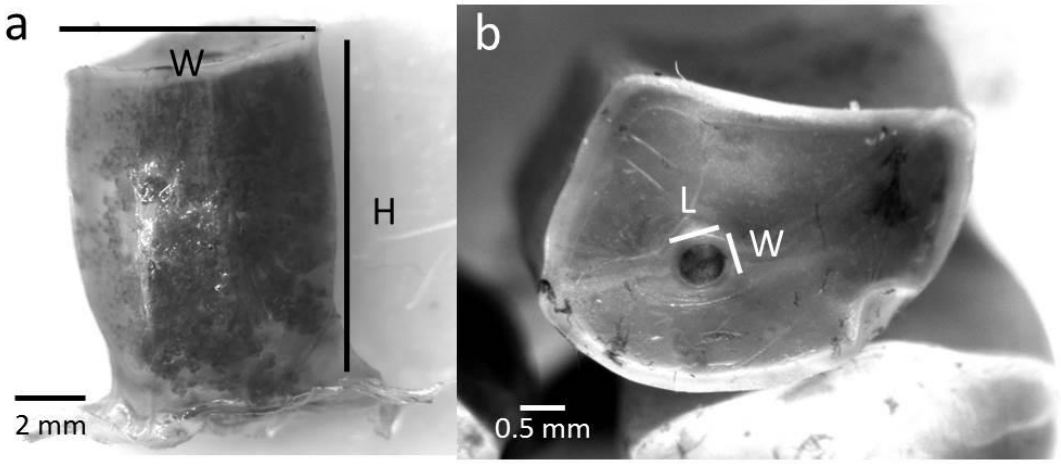

Figure 3.2. Morphometry of *Dicathais orbita* egg capsules; a) Capsule height (H) and width (W), b) aperture length (L) and width.

3.3.3 Larval development

Freshly deposited egg masses in the broodstock tanks were isolated in small seawater aquaria until larval hatching occurred. Newly hatched larvae were collected and reared continuously from hatching at 22°C on a diet of mixed green microalgae; *Nannochloropsis oculata* and *Tetraselmis suecica* and diatoms; *Isochrysis galbana* (Chapter 4). Initial larval density was 0.2 larvae mL^{-1}. Larvae were removed from culture containers and observed every 2-4 days for 41 days under a light microscope. Larvae (n = 10 per sampling day) were photographed and the images downloaded to image analysis software (Motic Images 2.0). Shell length, shell width, and the area of each velar lobe were measured to the nearest 0.1 µm, after calibration of the image software. For descriptive purposes, veligers were assigned to one of five groups on the basis of visible morphological features along the continuum of development. A

scheme developed by Davis *et al.* (1993) to describe differences in development between three strombid species was adapted by Harding (2006) to describe larval development of the muricid, *Rapana venosa*. The following briefly describes the scheme adopted for the present study; stage 1 represented newly hatched larvae (protoconch I), external features such as the bilobed velum, translucent foot and eyestalks are visible, while the beating heart can be seen through the protoconch I. Stage 2 larvae were characterised by an elongated beak and formation of protoconch II and siphonal canal. Stage 3, protoconch II had increased in size, the bifurcation of the velum and the siphonal canal elongated. Stage 4 beaks had diminished to a small point. Stage 5 protoconch II more than full whorls and foot showed strong contractions. Means for shell morphometrics corresponding to development stages were used in all analyses.

3.3.4 Statistical analysis

Shell length and GSI data were analysed between sexes and months using a 2 factor permutational univariate analysis for nonparametric data (Primer v6 + PERMANOVA add on) (Anderson *et al.*, 2008). A resemblance matrix was created using Euclidean distance then analysed using 9999 unrestricted permutations of the raw data. Pairwise comparisons using Gosset's t statistic were used to determine significant differences between sexes within months (Primer v6 PERMANOVA). The number of egg capsules in groups from wild and broodstock egg masses, the number of eggs per capsule and the size of eggs from the different sources were all analysed using independent samples t-test, after satisfying the assumptions for equality of variances using Levene's test (SPSS v.21).

One factor permutational MANOVA was used to examine differences between stages for larval morphometric data (Primer v6 PERMANOVA). Canonical analysis

of principal coordinates was used to explore the relationship between larval morphometry over the stages of development (Primer v6 PERMANOVA).

3.4 Results

3.4.1 Reproductive cycle

A total of 60 male and 60 female *Dicathais orbita* were assessed for reproductive condition over an annual cycle. The mean shell length of 81.54 ± 12.1 mm for sampled females and 81.41 ± 8.6 mm for males was not found to be significantly different between sexes (df =1, F = 1.066, P = 0.392), or between months (df = 11, F = 0.353, P = 0.553) , and there was no interaction between these factors (df = 11, F = 0.974, P = 0.477). The mean SBwt was not significantly different between females (29.64 g) and males (28.97 g) (df = 1, F = 0.0004, P = 0.981) and there was no interaction between sex and time of year (df = 11, F = 0.428, P = 0.941). However, SBwt was significantly different between months (df = 11, F = 3.305, P = 0.001). Post hoc analysis revealed the SBwt was significantly greater in autumn and winter months than in spring and summer months (Figure 3.3). SBwt was lowest during the peak spawning season in November and December and began increasing in January (Figure 3.3).

The gonadosomatic index of *D. orbita* showed considerable monthly variation throughout the study period (Figure 3.4). GSI was significantly higher for males than females throughout the year, apart from during peak spawning (df = 1, F = 109.2, P < 0.001) and there was a significant interaction between sex and month (df = 11, F = 8.137, P < 0.001) (Figure 3.4). Males had a significantly higher GSI than females from late summer (February) through to early spring (September) (Figure 3.4).

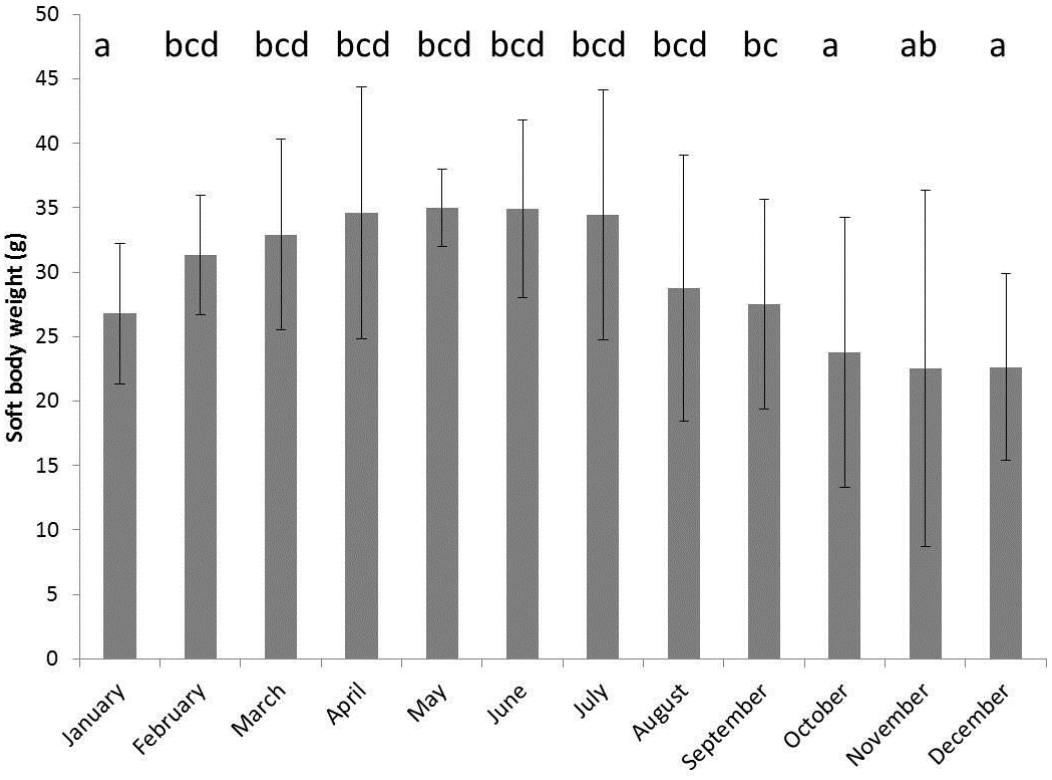

Figure 3.3. Soft body weight of *Dicathais orbita* collected over an annual cycle. The data shows the mean for females and males (n = 10). Error bars represent the standard deviation. Different letters represent statistical differences between months.

Males showed a steady increase in GSI throughout summer, autumn and winter, peaking in November (43.2 ± 7.7 %) then dropping significantly in December and January to a minimum of 7.4 ± 2.3 % (Figure 3.4). Females also showed maximum GSI in November (41.5 ± 12.5 %) before significantly dropping in December and January, however the GSI in females then plateaued, reaching a minimum in March and April (5.3 ± 1.8 %) (Figure 3.4). The GSI in females started to recover slowly through winter before significantly increasing in spring (Figure 3.4).

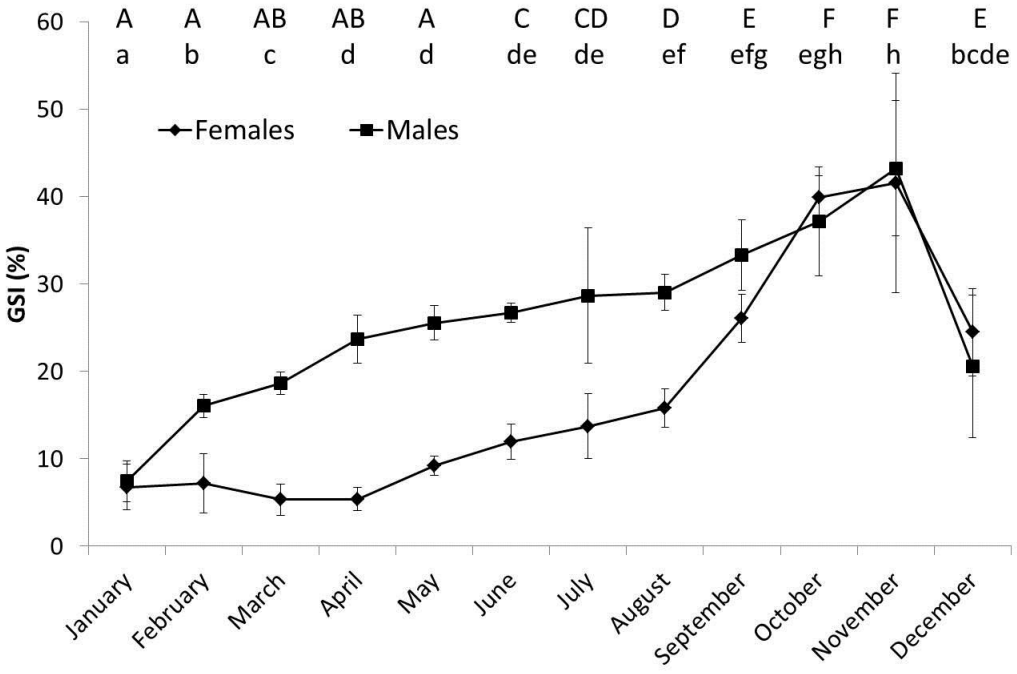

Figure 3.4. Monthly variation of the reproductive condition of *Dicathais orbita* as estimated by the gonadosomatic index (GSI). The data shows the mean for females (n = 5) and males (n = 5); error bars represent the standard deviation; different capital letters indicate significant differences between months for females, whereas different small letters indicate significant differences between months for males.

3.4.2 Breeding and egg capsules

Copulating pairs were observed in every month except February at field sites and every month except February, April and December in the broodstock population (Table 3.1). Most copulating pairs at field sites and in broodstock tanks were observed between April and August (Table 3.1). Spawning aggregations involved in egg capsule deposition were evident from August and peaked in December (Table 3.1). Aggregations of 2-9 females were observed depositing egg capsules in communal masses on vertical rock walls and under large boulders and rocky overhangs on natural shallow subtidal reefs, as well as on concrete jetty pilings, the sides of the broodstock tanks and on the shells of conspecifics. Fresh egg capsules

were creamy white and became purple over time. Newly deposited egg masses were often found adjacent to older, empty egg masses (Figure 3.5a) and most egg capsules were empty by February. Capsules laid in a single session by an individual female were connected by a continuous basal layer (Figure 3.5b) and were often observable as a distinct group within the larger mass, with colour and position in the mass indicating relative timing of deposition. That is, more recently deposited capsules were lighter in colour and were often positioned on the edge of the mass (Figure 3.5a). At the jetty location, capsules were deposited mostly towards the base of jetty pilings, although a few small masses were below 0.41 m Prediction Datum (BOM, 2007) in the intertidal zone, depending on the availability of space among other sessile organisms. The egg masses were predominantly deposited on the shoreward side of the pilings. At Marino Rocks, egg capsules were deposited on the underside of rocks or on vertical surfaces mostly protected from direct sunlight and prevailing waves. All egg capsules within a mass were attached to adjacent capsules at the basal membrane and once the edge of the mass has been detached from the substrate, the egg cluster can be carefully peeled to remove large sections of the mass without damaging the egg capsules (Figure 3.5b).

The mean number of egg capsules in a single group within an egg mass ranged from 13–72 and was not significantly different at field locations and in the broodstock population ($df = 44$, $t = 0.057$, $P = 0.955$) (Table 3.2). The capsules were on average 6.92 ± 1.1 x 6.04 ± 0.7 mm and neither capsule height ($df = 28$, $t = 1.99$, $P = 0.056$) nor width ($t = -1.757$, $P = 0.09$) were significantly different in wild populations and broodstock populations (Table 3.2). The capsule aperture was approximately 0.6 x 0.5 mm and again there were no significant differences in aperture length ($t = 1.642$, $P = 0.112$) or aperture width ($t = -0.595$, $P = 0.346$) from wild and broodstock populations (Table 3.2).

Table 3.1. Numbers of copulating pairs and individual females observed depositing egg capsules in wild populations and broodstock population over the course of the study.

Month	Copulating pairs		Egg capsule deposition	
	field	broodstock	field	broodstock
January	2	1	2	3
February	0	0	0	0
March	1	2	0	0
April	4	0	0	0
May	5	3	0	0
June	5	1	0	0
August	7	2	0	2
September	1	2	22	7
October	3	1	61	6
November	2	2	57	13
December	2	0	64	13

The number of eggs per capsule ranged from 3519-5908 and was not significantly different in broodstock and wild egg masses (df = 148, t = 0.615, P = 0.539). There was no interaction between the factors when comparing the number of eggs and the number of larvae hatching from wild and broodstock populations (df = 1, f = 0.585, P = 0.446). The mean number of larvae hatching ranged from 5430–5460 and did not differ significantly from the mean number of eggs (df = 1, f = 0.866, P = 0.363) and there was no difference between wild and broodstock populations (df = 1, f = 0.212, P = 0.645). The mean size of eggs from wild populations and from the broodstock population were also not significantly different (df = 148, t = -1.159, P = 0.114) and ranged from 49–150 µm (Table 3.2).

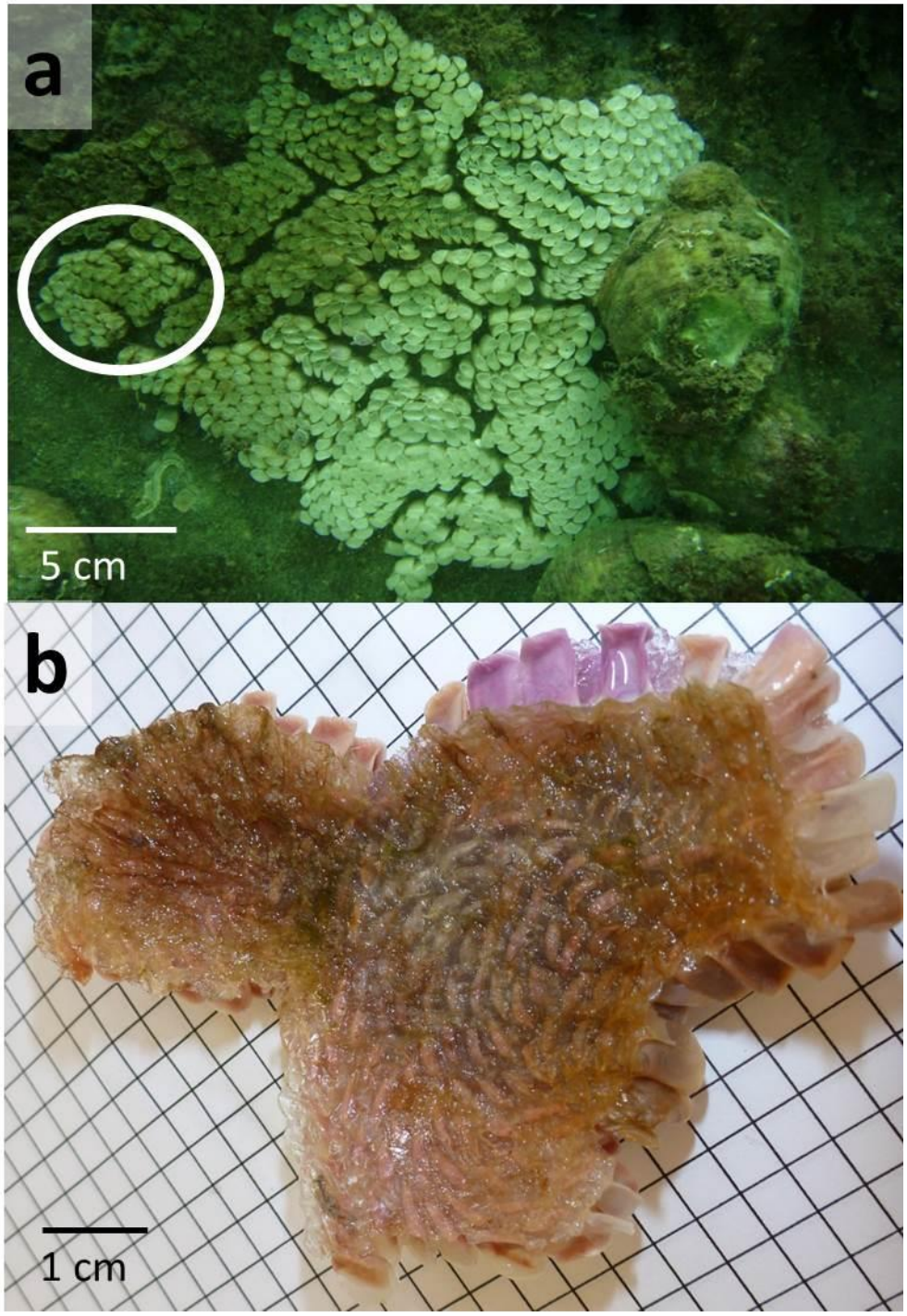

Figure 3.5. Egg masses of *Dicathais orbita*: a) separated into groups of eggs laid by individuals in one session (circled) with capsules added more recently visibly lighter in colour; and b) all capsules in an egg mass joined by a common basal membrane.

Table 3.2. The number of egg capsules spawned in one session, capsule dimensions, number of eggs per capsule and egg size for newly spawned *Dicathais orbita* egg masses. Data shown is from Broodstock (n = 17 capsules) and Wild populations (n = 29 capsules) and reported as mean ± St. Dev.

Source	No. capsules/group	Height (mm)	Width (mm)	Aperture length (mm)	Aperture width (mm)	Eggs capsule^{-1}	Egg size (µm) n = 150
		n=15					
Broodstock	48.8 ± 16.1	6.55 ± 0.91	6.19 ± 0.69	0.61 ± 0.04	0.51 ± 0.04	5607.6 ± 511.0	104.4 ± 20.6
Wild	49.1 ± 16.8	7.30 ± 1.08	5.88 ± 0.62	0.63 ± 0.03	0.49 ± 0.04	5477.2 ± 672.2	106.1 ± 19.3

3.4.3 Larval development

Distinct characteristics were identified throughout each stage of larval development. Veligers at stage 1 of development from hatching, to 7 dph, have a translucent protoconch, consisting of 1.5 whorls with small, tuberculate ornamentation (nodules). The vitellus and digestive gland can be identified through the shell (Figure 3.6a). The podium and operculum are also translucent and the bilobed ciliated velum is fully retractable into the shell when the larvae are not swimming. When swimming, the shell was generally directed downwards with the aperture and extended velum oriented upwards and in this position pigmented eye spots can be identified (Figure 3.6b).

Stage 2, after approximately 7 dph, shows the shell has approximately two full shell whorls with visible growth markings at the shell beak. The nodules present on the pre-hatching shell are not present on the new shell. The first sign of the siphonal canal is evident, increasing the width of the shell.

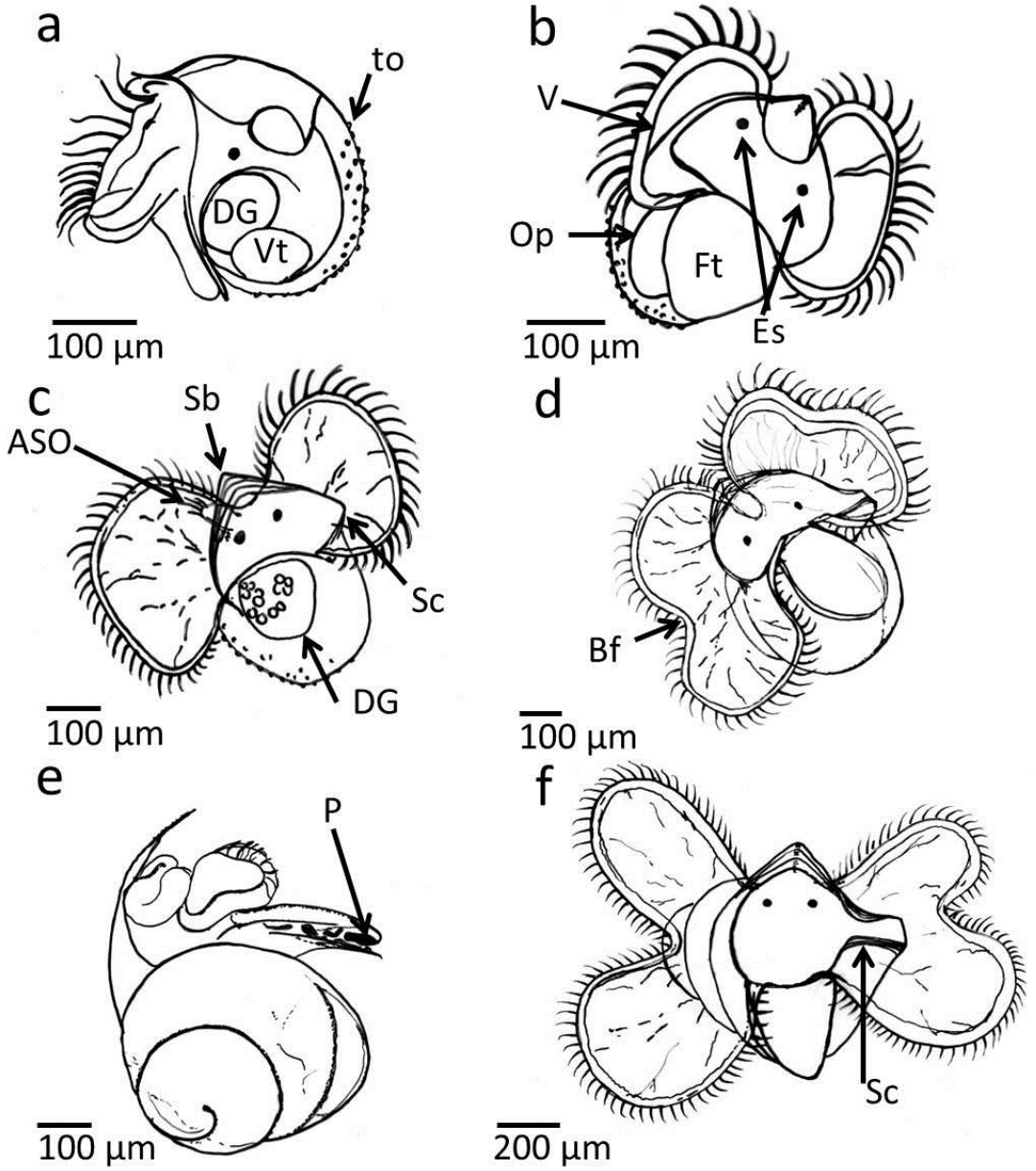

Figure 3.6. Developmental stages of *Dicathais orbita* through 6 weeks of post hatching development: a) ventral view of 1st stage development, b) lateral view of 1st stage development, c–f) 2nd to 5th stages of development. Tuberculate ornamentation (to), digestive gland (DG), vitellus (Vt), velum (V), foot (Ft), operculum (Op), eyespots (Es), shell beak (Sb), siphonal canal (Sc), bifurcation (Bf), pigmentation (P). Scale bars indicate approximate size from dissecting microscope images.

Contractions of the foot become obvious and consumed phytoplankton cells can be seen in the digestive gland (Figure 3.6c).

At Stage 3, around 15 dph, the siphonal canal is noticeably more developed. The first sign of velum bifurcation also develops (Figure 3.6d). At stage 4, more than 20 dph, the shell has approximately three shell whorls. The velum is prominently bifurcated. Pigmentation is evident on the dorsal surface of the foot which is capable of strong contractions (Figure 3.6e). At stage 5 (about 30 dph), the shell has approximately 3.25 shell whorls and pigmentation on the foot has increased. Bifurcation of the velum continues with the right lobe larger than the left (Figure 3.6f). The siphonal canal is well developed showing growth increments (Figure 3.6f).

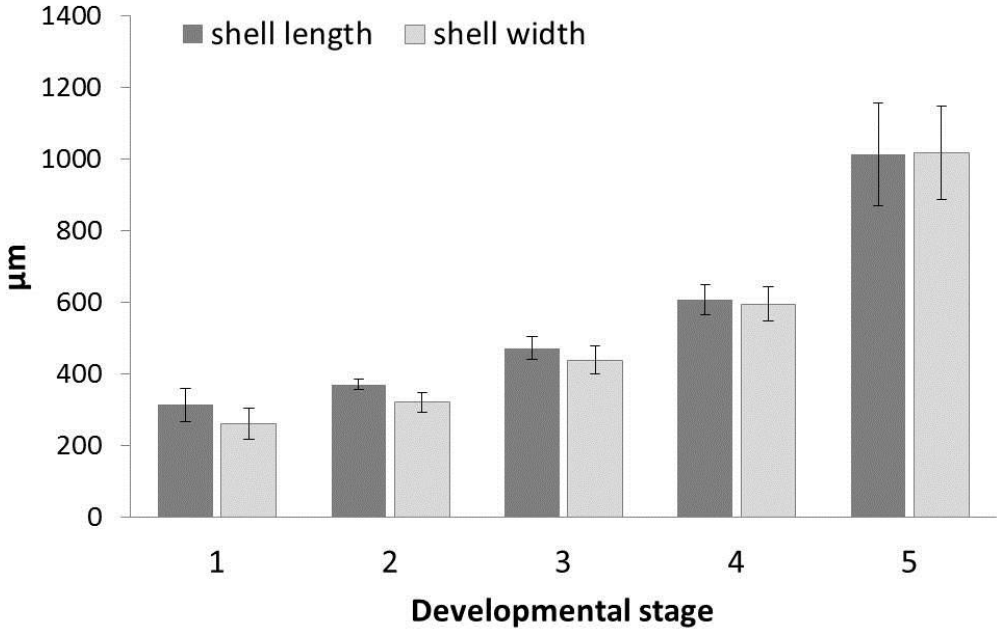

Figure 3.7. Shell length and shell width (µm) of *Dicathais orbita* larvae over 5 stages of development. The data represents means (n=10) from 3 replicate cultures. Error bars represent the standard deviation.

Growth did not proceed linearly for either shell length or width (Figure 3.7) and shell morphology changed considerably throughout development. Mean larval shell length varied from 253 µm at hatching to 974 µm at stage 5, and shell width varied from 204 µm at hatching to 981 µm at stage 5. The mean velar lobe area varied from 167745 μm^2 at stage 1 to 593889 μm^2 at stage 5 for the right velar lobe and 164544

μm^2 at stage 1 to 443442 μm^2 at stage 5 for the left velar lobe (Figure 3.8). The metrics for larval development were significantly greater for each successive stage of development (df = 4, F = 498.68, P < 0.001) (Figure 3.7 & 3.8). Multivariate analysis combining all larval morphometrics illustrates the changes in morphology over five stages of development.

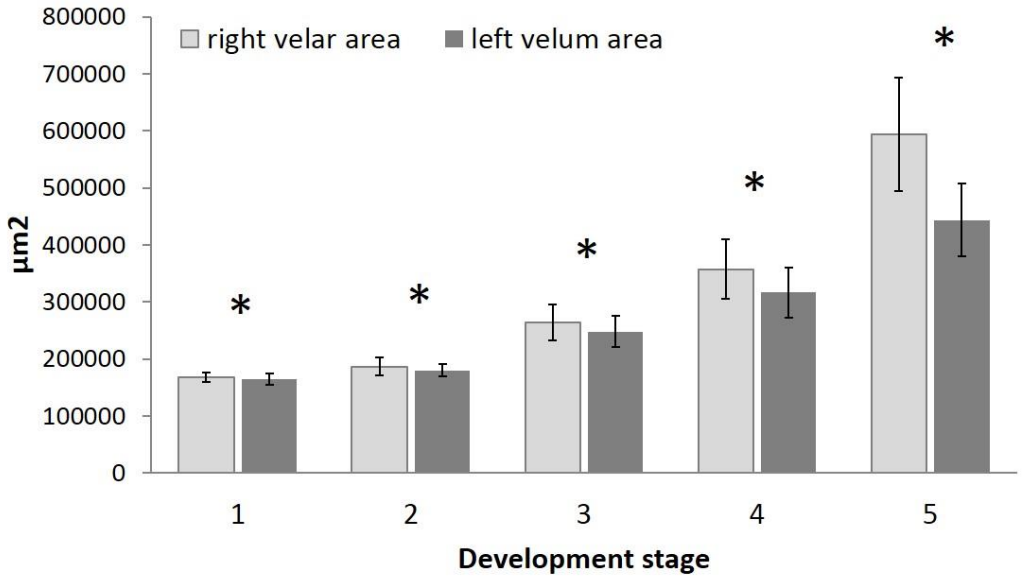

Figure 3.8. Area of the right and left velar lobes *Dicathais orbita* larvae over 5 stages of development. The data represents means (n = 10) from 3 replicate cultures. Error bars represent the standard deviation. * denotes significant differences between the stages.

The correlation coefficients of the first two eigenvalues determined by canonical analysis of principle coordinates show that over 96% of the variation in the data is explained by the differences in shell length and width between stages (δ^2_1 = 0.969) and 23% is explained by differences between the left and right velum area (δ^2_2 = 0.232) (Figure 3.9). The increasing spread of points along CAP2 illustrates that as larval size increases progressively through the stages, the larval shell shape and velum area also become more variable (Figure 3.9).

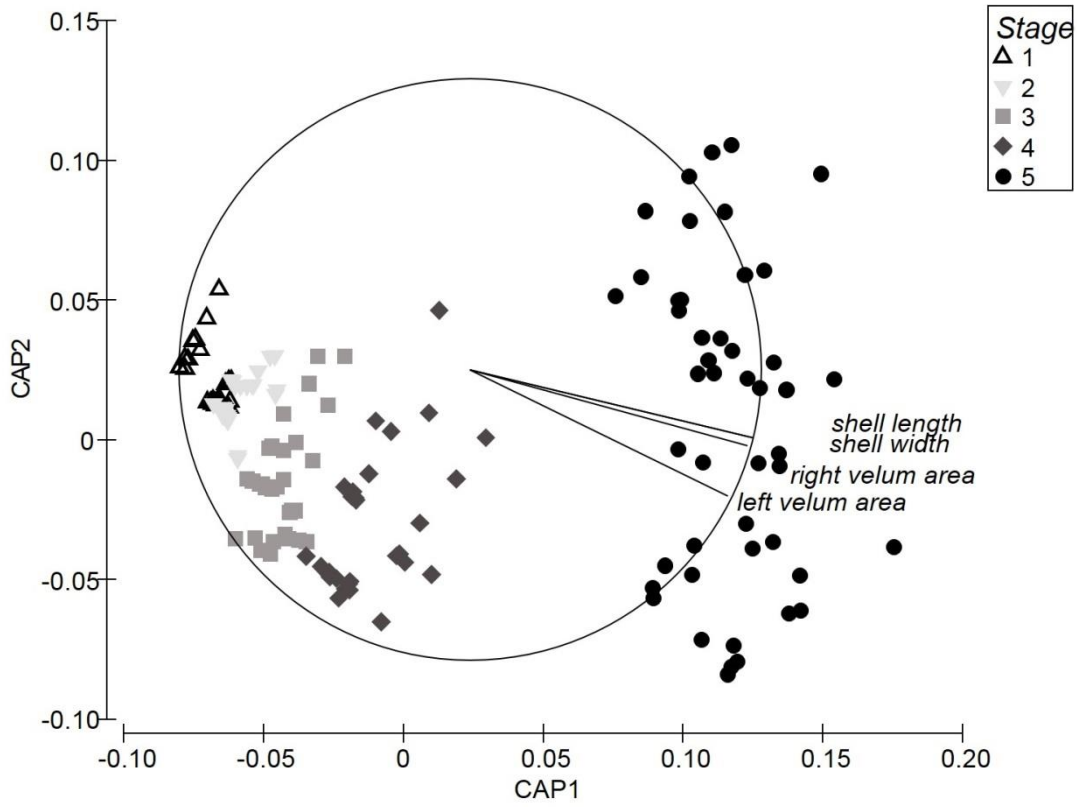

Figure 3.9. Canonical analysis of principal coordinates for larval shell and velum morphometrics.

3.5 Discussion

This study contributes to our knowledge of the reproductive cycle and larval development of the Australian muricid *Dicathais orbita*, a species identified as a model for natural products research and potential nutraceutical development (Benkendorff, 2013). The GSI, as determined by the proportion of the gonad area to the cross section of the gonad and digestive gland complex, was used to follow the reproductive cycle of *D. orbita*. This method has previously been used to follow the reproductive cycle of haliotids (Gurney & Mundy, 2004) and muricids (Vasconcelos *et al.*, 2008c). Using this index, the present study shows that reproduction in male and female *D. orbita* follows an annual cycle, with peak reproductive capacity between September and December in South Australia. These results are supported by observations of the greatest number of females spawning and the greatest number of

egg masses found during austral spring, in wild and broodstock populations. This coincides with increasing water temperatures for spring and summer along the metropolitan coast of South Australia (IMOS, 2011). The pattern of increasing reproductive condition following rising water temperatures in spring and summer is similar to other muricids (Naegel & García-Domínguez, 2006; Vasconcelos *et al.*, 2008b; Lahbib *et al.*, 2011).

Although the GSI was higher throughout most of the year for males than females, the GSI of both sexes synchronized during peak spawning. This is consistent with the pattern observed for a range of other muricids (Naegel & García-Domínguez, 2006; Vasconcelos *et al.*, 2012). Regardless of sex, the GSI also showed a post spawning decrease similar to other muricid species from across a range of latitudes in the northern hemisphere (Naegel & García-Domínguez, 2006; Vasconcelos *et al.*, 2008b; Lahbib *et al.*, 2011) and reflects the loss of gametes at spawning (Carrasco *et al.*, 2006). However, females took considerably longer to recover after spawning, then rapidly accumulated gonad mass in the months immediately prior to reproductive season. In the muricid *Hexaplex trunculus*, female GSI remained at around post spawning level for 5 months, whereas male GSI began increasing steadily after only a month (Vasconcelos, 2008b). Similarly, female *Neptunea arthritica* (Buccinidae) undergo a 4 month recovery period post spawning, and males only 3 months (Power & Keegan, 2001). Conversely, ripening of gonads in *P. pansa* occurred over 3 months for females after the recovery period, whereas ripening took up to a month longer for males (Naegel & García-Domínguez, 2006).

The decrease in GSI was accompanied by a decrease in soft tissue body weight. The energetic cost of mating (Hughes, 1986) and reproductive conditioning (Westley *et al.*, 2010b) can be substantial, requiring gastropods to draw on reserves in other

somatic tissues (Belisle & Stickle, 1978). For instance, Stickle and Mrozek (1973) reported a mean decrease of 34 % of pre-spawning soft body weight for the temperate muricid *Thais lamellosa*. Similarly, soft body weight of *H. trunculus* decreased by around 30% in the two months following peak spawning (Vasconcelos, 2008b; Vasconcelos *et al.,* 2009). We found that soft body weight of *D. orbita* fluctuated by nearly 49 % between a peak in late autumn to early winter and the post-spawning minimum in early summer. This emphasises the significant cost of reproduction in gastropods that mate and deposit benthic egg masses.

Some *D. orbita* were observed copulating in the field all year round. Histological studies by Westley *et al.* (2010b) revealed that *D. orbita* has the ability to store sperm for > 10 months. The fresh egg capsules of *D. orbita* were visible from August in South Australia, with the peak of the reproductive cycle occurring between November and December and most egg capsules being empty by late January. Phillips (1969) noted that peak spawning of *D. orbita* occurs during September and October at Rottnest Island in Western Australia. On the east coast of NSW, Australia, peak spawning is between August and October (Przeslawski, 2008). This earlier onset of peak spawning is consistent with the lower latitude and the Leeuwin and East Australian currents which bring warm water south along the west and east Australian coastlines respectively, in late winter – spring. In comparison the semi enclosed sea of Gulf St Vincent (GSV), South Australia, is relatively shallow and warmer temperatures result mostly from increasing insolation in spring to summer. Once deposited, embryonic development proceeds for approximately 4 weeks (Phillips 1969), indicating peak hatching of planktotrophic veligers in January. A number of species of phytoplankton consumed by *D. orbita* larvae (Phillips, 1969; Chapter 4) are prolific in GSV at that time of year (Van Ruth 2008) suggesting that spawning may be timed to take advantage of this food availability in the plankton.

Females were gregarious when spawning with one or more individuals simultaneously spawning, as well as others surrounding the egg mass. Communal spawning behaviour of *D. orbita* occurs throughout South Australia (WN pers. obs), NSW (KB pers. obs.) and in Western Australia (Phillips, 1969), and has been reported for other muricid species around the world (Romero *et al.*, 2004; Sağlam & Düzgüneş, 2007; Gallardo *et al.*, 2012; Jagadis *et al.*, 2013). Multiple spawnings may also occur from an individual over the spawning season, as is the case for *Rapana* v*enosa* (Valenciennes, 1846), which spawns hundreds of capsules in groups of less than 30 at a time (Sağlam & Düzgüneş, 2007).

It is well established that fecundity increases with increasing female size (Spight, 1976; Phillips, 1969). The mean shell length of *D. orbita* in the present study is 81.5 mm, with an average of 49 capsules deposited by an individual in one session. By comparison, Phillips (1969) reported a mean shell length of 56.6 mm in a Western Australian population, and the mean number of egg capsules spawned in a session was 40. The number of eggs per capsule also conformed to the same pattern with a range of 3519 to 5908, with a mean of 5542 for the present study. In comparison, Phillips (1969) reported a range from 730 to 7180 eggs per capsule and the mean spawned by one individual of 4620 for Western Australian populations. Both the size of the egg capsules and the size of the eggs dictate how many eggs can be contained within each egg capsule (Sağlam & Düzgüneş, 2007; Gonzáles & Gallardo, 1999; Gallardo & Gonzáles, 1994; Romero *et al.*, 2004). The size of eggs varies substantially for muricid species (Gallardo & Gonzáles, 1994, Romero *et al.*, 2004). We found egg size for *D. orbita* to range from 49 µm to 150 µm with a mean of 105 µm. Other muricid species with planktotrophic larvae, *C. concholepas* and *R. venosa*, spawn eggs between 158 to 169 µm (Gallardo & Gonzáles 1994) and 100 µm (Sağlam & Düzgüneş, 2007), respectively, while *P. pansa* and *Drupella cornus*

spawn eggs of 149 µm (Romero *et al.,* 2004) and between 160 to 180 µm (Turner, 1992).

The five stages of development show similar morphological changes to other muricids, although the duration of each stage and the total duration of larval development varies according to species. During the first stage of *D. orbita* post hatching development, the shell was translucent with approximately 1.5 shell whorls with a small shell beak and small nodules on the shell. The velum was bilobed and the foot and operculum were translucent. The number of shell whorls during the first stage was greater than *R. venosa,* which only had one whorl by day 6 (Harding, 2006). The translucence of the protoconch, the protoconch beak and a bilobed velum are features shared with other muricids; *R. venosa* (Harding, 2006), *C. concholepas* (Di Salvo, 1988) and *P. pansa* (Naegel *et al.,* 2003). By stage 2, *D. orbita* had two shell whorls, the shell beak had become more prominent, the siphonal canal had begun to form and both the protoconch and foot remained translucent. The siphonal canal of *C. concholepas* also becomes evident at the end of the first week, while the shell beak continues to extend (Di Salvo, 1988). In contrast, *R. venosa* has only developed one complete shell whorl in stage 2, but the foot is becoming opaque (Harding, 2006).

Development proceeds rapidly during stage 3. This is the first sign of the velum beginning to bifurcate for *D. orbita*, *R. venosa* (Harding, 2006) and *P. pansa* (Naegel *et al.,* 2003) alike. The shell of *R. venosa* increases to two full shell whorls (Harding 2006), whereas the shell of *D. orbita* has around 2.5 whorls during this stage. At stage 4, the foot of *D. orbita* shows stronger contractions and pigmentation is also evident. The shell consists of three whorls with the shell beak and siphonal canal continuing to elongate. The shell of *R. venosa* also has three whorls by this stage and

the siphonal canal is also continuing to elongate, but the shell beak has become less elongated (Harding, 2006). Di Salvo (1988) also noted that the shell beak of *C. concholepas* began to decrease in prominence during stage 4. The four lobes of the velum undergo elongation, considerably increasing the area of the velum during stage 4 for *D. orbita*. The same pattern of velum growth occurs for *R. venosa* (Harding, 2006) and *C. concholepas* (Di Salvo, 1988). The elongation of the velum and the siphonal canal continues through stage 5, when veligers become competent to settle. By this stage *D. orbita* has 3.25 shell whorls whereas *R. venosa* has only three shell whorls (Harding, 2006). The time to develop to stage 5 differs substantially between species, with *D. orbita* and *R. venosa* reaching this stage after about 30 days, whereas *C. concholepas* developed to this stage after 14 weeks (Di Salvo, 1988) and *P. pansa* took 20 weeks to reach this stage (Naegel *et al.*, 2003).

Once hatched, the growth of *D. orbita* larval shell proceeded rapidly and more than tripled in size during nearly 6 weeks of planktonic development. This increase is the same as that of *C. concholepas*, which also increased in size approximately 3 times over 6 weeks (Di Salvo, 1988). The increase shell length of *D. orbita* is relatively comparable to shell length observations of the muricid species *P. pansa*, which also more than tripled, but took over 8 weeks (Naegel *et al.*, 2003). For the present study, shell width was initially less than shell length. However, as development progressed, shell width eventually equalled and then over took shell length in the later stages. In a study of larval development of the whelk, *Babylonia spirata*, Sreejaya *et al.* (2004) found that the difference between shell length and shell width measurements also decreased, although in that species shell width never overtook shell length. The velar lobes increase proportionally in size relative to the shell (Klinzing & Pechenik, 2000). Throughout the present study the left velar lobe was larger than the right and the difference increased with increasing shell morphological changes. Uneven lobe

size was also noted for *C. concholepas* (Di Salvo, 1988) and may be in response to uneven distribution of weight as shell morphology changes.

Marine gastropod life cycles may involve complex structural and morphological changes at different intervals. This study provides baseline information on the reproductive cycle, and a description of post-hatching larval development of the muricid gastropod *D. orbita*. Understanding the reproductive cycle is imperative in managing broodstock populations for aquaculture, as is an understanding of larval developmental changes. Further work in the pursuit of sustainable aquaculture of this species should focus on optimal conditions for broodstock conditioning and factors affecting larval growth through to metamorphosis.

Acknowledgments

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CHAPTER 4: Growth, settlement, and survival of *Dicathais orbita* (Neogastropoda, mollusca) larvae in response to temperature and settlement cues

Noble, W. J., Benkendorff, K., Harris, J. O., *Aquaculture Research* (2013). In Press, DOI: 10.1111/are.12298.

4.1 Abstract

The southern Australian whelk, *Dicathais orbita*, is a potential candidate for aquaculture, as both seafood and for bioactive compound production. Larval rearing experiments to determine the effects of temperature and diet on the growth and survival of *D. orbita* larvae under laboratory conditions comprised five different unicellular algal diets of two brown algal species; *Isochrysis galbana* and *Chaetoceros muelleri*, two green algae; *Tetraselmis seucica* and *Nannochloropsis oculata*, and a mixture of all four strains for larvae maintained at 16°C and 22°C. Absolute growth, specific growth rate (SGR) and survival were determined regularly. Larvae reared at 22°C on a mixed diet, or brown algae, performed significantly better than those reared on green algal diets alone. Trials with settlement cues were undertaken on different aged larvae to determine when larvae become competent. An array of natural cues (carrion, *Xenostrobus pulex*, adult mucus and *Ulvella lens*), as well as concentrations of KCl were tested. 20 mM KCl induced the greatest settlement, however no larvae metamorphosed under the conditions provided. This study confirms long-lived planktotrophic larval development for *D. orbita* with higher development rates at the higher water temperatures. Further studies will optimise culture conditions and cues for settlement and metamorphosis.

4.2 Introduction

There is a global trend in aquaculture towards diversifying the number of farmed species (FAO, 2012). The span of products derived from aquaculture is also growing, with the lucrative pharmaceutical and "nutraceutical" industries looking to aquaculture to provide significant quantities of novel therapeutics (Benkendorff, 2009). In South Australia there is a significant molluscan aquaculture industry centred mostly on abalone, mussels and oysters. A number of farmers have expressed interest in diversifying their practices to include other species (pers. comms.). The muricid whelk, *Dicathais orbita*, may be an attractive addition to the current aquaculture industry in southern Australia, as it is endemic to southern Australian rocky shores and reefs (Gowlett-Holmes, 2008) and has been shown to produce anticancer compounds (Benkendorff *et al.,* 2011; Edwards *et al.,* 2012; Benkendorff, 2013) that can be sustainably harvested en masse from adult animals (Chapter 2).

The term whelk is a generic name given to various families of predatory caenogastropods, including the Muricidae, Buccinidae, Babylonidae, and Melongenidae, which are harvested widely around the world. Global whelk production is presently relatively small in comparison to other mollusc fisheries and aquaculture, although both production and the number of species being targeted are increasing (FAO, 2012). The need to manage and create a regular supply of whelks for seafood markets has been the impetus for research into techniques to culturing a number of whelk species (Di Salvo, 1988; Nugranad *et al.,* 1994; Chaitanawisuti & Kritsanapuntu, 1997; Carrasco *et al.,* 2006). Much of this research is aimed at the larval phase as mortality can be orders of magnitude higher at this stage than post settlement and metamorphosis (Pechenik, 1999). Despite the obstacles of larval rearing, considerable success has been achieved in aquaculture of planktotrophic (Di Salvo, 1988; Chaitanawisuti & Kritsanapuntu, 1997), lecithotrophic (Gonzáles &

Gallardo, 1999, Gallardo & Sánchez, 2001) and direct developing (Nugranad *et al.*, 1994) whelk species.

Although previous attempts to maintain *D. orbita* larvae under lab conditions have reported the use of microalgae as food, larvae hatch with an internal yolk (Phillips, 1969). Other whelks that develop through a planktotrophic phase show considerable tolerance to starvation due to their endogenous yolk material (Zheng *et al.*, 2005). Consequently we can not rule out the possibility that this species develops via a lecithotrophic strategy, exploiting a planktotrophic strategy if resources permit. In the case that *D. orbita* is primarily a planktotrophic species, it is likely that food supply and food quality will have important implications for planktonic larval growth rates, pre-settlement mortality, size at settlement and subsequent post-metamorphic growth rates (Vargas *et al.*, 2006).

Temperature is considered to be one of the most important variables controlling development of poikilotherms, including marine gastropods (Hoegh-Guldberg & Pearse, 1995). Przeslawski (2004) reviewed the effect of temperature on encapsulated molluscan larvae and found that development time generally decreases with increasing temperature, within a tolerable range. Lower temperatures can have significant effects on gastropod larval development, to the point of preventing larvae from ever completing settlement (Kingsley-Smith *et al.*, 2005). The most suitable temperature for optimal growth, development and survival is likely to be species specific.

Settlement and metamorphosis of invertebrate larvae is an important transition between life stages that can include changes in form, habitat and diet. The cues that induce larvae to begin this stage depend firstly on larvae being sufficiently developed to be competent for the change. A number of studies have used excess potassium

ions to test for competency and induce settlement and metamorphosis in whelks (Ke *et al.*, 2000; Gallardo & Sánchez, 2001), including the planktotrophic muricid *Concholepas concholepas* Bruguière (Campos *et al.*, 1994). However, other cues such as the mucus of adult conspecifics, microalgal biofilms (Rodríguez *et al.*, 1995) and adult prey (Avila, 1998) may signal suitable habitat for larvae to settle and metamorphose.

No comprehensive study on *D. orbita* larvae has been published to confirm a planktotrophic strategy, or to assess the effect of different microalgae diets combined with temperature on larval development rates. Similarly, the age at which competence for settlement is achieved and the cues that may induce settlement have not been previously investigated for this species. Using a range of readily available phytoplankton as feed and a temperature range typical of South Australian coastal waters during spring and summer (IMOS, 2011), it was the aim of this study to assess the effects of temperature and diet on the growth and survival of *D. orbita* larvae. Once the most successful combination of temperature and diet had been ascertained, this combination was used to investigate the effect of various settlement cues at different ages of larvae. The results of this study provide an important assessment of *D. orbita* larval development and a basis for closing the life cycle of this new gastropod species with excellent potential for aquaculture.

4.3 Methods

4.3.1 Larval culture methods

During February to March 2009, egg capsules and larvae were cultured in 10 L white, polypropylene plastic (Australian Standard 2070:1999) buckets (265 mm diameter x 235 mm deep, 244 mm base diameter) (NCI packaging). Water volume was kept at 8 L with a depth of 160 mm. Salinity (35-37 g L^{-1}), dissolved oxygen

(7.8-8.5 mg L^{-1}) and pH (8.0-8.4) were monitored (YSI 556) every other day, as was ammonia (0.01-0.08 mg L^{-1}), nitrite (<0.1 mg L^{-1}) and nitrate (<0.1 mg L^{-1}) (Hach FF-1A).

Egg capsules were obtained from various locations along the metropolitan coast of South Australia, or deposited by the specimens maintained in recirculating aquaria at Flinders University, rinsed in fresh water and held in constantly aerated, 0.45 µm FSW. Hatched larvae were removed daily with a 150 µm nylon sieve and transferred to clean holding containers with 8 L of gently aerated FSW and kept at 19-20˚C. Larvae were homogenously distributed in the water column before larval density was determined from the average of 3 replicate, 100 mL subsamples of water. Based on the larval density, the correct volume of water was then removed from the buckets to transfer a total of 1600 larvae to culture containers with 2 L of FSW, kept with the same attributes as the holding containers to minimise thermal and osmotic shock during transfer. Water was then added to make up a final volume of 8 L and kept aerated via airline and airstone. Larval density was then rechecked to ensure that larvae were at a density of 0.2 larvae mL^{-1}, which is a similar density to that used in the successful culture of other whelks (Di Salvo, 1988; Nugranad *et al.,* 1994; Chaitanawisuti & Kritsanapuntu, 1997; Gallardo & Sánchez, 2001).

4.3.2 Diet, temperature, and survival

Diet and temperature treatment combinations were undertaken in triplicate using water kept at either 16°C or 22°C. The two temperatures chosen are representative of the range experienced along South Australia's coasts during Spring and Summer (IMOS, 2011), when *D. orbita* egg capsules are found (Chapter 3). Water was allowed to cool to ambient temperature (16°C) in the culture facility, or was heated to 22°C for the experiment by the use of an immersed 50W water heater. Larvae

were fed 2 x 10^4 cells mL^{-1} of either *Isochrysis galbana* Parke 1949, *Chaetoceros muelleri* Lemmermann 1898, *Tetraselmis suecica* (Kylin) Butcher 1959, *Nannochloropsis oculata* Droop 1955, or a mixture in equal proportions of all four algal species, every two days. At each temperature, cultures of larvae were maintained without food to check for lecithotrophic development from stored yolk reserves.

Algae were continually cultured in 20 L carboys with f2 media (Guillard & Ryther, 1962) under 12:12 light:dark regime. Algal cell density in stock solutions was determined the day of addition to larval culture containers by way of 3 replicate counts using a compound microscope (Olympus, model: CX40RF200) and haemocytometer (40 x magnification) before dilution and addition to each treatment. Equal proportions of streptomycin and neomycin (Sigma-Aldrich®) were dissolved in a small amount of FSW and added to culture containers to a final nominal concentration of 10 mg L^{-1}.

In each larval culture bucket, water was changed every second or third day and larvae were removed on a 150 µm nylon sieve for observation and measurement. Larvae that were not actively swimming and were on the bottom of the culture containers were placed in a petri dish and examined under a dissecting microscope. The number of dead larvae were recorded and removed; those that showed movement by extension of the velum were returned to the culture container. Live larvae in each treatment were enumerated and the percent survival was calculated over time, commencing with 100 % at day 0 (day of hatching). This value was used to determine the density of larvae in each replicate throughout the experiments.

The shape of the larval shell changed considerably over the course of development (Figure 4.1, Chapter 3). To determine whether shell length alone was an adequate

measure of size for larvae reared under various experimental conditions, both the lengths and widths of larvae (Figure 4.1) were measured on the day of hatching (Day 0) and every 4-5 days thereafter to correspond to alternate water changes, for up to 10 larvae from each treatment throughout the experiment. Larvae (n = 287) were photographed under a dissecting microscope and images downloaded into image analysis software (Motic Images 2.0) for size determination to the nearest 0.1 µm.

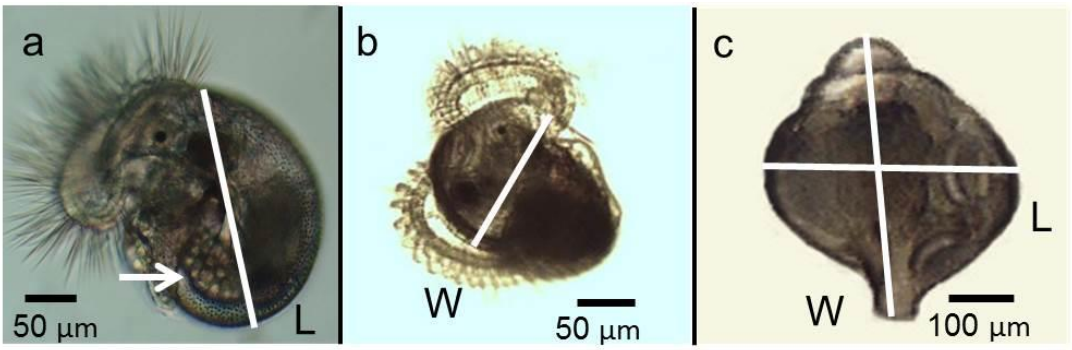

Figure 4.1. The larvae of *Dicathais orbita* (Muricidae, Gastropoda), showing two different stages of development post hatching from the benthic egg capsules. Shell dimensions changed considerably over the course of 38 days. Shell length (L) and width (W) were measured throughout the trial to determine whether shell length was an appropriate measure of growth over the entire study interval: a) lateral view of larvae < 7 days old, with algal cells in the gut indicated by an arrow; b) ventral view of newly hatched larvae; c) ventral view of 38 days old larvae.

Based on the correlation between length and width of the shell ($r^2 = 0.9955$, $p < 0.05$) the mean shell length from each replicate tank was used in analysis of absolute growth and SGR. Specific growth rate (SGR) was determined as the percentage increase per day (% day^{-1}) using the function described by Reaburn & Edwards (2003):

$$SGR = (\ln y_2 - \ln y_1)/\Delta T \times 100$$

where y is the shell length, and ΔT is time between measurements.

4.3.3 Settlement cues

Competency for invertebrate larvae can be considered as the point of development at which larvae are sufficiently developed to settle and metamorphose (Hadfield, 1984). Size has been demonstrated to be a reasonable indicator of metamorphic competence for a number of species of gastropod larvae (Ke *et al.,* 2000). Experiments indicated better growth of larvae reared at 22°C on a mixed diet than for other experimental treatments; hence settlement experiments used larvae reared under these conditions.

All settlement experiments were conducted in 5 mL plastic microscope plates and 10 larvae were used in each replicate (n = 3). The experiment was run with batches of larvae at different stages of development: the day of hatching (day 0), 10 dph (day 10), 20 dph (day 20) and at 30 dph (day 30). The number of larvae responding to settlement cues in each batch was monitored for 10 min h^{-1} for the first 7 h and again at 18, 24, 48, 36, 60 and 72 h. Settlement observations focused on pre-metamorphic cues, such as sinking and creeping behaviour, which have been noted in other species of gastropod larvae prior to metamorphosis (Di Salvo, 1988; Davis, 1994; Naegel *et al.*, 2003). Five settlement cues were trialled in this study: KCl, live and dead (carrion) prey (*Xenostrobus pulex* Lamarck), an algal biofilm of *Ulvella lens* Crouan & Crouan and the mucus of adult conspecifics. Negative controls consisted of seawater with no added cue. KCl was tested at four concentrations: 20, 30, 40 and 50 mM, to establish the most suitable positive control for comparison with the natural cues.

Test solutions of KCl (Sigma-Aldrich®) were prepared as 0.5 M stock solutions in deionised water. This stock solution was diluted with 0.45 µm FSW to achieve the desired concentrations of 20, 30, 40 and 50 mM. Ionic concentration of each test solution was confirmed by flame photometry (Eel Flame Photometer).

Metamorphosis of planktotrophic larvae requires a shift in diet from planktonic organisms to benthic prey. Juvenile *D. orbita* have often been seen to feed on the small mussel, *X. pulex* (pers. obs.), which can occur in large mussel beds on intertidal rock platforms and subtidal reefs in South Australia. Hence, *X. pulex* were offered as a potential settlement substrate to larval *D. orbita*. Patches of *X. pulex* were collected (permit number S23101-1) from rocky intertidal regions at Marino, South Australia (-35.04385°, 138.50810°) and sorted under a dissecting microscope to select the smallest individuals from the mass. Mean shell length of *X. pulex* used in settlement experiments (n = 9) was 486.6 ± 36.8 µm. Two juvenile mussels were used in each experiment and allowed to attach byssal threads before larvae were introduced.

Whelks are widely recognised as scavengers known to feed opportunistically on a wide range of carrion (Morton & Jones, 2003). Studies on juvenile *D. orbita* found that specimens grew faster when fed a diet of fresh carrion, as opposed to other diets (Woodcock & Benkendorff, 2008). For the present study, carrion treatments were made by crushing a single, live juvenile *X. pulex* on the bottom of the test container.

The presence of adult conspecific mucus has been shown to induce settlement in the temperate muricid, *C. concholepas* (Rodríguez *et al.,* 1995). Plastic microscope plates used to observe settlement experiments were coated with adult mucus by placing adult *D. orbita* on the plates and allowing them to crawl off. This process was repeated several times the day before being used for experimental observations and kept hydrated in FSW until required.

Ulvella lens is biofilm forming microalgae commonly used on settlement plates in South Australian abalone farms, where *D. orbita* have been found. It is likely that larvae are brought on to the farm through water intake pipes and settle according to natural cues available on the abalone farm. Thus, plastic settlement plates with 100%

covering of *U. lens* were used as another settlement cue, with ~1 cm squares of the plates placed in the bottom of each settlement dish.

4.3.4 Statistical analysis

A correlation analysis was used to test the relationship between shell length and width measurements (SPSS v21). As the larvae in the control treatment had all perished by day 19, analysis of absolute growth was split into two parts; day 0-15 including the control, and day 19-38, without the control. The absolute growth data was analysed using permutational univariate analysis for nonparametric data, at different temperature and diet combinations (Primer v6 + PERMANOVA add on, Anderson *et al*., 2008). A resemblance matrix was created using Euclidean distance then analysed using 9999 unrestricted permutations of the raw data. Pairwise comparisons using Gosset's t statistic were used to determine significant differences between treatments within diets (Primer v6 PERMANOVA).

SGR data was split for comparison into three intervals corresponding to apparent changes in growth rate (Stage 1 & 2, Stage 3 & early 4, Stage late 4 & 5, Chapter 3), Interval 1: day 0–day 15, Interval 2: day 15–day 24, and Interval 3: day 24–day 38 and the mean growth per day for each treatment interval was analysed using a nonparametric 2 factor PERMANOVA (Primer v6). Survival data was analysed for day 15, 24 and 38 using a 3-factor repeated measures ANOVA for the effects of diet and temperature, with day as the repeated measure (SPSS v21).

A nonparametric 2 factor PERMANOVA (Primer v6) was used in the settlement experiments to determine the optimum larval age and concentration of KCl for settlement. Based on the high mortality after 7 h of exposure to KCl, the analysis was performed only for this time point. Pairwise tests were performed to establish significant differences between different concentrations of KCl and the responses of

larvae of different ages. Differences were determined to be significant for $P \leq 0.05$ for all statistical analyses.

4.4 Results

D. orbita larvae were observed feeding on all the microalgal cultures tested and algal cells could be observed in the stomach of some larvae (e.g. Figure 4.1a).

4.4.1 Absolute shell length

The strong correlation between shell length and shell width ($R = 0.9989$, $P < 0.001$) indicates that shell length is a suitable measure to assess growth throughout larval development in this species (Figure 4.2). The mean larval size at hatching was 244.8 µm ($n = 110$). Size increased slowly for the first 15 days, but larvae grew faster from day 19 onwards, regardless of the temperature (Figure 4.3). On average, larvae in the unfed group grew only 15.4 % and 16 % larger than the mean hatching size for the 16°C and 22°C treatments respectively (Figure 4.3). The greatest absolute growth of 74.4 % by day 15 was observed for larvae maintained at 22°C on a mixed diet (Table 4.1). At day 38 the mean larval size was also the greatest at 22°C fed on a mixed diet (Table 4.1) and equated to a 427.1 % increase in size from initial mean hatching size. At this point the maximum shell length for the 16 °C treatment was also found in the mixed diet treatment (Table 4.1), but only equated to an increase in shell length of 258.5 % over initial mean shell length. The higher temperature of 22°C consistently led to greater growth than the 16°C treatment, regardless of diet (Figure 4.3).

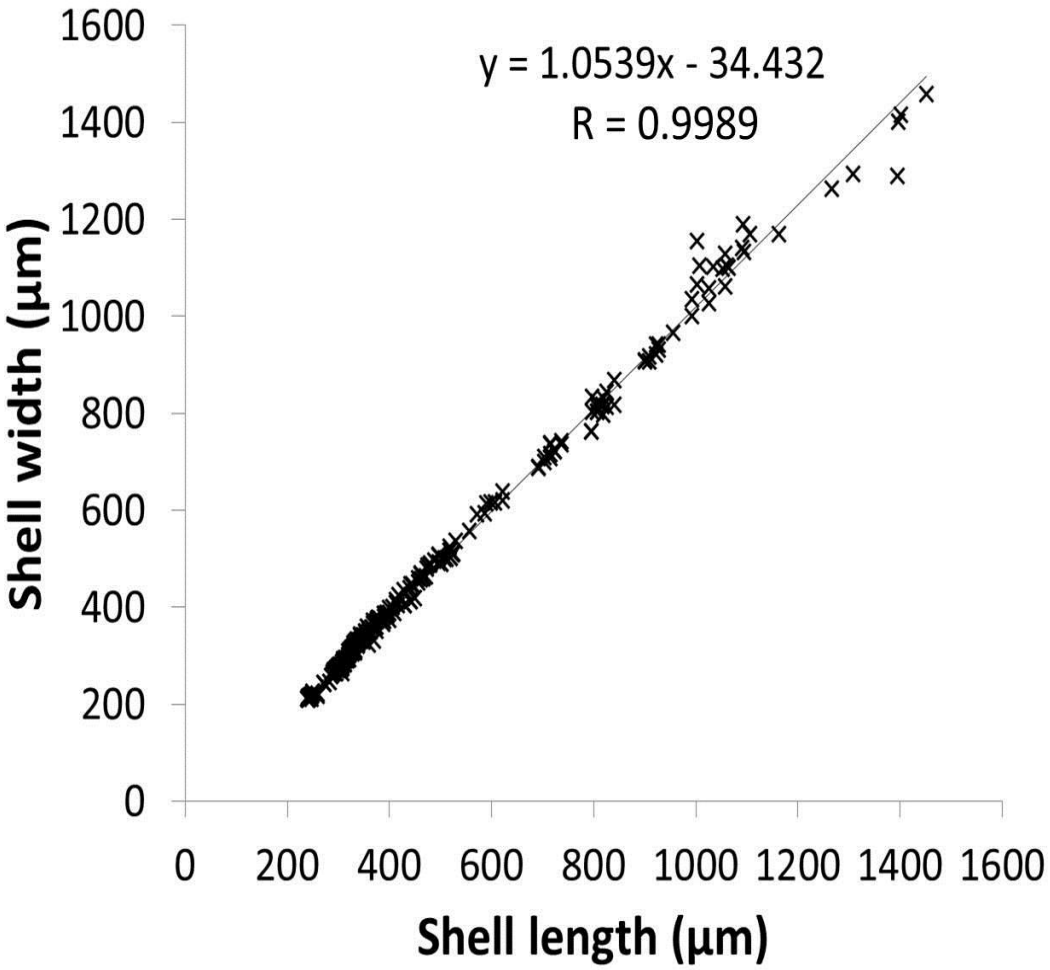

Figure 4.2. The correlation (P < 0.000) between *Dicathais orbita* shell length and width throughout 38 days of growth (n = 287).

Results from the univariate PERMANOVA indicate that at day 15 there was no interaction between temperature and diet (df = 5, F = 0.176, P = 0.97), but both temperature (df = 1, F = 8.87, P = 0.004) and diet (df = 5, F = 18.81, P = 0.001) had significant effects on absolute shell length. Pairwise tests revealed that at day 15, the larvae in the unfed group were significantly smaller than larvae in all other diet treatments (Table 4.1). Larvae fed on *N. oculata* were also significantly smaller than those fed other algal diets and larvae fed on *T. suecica* were smaller than those fed on a mixed diet or *C. muelleri* after 15 days (Table 4.1).

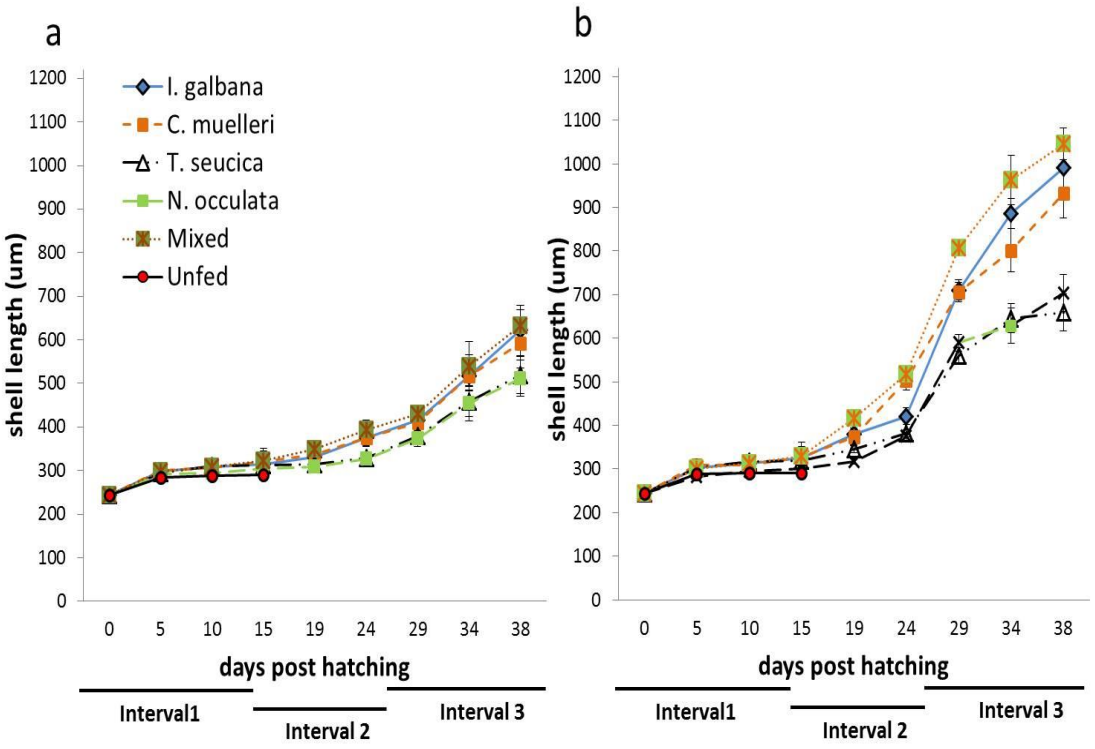

Figure 4.3. Mean absolute growth (±St. Dev.) of *Dicathais orbita* larvae over a 3 intervals post hatching for a) 16°C & b) 22°C fed on different microalgal diets. The data represents means from three independent replicate larval cultures.

Table 4.1. Mean shell length (± St. Dev.) of post hatching *Dicathais orbita* veligers maintained at 16°C & 22°C on different microalgal diets. All larvae in the unfed group had perished by the next assessment (Day 19). The data represents means from three independent replicate larval cultures. Small subscript letters indicate significant differences between diets at 16°C, whereas the capital letters denote significant differences between diets at 22°C.

Diet	Day 15		Day 38	
	16°C	22°C	16°C	22°C
Isochrysis galbana	314.6 ± 36.9 ^{ab}	324.4 ± 16.4 ^{AB}	621.6 ± 57.9 ^{a}	991.6 ± 106.8 ^{A}
Chaetoceros muelleri	320.4 ± 23.8 ^{a}	327.9 ± 32.6 ^{A}	591.9 ± 55.6 ^{b}	931.9 ± 105.3^{B}
Tetraselmis suecica	312.2 ± 7.7 ^{b}	319.2 ± 15.7 ^{B}	518.8 ± 42.1 ^{c}	659.1 ± 70.3 ^{C}
Nannochloropsis oculata	303.7 ± 7.2 ^{c}	310.5 ± 11.3 ^{C}	511.8 ± 41.1 ^{c}	704.2 ± 66.2 ^{D}
Mixed	322.7 ± 9.7 ^{a}	328.7 ± 11.5 ^{A}	632.8 ± 39.9 ^{a}	1045.6 ± 81.6 ^{E}
Unfed	289.5 ± 1.4 ^{d}	291.5 ± 7.5 ^{D}	-	-

At day 38, significant differences were observed for temperature and diet ($P < 0.001$), and the two factors had a strong interaction ($df = 4$, $F = 42.06$, $P < 0.001$). Due to this interaction between temperature and diet at day 38, pairwise tests were performed separately for each temperature. At day 38 in the 16°C treatments the larvae obtained significantly different shell lengths in all diet groups with two only exceptions; larvae fed *I. galbana* and the mixed diet did not differ, but were significantly larger than all other groups; whereas larvae fed *T. suecica* and *N. oculata* were not significantly different from each other, but were smaller than all other larval groups (Table 4.1). However at 22°C, absolute shell length was significantly different for all pairs of diets (Table 4.1) with the largest shell length obtained on the mixed diet, followed by *I. galbana* and *C. muelleri*.

4.4.2 Specific growth rate

SGR across both temperatures ranged from 0.13% day^{-1} at day 10, maintained at 22°C and fed on *N. oculata*; to 1.19% day^{-1} at day 29, maintained at 22°C, and fed on *I. galbana* (Figure 4.4). Temperature was not a significant factor influencing the growth rate of larvae for interval 1 ($F = 0.842$, $P = 0.359$), but SGR was significantly higher at 22°C than at 16°C for interval 2 ($F = 18.576$, $P < 0.001$) and interval 3 ($F = 23.87$, $P < 0.001$). Diet did not have a significant effect for any interval (interval 1: $F = 1.566$, $P = 0.172$, interval 2: $F = 1.234$, $P = 0.303$, interval 3: $F = 0.762$, $P = 0.542$) and there was no interaction between diet and temperature for any interval (interval 1: $F = 0.134$, $P = 0.983$, interval 2: $F = 0.811$, $P = 0.521$, interval 3: $F = 0.295$, $P = 0.88$).

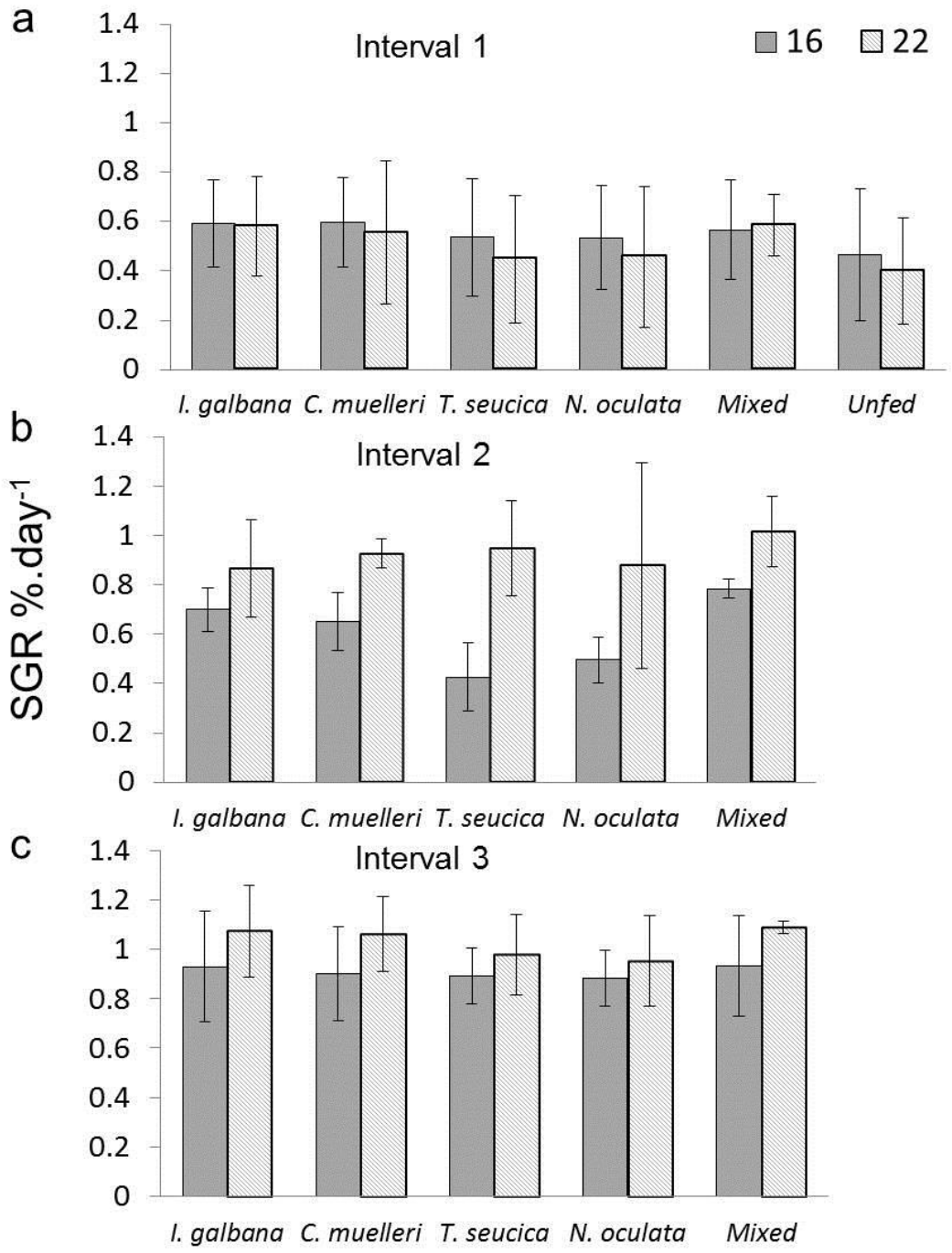

Figure 4.4. Specific Growth Rate (SGR) (±St. Dev.) of *Dicathais orbita* larvae over 38 days post hatching (dph) maintained at 16°C or 22°C and fed on different microalgal diets; a) interval 1: 0-15 dph; b) interval 2: 15–24 dph; and c) interval 3: 24–38 dph. The data represents mean SGR % day^{-1} (n = 30) from three independent replicate larval cultures sampled every 2-3 days from within each temperature and diet treatment.

4.4.3 Survival

In all treatments most larval mortality occurred within the first 15 days and all larvae in the unfed treatment had perished by day 19 (Figure 4.5). By day 38, survival was less than 20 % in all other treatments, and no larvae were found alive at the next observation, 42 dph, in any treatment. Throughout the study, the larvae fed the mixed diet had a higher survival than all other diets (Figure 4.5). Apart from days 34 and 38, mean survival was higher in the 16°C treatment for all diets at all time points. Three factor repeated measures ANOVA revealed that the differences in survival of larvae were not significantly influenced by temperature ($F = 0.371$, $P = 0.543$), but were significant for diet ($F = 4.15$, $P = 0.002$) and day ($F = 70.4$, $P < 0.001$) and there was no significant interaction between temperature and diet ($F = 6.443E^{-2}$, $P = 0.996$), temperature and day ($F = 6.194E^{-2}$, $P = 0.943$), diet and day ($F = 0.12$, $P = 0.999$) or temperature, diet and day ($F = 1.916E^{-2}$, $P = 0.997$). Pairwise comparisons at the level of diets indicated that larvae in the unfed group had a significantly lower survival than larvae fed on *I. galbana* ($P = 0.006$), *C. muelleri* ($P = 0.009$) and the mixed diet treatment ($P < 0.001$) (Figure 4.5). In addition, larvae fed the mixed diet had a significantly higher survival than those fed on *T. suecica* or *N. oculata* (Figure 4.5). Pairwise comparisons of intervals confirmed that mean survival of larvae was significantly less for each consecutive interval ($P < 0.001$ for all pairs).

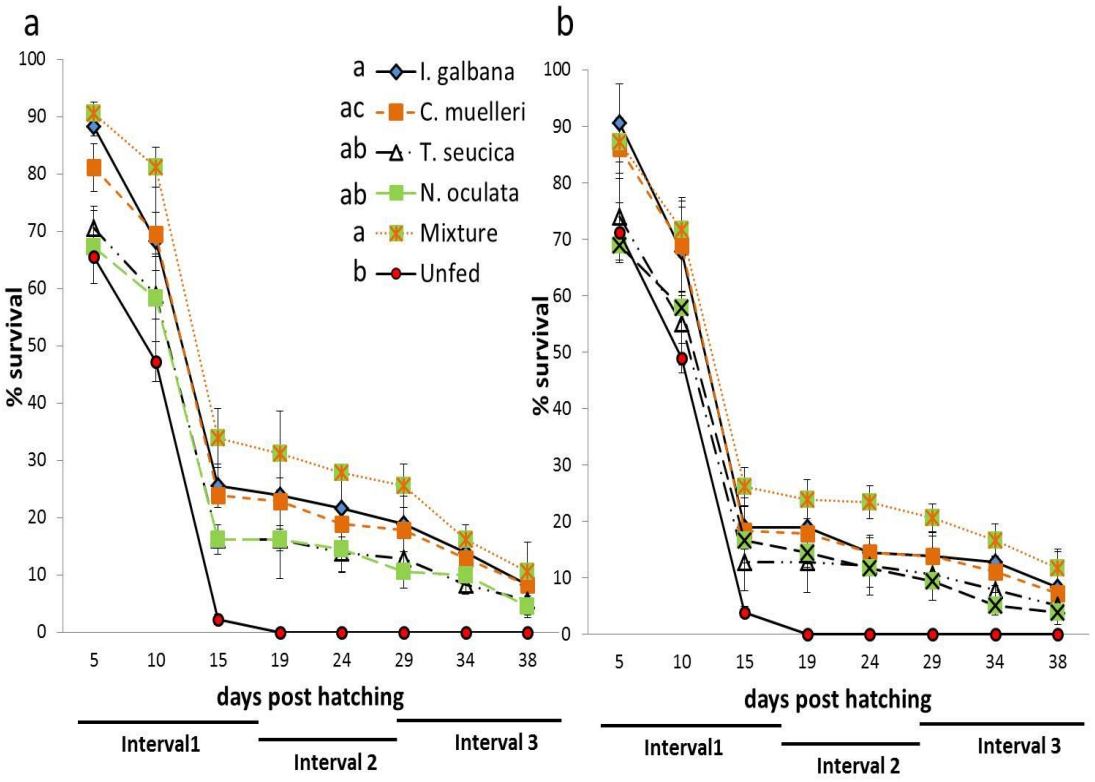

Figure 4.5. Mean survival (±St. Dev.) of *Dicathais orbita* larvae fed on different diets and maintained at two different temperatures; a) 16°C & b) 22°C fed on different microalgal diets. The data represents means from three independent replicate larval cultures. Different letters indicate significant differences (P < 0.05) between diets in the key.

4.4.4 Settlement

Larvae settled on the bottom of the settlement containers on their dorsum with velum extended or partially retracted. This behaviour was observed in all age classes and was observed to be temporary on a number of occasions with larvae resuspending during the 10 min observation interval. Larvae were considered to have perished when no movement could be seen within the shell, which stayed translucent throughout the development interval.

Larval mortality throughout KCl settlement experiments was consistently lowest in the control group, followed by KCl 20 mM (

Figure 4.6). Larval mortality increased with increasing exposure to KCl. All KCl concentrations >20 mM led to 100 % mortality after 18 or 24 h of exposure, except at 30 mM KCl where larvae 30 dph survived up to 48 h exposure (Figure 4.6).

The most successful concentration of potassium chloride for settlement was also 20 mM, especially for 20 and 30 day old larvae (Figure 4.7). Up to 73.3% of 30 dph larvae settled after 36 h of exposure to this concentration and remained at that level up to 48 h of exposure, beyond which mortality occurred (Figure 4.7). Settlement success was strongly influenced by larval age, with only a few newly hatched larvae settling in any treatment (Figure 4.7). No larval settlement occurred for newly hatched (Day 0) larvae in the control group and a maximum of 20 percent settlement occurred in the larvae that were 30 dph (Figure 4.7). Increased duration of exposure to KCl generally led to greater settlement of larvae of all ages (Figure 4.7), until the point where high mortality occurred.

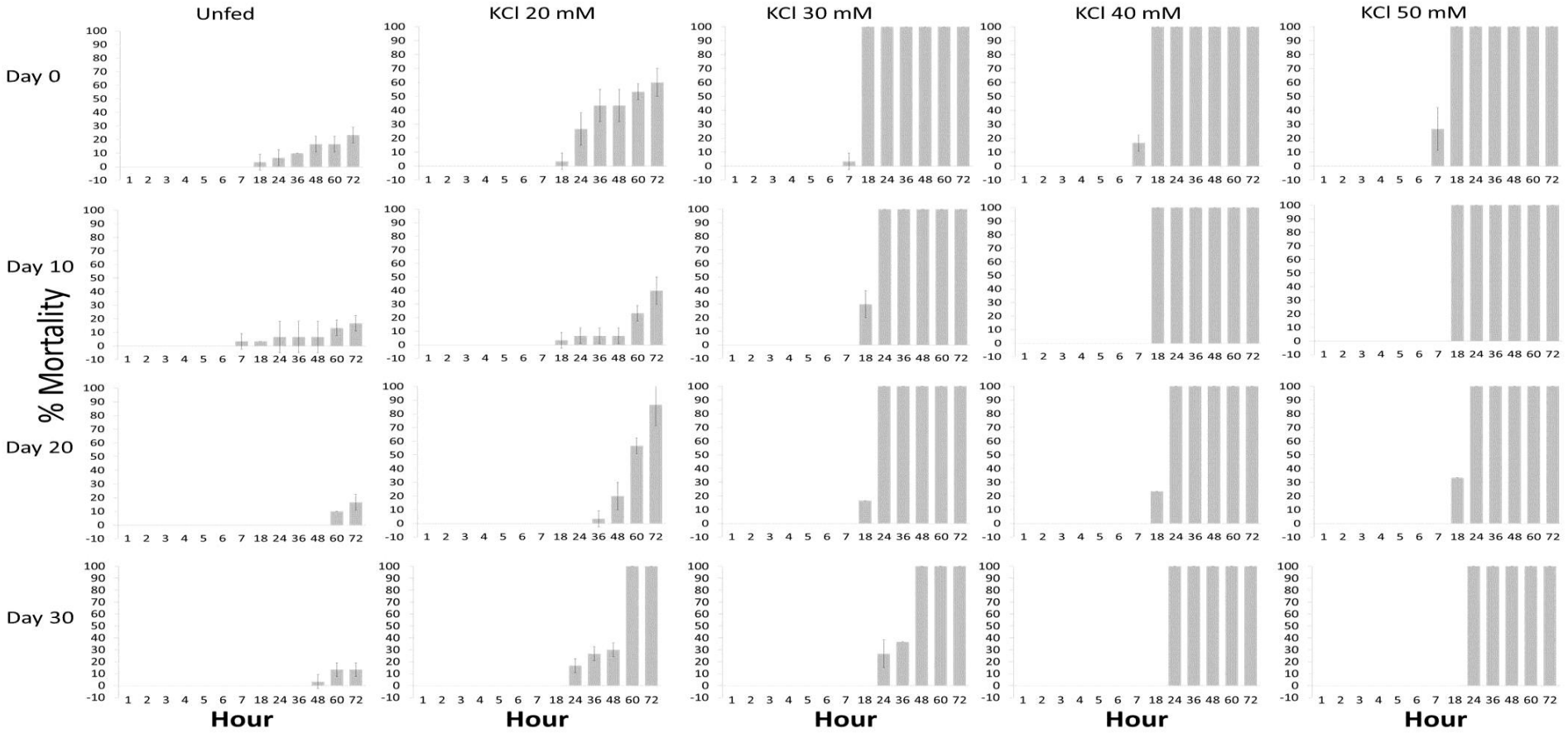

Figure 4.6. Mean mortality (±St. Dev.) of *Dicathais orbita* larvae in response to various concentrations of potassium chloride. Larvae were tested on the day of hatching (Day 0), 10; 20 and 30 days post hatching. The data represents means from three independent replicate larval cultures at each time point after exposure to KCl in the settlement plates.

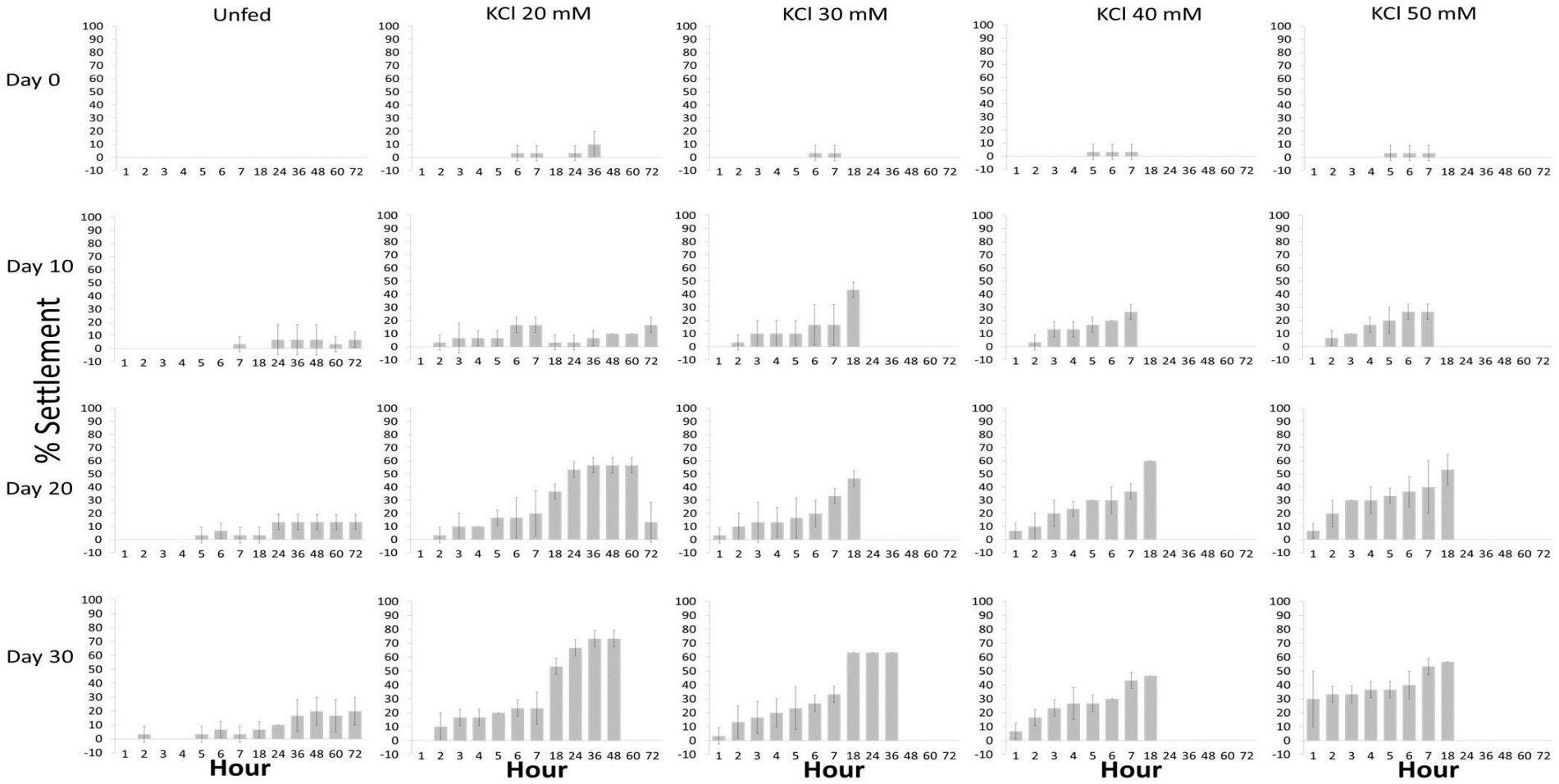

Figure 4.7. Mean settlement (±St. Dev.) of *Dicathais orbita* in response to various concentrations of potassium chloride. Larvae were tested on the day of hatching (Day 0), 10; 20 and 30 days post hatching by exposure from 1 h up to 72 h. The data represents means from three independent replicate larval cultures.

Larval settlement and mortality of thirty day old (Day 30) larvae exposed to 20 mM KCl for 36 h was chosen as the optimal combination to compare to other cues. Settlement was more than 3 times greater for this positive control than for that of the adult mucus, which induced the next greatest settlement (Figure 4.8a). PERMANOVA revealed a significant difference in both larval settlement ($F = 61.366$, $P < 0.001$) and mortality ($F = 4.72$, $P = 0.025$) according to the settlement cue. Pairwise tests revealed that potassium chloride (20 mM) led to significantly more settlement than the negative control or any other cue (Figure 4.8a). Settlement for any of the other cues was not significantly different to the negative control (Figure 4.8a). All treatments led to significantly greater mortality than the negative control (Figure 4.8b). Larval mortality was greatest for KCl (20 mM) and differed significantly from all groups (Figure 4.8b).

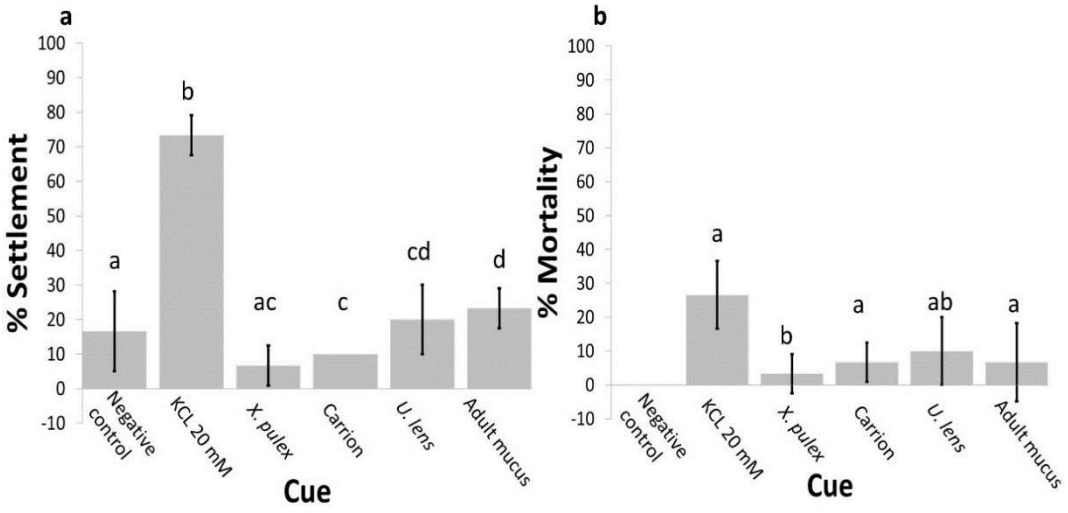

Figure 4.8. Mean a) settlement and b) mortality (±St. Dev.) of 30 day old *Dicathais orbita* in response to various settlement cues after 36 h of exposure. The settlement cues include a negative control (no cue), positive control (20 mM KCl), juvenile prey (live *Xenostrobus pulex*), carrion (crushed *X. pulex*), biofilm (*U. lens)* and adult *D. orbita* mucus. The data represents means from three independent replicate larval cultures. Different letters indicate significant differences (P < 0.01) between treatments.

4.5 Discussion

The results of this study confirm that *D. orbita* larvae are planktotrophic (Figure 4.1a). Furthermore, growth and survival is significantly affected by both temperature and diet under laboratory conditions, with greater growth and final size achieved when maintained at 22°C than at 16°C. Larvae fed on a mixed diet of brown and green microalgae consistently attained the greatest size, followed by diets comprised solely of brown microalgae, which in turn performed better than larvae fed on diets comprised of green microalgae alone. In gastropods, the quality and quantity of food (Phillips, 1969; Zheng *et al.*, 2001) and temperature (Hoegh-Guldberg & Pearse, 1995; Przeslawski, 2004) have been determined as key factors in growth and mortality. Planktotrophic larvae continue to grow and develop until they obtain competency for settlement and metamorphosis. Our settlement experiments show

that the majority of *D. orbita* larvae only commence settlement after at least 20 dph. They were observed to settle in response to various natural cues, but KCl at a concentration of 20 mM is the most suitable artificial treatment of those trialled in this experiment for inducing settlement. However, the conditions provided throughout culture or in settlement containers were not suitable for metamorphosis and led to high mortality. The results obtained in this study can be compared to those of Phillips (1969) who reported a shell length at hatching of 240 µm for *D. orbita* from Western Australia and an average size of 300 µm at day 10 and 360 µm at day 20. In the present study, we found that mean larval shell length is 244.8 µm ± 3.103 St. Dev. (n = 110) for newly hatched larvae from South Australia and reaching an average size of 293 µm at day 10 and 317 µm at day 20. However, Phillips (1969) provides no information on the temperature or density at which larval experiments were conducted. The hatching size of *D. orbita* is typical of other muricids in the subfamily Rapaninae with planktotrophic development (reviewed by Romero *et al.*, 2004), for example; *C. concholepas*, *Thais* (*Stramonita*) *chocolata* and *P. pansa* Gould larvae have hatching sizes at ~ 250 µm (Di Salvo, 1988), 225 µm (Romero *et al.*, 2004) and ~ 280 µm (Naegel *et al.*, 2003), respectively. Fifteen dph *C. concholepas* was ~ 300 µm and had increased to 520 µm after 20 days; by 120 days the larvae had attained a shell length of ~ 1600 µm (Di Salvo, 1988). *T. chocolata* attained a size range of 1450-1740 µm after 4 months (Romero *et al.*, 2004). *P. pansa* grew to ~470 µm in the first 28 days and had a shell length of more than 1000 µm after 105 dph (Naegel *et al.*, 2003). This indicates that there could be much potential for further growth of *D. orbita* larvae if they can be maintained in culture for longer intervals of time.

The size and number of larvae at hatching can indicate the duration of planktonic development for muricid larvae. Romero *et al.* (2004) summarised the hatching size

of a number of muricids with planktotrophic, lecithotrophic and direct developing larvae. Direct developing species such as *Nucella crassilabrum, Trophon laciniatus* and *T. geversianus* produced relatively few large larvae that hatch as crawling juveniles at 1148 µm, 1800-3300 µm and 3000 µm. Lecithotrophic species, such as *C. giganteus*, produce about 40-150 hatching larvae per capsule with an average size of ~1000 µm (Gonzáles & Gallardo, 1999) These larvae can settle and metamorphose in as little as 3-4 days after a maximum of 72 days observed in the plankton. By comparison, planktotrophic species such as *T. chocolata* and *C. concholepas* produce ~700->3000 larvae with a size at hatching of 220 µm and 260 µm respectively, and a long planktonic developmental period to reach a size of ~1450-1750 at settlement (Romero *et al.*, 2004). The similarity in hatching size (~245 µm) and the high fecundity (730-7000, Philips, 1969, Chapter 3) of *D. orbita* to other planktotrophic Rapaninae further confirms planktotrophic development for this Australian species. Hence the full planktonic development phase for *D. orbita* is likely to be similar to these other temperate Rapaninae, with approximately 4 months noted for *T. chocolata* (Romero *et al.*, 2004) and *C. concholepas* (Di Salvo, 1988).

As is the case for other planktotrophic larvae, diet exerts a profound influence on larval development in *D. orbita*. The value of different diets to the success of larval culture is related to digestibility, feeding efficiency and the nutritional composition (Zheng *et al.*, 2001). Marty *et al.* (2003) hypothesized that differences in microalgal species fed to *Crepidula fornicata* Lamarck 1799 larvae could be responsible for the different growth rates reported by Pechenik (1984) and Le Gall (1995) for the same species. Phillips (1969) stated that development of appropriate food supplements was a major problem in the continued experimentation on *D. orbita* larval intervals. In that study, larvae were provided with either a brown (*Phaeodactylum tricornutum* Bohlin 1897), or green (*Dunaliella tertiolecta* Bucher 1959), unicellular algal diet. In

the present study, diets comprised of mixed brown and green microalgae and brown microalgal diets resulted in significantly greater absolute growth and survival than a diet of the green microalgae, *T. suecica* or *N. oculata* alone. These results support findings from similar studies that concluded diets composed of brown and green microalgae are the most appropriate food for a number of molluscan larvae (Kingsley-Smith *et al.,* 2005; Vargas *et al.,* 2006), including the temperate muricid gastropod *C. concholepas* (Di Salvo, 1988).

Metamorphosis of the larva into the juvenile/adult typically occurs in concert with, or directly following, settlement out of the water column (Jackson *et al.,* 2002). The present study identifies that KCl at a concentration of 20 mM is the most suitable treatment to use to induce settlement of *D. orbita* larvae, but high mortality was observed for higher concentrations after 18 h exposure. This trend of low KCl concentration leading to good settlement response and higher concentrations leading to high mortality after a reasonably short exposure time is consistent with results from other studies on whelk larvae. Up to 90 % of *Babylonia formosae* Sowerby 1866 and *B. areolata* larvae were induced to settle after 24 h exposure to 12 mM KCl, yet 15 mM KCl concentration for the same duration led to high mortality (Ke *et al.,* 2000). Less than 60 % of *C. concholepas* were induced to settle in response to 20 mM KCl after 64 h, and larvae exposed to 25 mM did not survive (Campos *et al.,* 1994). Later studies on the same species produced slightly different results with 20 mM KCl inducing settlement and 30–40 mM being toxic (Gallardo & Sánchez, 2001). Naegel *et al.* (2003) found that *P. pansa* settled in response to approximately 25 mM KCl. Overall, whilst lower concentrations of KCl (20 mM) can be used to artificially induce settlement of Muricidae larvae, it would be preferable to identify natural settlement cues.

Successful transition from planktotrophic larvae to benthic juvenile is linked to the availability of larval food (Naegel *et al.,* 2003), suitable settlement cues in the form of habitat (Davis, 1994; Kingsley-Smith *et al.,* 2005), food for post-metamorphic juveniles (Manríquez *et al.,* 2004), the origin of egg capsules (Gallardo & Sánchez, 2001), turbulence (Pechenik & Gee, 1993) and temperature (Przeslawski, 2004). Any of these factors or a combination could have contributed to conditions provided throughout growth and development, or in settlement containers, being unsuitable for metamorphosis, thus resulting in mortality. The settlement trial was conducted in still water and this may have limited the success of the natural inducers, *U. lens*, *X. pulex*, carrion and adult mucus, since natural inducers perform better in conjunction with turbulence (Pechenik & Gee, 1993, Naegel *et al.,* 2003). Phillips (1969) reported settlement of *D. orbita* larvae at 20 dph, but no information is available on mortality or whether metamorphosis occurred. Greater planktonic development may have occurred if appropriate food had been provided to larvae for longer. Larvae of some species of gastropod can be maintained in laboratory conditions for months (Naegel *et al.,* 2003, Kingsley-Smith *et al.,* 2005) or years (Strathmann & Strathmann, 2007).

This is the first published study on the growth and survival of *D. orbita* larvae since Phillips (1969). Although no spontaneous larval settlement was observed in this study, larval growth and development was maintained for 38 days. The optimal conditions of higher temperature and mixed algal diet for larval growth, along with the assessment of settlement cues provides vital, baseline information for future studies aimed at closing the life cycle of this medicinal mollusc (Benkendorff, 2013). Future studies relating to dietary preference at specific developmental stages and stocking density of larvae would add value in closing the life cycle of this species.

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CHAPTER 5: General discussion

This study provides significant progress towards understanding the life history of *D. orbita*, thus contributing to the potential for novel marine resources from the southern Australian muricid whelk, to be further developed through aquaculture. Whelks are widely fished and some species have been successfully developed for aquaculture for food throughout the world (Appendix A). Many of the emerging pharmaceutical leads are coming from marine species (Hunt & Vincent, 2006) and offer potential to add value to the fisheries and aquaculture industries. Aquaculture offers a virtual panacea for sustainable supply of both food and bioactive compounds (Benkendorff, 2009), but techniques for sustainable aquaculture are species specific. Hence, each new lead often requires solutions to novel problems.

In the case of *D. orbita*, anaesthetics proved useful to non-destructively identify the sex in this gonochoristic species, as well as to extract bioactive compounds (Chapter 2). Magnesium chloride was effective in anaesthetising animals enough to pull the operculum open and observe the sex of the animals via presence or absence of a penis. This technique can be used to assess the sex ratio of wild populations. Anaesthetisation is also useful for manipulating aquaculture stock (Acosta-Salmón & Davis, 2007; Butt *et al.*, 2008) and could be used to manage broodstock of *D. orbita*.

Although less effective as an anaesthetic, ethanol was found to be useful to chemically manipulate *D. orbita* and sustainably extract bioactive compounds en masse from the species. This technique of 'milking' animals could be used to provide a supply for ongoing preclinical and clinical trials (Chapter 2). This is the first known application of mass milking using a chemically induced stress response. Mechanical stimulation has been used to repeatedly collect Tyrian purple from the central American muricid, *P. pansa* (Michel-MorfÍn & Chávez, 2000), but the method has

been implicated as one of the causes for declines in wild populations (Naegel & Lopez-Rocha, 2006). Studies on captive animals show that mortality accompanies declines in productivity if milking is conducted less than every 3-4 weeks (Naegel, 2005). The mortality was attributed to the effect of handling (Michel-MorfÍn & Chávez, 2000; Naegel, 2005). The use of chemical methods to extract bioactive compounds requires less handling and may assuage mortality, but further studies will be required to optimise yield and determine the effects of repetitive extraction.

Creating a sustainable supply of *D. orbita* from aquaculture that does not place direct pressure on wild stocks is imperative if the severe effects of overexploitation experienced by other whelk fisheries are to be avoided (Castilla, 1997; Leiva and Castilla, 2002; Rosenberg, 2003). Aquaculture also offers the opportunity to milk animals for bioactive compounds before harvesting for meat, thus diversifying and value-adding to the product. Studies on the proximate composition of the species show that it has high protein content, suggesting value as a nutritional seafood (Woodcock & Benkendorff, 2008). In some countries the hypobranchial gland, the organ responsible for the production of bioactive compounds, is consumed along with the rest of the meat (Cooksey, 2013). This could be used to enhance the marketability through promotion of *D. orbita* as a functional food (Milner, 1999; Benkendorff, 2013). This relatively new class of foods are increasingly gaining attention for the potential to reduce the incidence of chronic disease (Lordan *et al.*, 2011).

However, before aquaculture of *D. orbita* can be realised on a commercial scale, an understanding of the reproductive cycle is needed in order to manage production. The present study revealed that in GSV, *D. orbita* has an annual reproductive cycle with spawning coinciding with an increase in water temperature and peak spawning

occurring in December (Chapter 3). Animals maintained in captivity in a recirculating system also followed the same cycle, readily spawning groups of egg capsules and adding to a communal egg mass with no significant difference between the number of egg capsules in a group, the size of egg capsules, or the number and size of eggs, to those spawned in the wild. This indicates that broodstock populations for aquaculture purposes are likely to be easy to maintain and predictable to manage. Given that some species of muricids show differing reproductive cycles in different locations it is possible that a broodstock population of *D. orbita* could be influenced to spawn outside of the natural spawning cycle observed in this study. An attempt was made to do this by manipulating water temperature and day length (Appendix C), however no spawning occurred during the 6 month experimental period.

Compared to other species of muricid whelks that produce planktotrophic larvae, *D. orbita* spawns a similar number of egg capsules and eggs (Table A.1) This study found that *D. orbita* produced ~700-7000 eggs per capsule (Chapter 3). In a previous study on the ecology of *D. orbita* in Western Australia, Phillips (1969) reported a range of 730-7180 eggs per capsule and the temperate muricids, *C. concholepas* and, *T. haemastoma* each produce ~ 600-14000 and 500-4000 eggs, respectively (Table A.1). There was no significant difference between the number of eggs in the capsules and compared to the number of larvae hatching (Chapter 3), it is clear that nurse eggs are not used as a nutrition source for embryonic development. The egg capsules of *C. giganteus* may contain over 1000 eggs, but relatively few planktotrophic larvae (<100) hatch due to consumption of nurse eggs during the embryonic phase (Table A.1). In general this leads to species with lecithotrophic development hatching relatively large larvae that are less dependent on the plankton to reach competency and have a short planktonic phase. In contrast, species with planktotrophic development are more complex to culture due to a longer planktonic phase and the

need to understand nutritional requirements for what is the most vulnerable stage of the lifecycle.

Temperature exerts a strong influence on larval development time for whelks (Table A.1). In the present study, post-hatching *D. orbita* larvae reared at 22°C attained greater size over 38 days than larvae reared at 16°C (Chapter 4). Within the same range of temperature for optimal *D. orbita* larval growth (21-23°C), the central American muricid, *P. pansa* had a planktonic larval period of 120 days (Table A.1). The temperate muricid, *T. chocolata* also had a planktotrophic development period of 120 days at 22°C, and the South American muricid, *C. concholepas* has similar planktotrophic period of between 90-124 days at temperatures between 15-18°C (Table A.1). Typically, muricids with planktotrophic development from more temperate regions have longer pelagic larval phases than those at lower latitudes. The latitudinal range for *D. orbita* in Australia extends from approximately 26° to 42° south. Hence, it may be possible to increase larval development rates using warmer water in conjunction with populations from the northern range extent.

APPENDIX A: **Global whelk fisheries production. F = Commercial Fishery, Aq = Aquaculture, aF = Artisanal Fishery.**

Activity	Region	Country	Family	Species	Production/Value	Reference
aF, F	Africa	Tunisia, Tanzania	Muricidae	*Chicoreus ramosus, Hexaplex trunculus, C. chicoreus*	NA	Barnes *et al.,* 1998, Muhando & Jiddawi, 1998, Gharsallah *et al.,* 2010
F	Africa	Tunisia	Buccinidae	*Bolinus brandaris*	NA	Elhasni *et al.,* 2013
aF, F	Africa	Tunisia, Tanzania	Fasciolariidae	*Pleuroploca trapezium*	NA	Barnes *et al.,* 1998, Muhando & Jiddawi, 1998
aF	Asia	South East: Thailand, Cambodia, Indonesia, Vietnam, Philippines	Muricidae	*C. ramosus, C. brunneus, C. torrefactus, Cymia lacera, Thais carnifera*	NA	Nugranad & Kerdpoom, 1995, Poutiers, 1998, Berthou *et al.,* 2009, Hylleberg, 2010
F, Aq	Asia	South East: Thailand, Cambodia, Indonesia, Vietnam, Philippines	Buccinidae	*Babylonia areolata*	8-11 AUD kg^{-1}	Chaitanawisuti *et al.,* 2002, FAO, 2012
F/A seeding	Asia	Japan	Buccinidae	*Neptunea polycostata, N. intersculpta, N. arthritica, Buccinum isaotakii, B. meddendirffi, B. bayani, Fusitron oregonensis*	300+ t.p.a 1977 onwards	Seto & Doi, 2000, Nashimoto *et al.,* 1995, Fujinaga *et al.,* 2006
F	Asia	Korea	Buccinidae	*B. opisoplectum dall*	NA	Park *et al.,* 2007
aF	Asia	China	Fasciolariidae	*Fusinus salisburyi, F. forceps, F. diandriensis, F. perplexus*	NA	Poutiers, 1998, Berthou *et al.,* 2009, Callomon & Snyder, 2009
Aq, F	Asia	China	Buccinidae	*B. formosae habei*, *B. areolata, B. lutosa*	NA	Poutiers, 1998, Berthou *et al.,* 2009, Callomon & Snyder, 2009, Zheng *et al.,* 2010

Activity	Region	Country	Family	Species	Production/Value	Reference
aF	Asia	Philippines	Muricidae	*C. rosarius, H. chicoreum*	NA	Poutiers, 1998, Hermosilia & Narido, 2007
aF, F	Asia	Philippines	Fasciolariidae	*P. filamentosa, P.trapezium*	NA	Poutiers, 1998
F	Central America	Mexico to Peru	Muricidae	*H. nigritus, H. brassica, H. radix, C. regius, P. pansa*	NA	Ríos-Jara *et al.,* 2001, Naegel & Lopez-Rocha, 2006
aF, F	Central America	Mexico	Fasciolariidae, Muricidae,	*Cantharus pallidus, F. dupetittouarsi, H. brassica*	NA	Cudney-Bueno, 2000, Ríos-Jara *et al.,* 2001
F	Eastern Europe	Turkey, Bulgaria, Russia, Ukraine	Muricidae	*R. venosa*	NA	NOAA, 1997, Altuğ & Güler, 2002, Duzgunes & Erdogan, 2008
F	Europe	Russia	Buccinidae	*B. bayani bayani, B. verkruzeni, B. bulbacea, N. constricta, N. lyrata lyrata, N. polycostata*	total for all species 829.1 t in 2008	Borulya & Bregman, 2002, Repina & Drobyanzin, 2009
F	Europe	Ireland	Buccinidae	*B. undatum*	3800-10000 t.p.a in 2003-2005	Fahy *et al.,* 2005
aF	Europe	Portugal	Muricidae	*H. trunculus, B. brandaris*	14-29 AUD kg^{-1}	Vasconcelos, 2008a, Vasconcelos *et al.,* 2012
F	Europe	France	Muricidae	*B. brandaris*	NA	Martín *et al.,* 1995, NOAA, 1997, Solé *et al.,* 1998, Ramón & Amor, 2002
F	Europe	Northern Europe: NE Atlantic- Iceland, Norway, Sweden, Ireland, France, Belgium, The Netherlands	Buccinidae	*B. undatum*	55795 t in 1994 from Iceland fishery, 45000-225000 t estimated harvestable stock from Swedish fishery	NOAA, 1997, Valentinsson *et al.,* 1999
F	Europe	Spain	Muricidae	*B. brandaris*	NA	Martín *et al.,* 1995

Activity	Region	Country	Family	Species	Production/Value	Reference
F	Europe	Italy	Muricidae	*H. trunculus*	NA	NOAA, 1997
F	Europe	Croatia	Muricidae	*H. trunculus*	NA	Benović, 1997, NOAA, 1997
F	Europe	UK (England, Scotland, Wales)	Buccinidae	*B. undatum*	55795 t in 1994	NOAA, 1997, Shelmerdine *et al.,* 2007
F	Europe	Georgia	Muricidae	*R. venosa*	3000 t in 2005	Duzgunes & Erdogan, 2008
aF, F	India	India	Fasiolariidae	*P. trapezium*	3-12 t.p.a	Shanthini & Patterson, 2005
aF, F	India	India	Muricidae	*C. ramosus, R. rapiformis, T. rudolfi, T. bufo, Cymia lacera*	NA	Poutiers, 1998, Ramadoss, 2003, Berthou *et al.,* 2009
F Aq	India	India	Buccinidae	*B. spirata, B. zeylanica*	Ave. 247 t p.a. 1999-2003, total between 2002-05 = 85871 kg	Sreejaya *et al.,* 2004, Mohamed, 2012, Mohan *et al.,* 2012
F	North America	Canada	Buccinidae	*B. undatum*		FAO, 2012
F	North America	US	Buccinidae	*Kelletia kelletii*	50 t.p.a	CDFG, 2010
F	North America	US	Buccinidae	*Busycon carica, B. carica, B. canaliculatus, B. spiratus, B. sinistrum*	1424 kg meat, 2008. 1400 t in 2012	Anderson & Eversole, 1993, Power *et al.,* 2009, Peemoeler & Stevens, 2013
F	North Atlantic	Belgium	Buccinidae	*B. undatum*	NA	Redant, 1997, Nasution & Roberts, 2005, Berthou *et al.,* 2009
F	South America	Peru/Chile	Muricidae	*Concholepas concholepas, C. ramosus, C. giganteus, T. chocolata, T. geversianus, Xanthochorus cassidiformis*	NA	Castilla & Gelcich, 2008, Berthou *et al.,* 2009
aF	South America	Argentina	Buccinidae	*Buccinops globosum*	20-9200 kg p.a	Narvarte *et al.,* 2007
aF	Southern Australia	Australia	Muricidae	*Dicathais orbita*	NA	Kingsford *et al.,* 1991

APPENDIX B: **Comparison of embryonic and larval development, diet and settlement cues for a range of whelk species. DD = Direct Development, LD = Lecithotrophic Development, PD = Planktotrophic Development, NA = Not Applicable, NT = Not Tested.**

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development (days)	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Muricidae												
Acanthina lapilloides				DD		800				NA	NA	Spight, 1976
Acanthina monodon	240		134-1116 eggs	DD	70-80 @ 9.7-10.6 °C	825-1300				NA	NA	Gallardo, 1979
Acanthina paucilirata			153 eggs, 8 embryos	DD						NA	NA	D'Asaro, 1991
Acanthina spirata	275		40-140 eggs, 20-39 embryos	DD		670				NA	NA	D'Asaro, 1991, Spight, 1976
Argobuccinum pustulosum	167	47-149	2463-5356 eggs	PD	54-56	261				NT	NT	Gallardo *et al.,* 2012

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development (days)	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Ceratostoma burnetti	300			DD						NA	NA	Spight, 1976
Ceratostoma burnetti	300			DD						NA	NA	Spight, 1976
Ceratostoma foliatum	720		47-80 embryos, 19-35 juveniles	DD		1500				NA	NA	Spight, 1976, D'Asaro, 1991
Ceratostoma nuttalli			15-20 embryos	DD						NA	NA	D'Asaro, 1991
Ceratostoma rorifluum			7-12 embryos	DD						NA	NA	D'Asaro, 1991
Chicoreus ramosus	287-355		178-214 eggs, 12-27 embryos	DD	40	1860				NA	NA	Mahmoud *et al.,* 2013
Chicoreus virgineus	510-608		100-380	DD	20	1652-1993				NA	NA	Jagadis *et al.,* 2013
Chorus giganteus	249		710-2120 eggs, 45-143 embryos	LD	60-72 @ 15.5 °C, 54-87 @ 12-18°C	1042-1226	3 to 5		1042-1226	NA	20-30mM KCl	Gonzales & Gallardo, 1999, Gallardo & Sanchez, 2001, Gallardo & Cancino, 2009

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Concholepas concholepas	158-169		668-14250 eggs	PD	36–50 @ 17-18°C, 69-128 @ 13.5-14.5°C	260	90-124	15-18	1500-1800	*Tetraselmis* sp., *Pavlova* sp.,*Chaetoceros* sp., *Isochrysis galbana (Tahitian) Pseudoisochyris* sp., *Chaetoceros californicum, Isochrysis* sp., *Monochyrysis* sp.	*Semimytilus algosus, excess K: 20mM =58 %, 15mM=0 %, 25mM=no surv, microalga Prasinocladus marinus,* adult mucus conspecific shells, microbial films	Castilla & Cancino, 1976, Gallardo, 1979, Di Salvo, 1988, Campos *et al.,* 1994, Di Salvo & Carriker, 1994, Rodruiguez *et al.,* 1995, Manríquez *et al.,* 2008
Crassilabrum crassilabrum	230		60-220 eggs	DD		402			900	NA	NT	Gallardo & Gonzales, 1994
Dicathais orbita			730-7180 eggs	PD		240	38-41	16-22	1024	*Tetraselmis seucica, Nannochloropsis oculata, Chaetoceros muelleri, Isochrysis galbana*	20 mM KCl	Phillips, 1969, this study

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development (days)	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Eupleura caudata	340-390			DD		700-1100				NA	NT	Spight, 1976
Forreria belckeri			1250-1500 eggs, 2 embryos	LD						NA	NT	D'Asaro, 1991
Nucella canaliculata	375-620		13-25 embryos	DD	90-150	1300				NA	NT	D'Asaro, 1991
Nucella cingulata			12-49 juveniles	DD						NA	NT	D'Asaro, 1991
Nucella dubia			10-17 juveniles	DD						NA	NT	D'Asaro, 1991
Nucella emarginata	180-210		300-1000 eggs, 20-33 embryos	DD	72 @ 9-11 °C, 80 @ 8-10 °C	1150-1190				NA	NT	Spight, 1976, D'Asaro, 1991
Nucella lamellosa	590-638		19-81 eggs	DD	29 @ 11.5-17 °C	1000				NA	NT	Spight, 1976
Nucella lapillus	187-240		500-1000 eggs	DD	120	1000				NA	NT	Spight, 1976; Pechenik *et al.,* 1984
Ocenebra aciculata	450			DD						NA	NT	Spight, 1976
Ocenebra erinacea				DD		960				NA	NT	Spight, 1976
Ocenebra inermicosta				DD						NA	NT	D'Asaro, 1991

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development (days)	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Ocenebra interfossa			3-5 embryos	DD						NA	NT	D'Asaro, 1991
Ocenebra japonica	170-200			DD		1400-1760				NA	NT	Spight, 1976
Ocenebra lurida			5-12 juveniles	DD						NA	NT	D'Asaro, 1991
Ocenebra sp.	225-250			DD		775-2025				NA	NT	Spight, 1976
Plicopurpura pansa	149		95-1092 embryos	PD	36-65 @ 21-23 °C	188-282	120	21-23	902	*I. galbana, Thalasiosira weissflogii*	15mM KCl	Romero *et al.,* 2004, Naegel *et al.,* 2003
Pteropurpura festiva			782-870 eggs	LD	21-28					NA	NT	D'Asaro, 1991
Purpura patula	240			LD		400				NA	NT	Spight, 1976
Rapana bulbosa	280–320			LD		420				NA	NT	Spight, 1976

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development (days)	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Rapana venosa	214	197-999	124–1,300 eggs;few embryos	PD	15-27 @ 25°C	600-700	24-42	22-26	1180-1240	*Pseudoisochrysis* sp., *Chaetocerous* sp., *Tetraselmis* sp.	NT	D'Asaro, 1991, Sağlam & Düzgüneş, 2007, Harding, 2006
Thais bufo	252		800-1400 embryos	PD	10-11 @ 24°C	334-367					NT	D'Asaro, 1991
Thais carinifera	200-230		140 eggs	PD	19	340-400					NT	Spight, 1976
Thais chocolata	130		1700-3200 eggs	PD	49 @ 13.6 °C	225	120	22	1450-1750	*I. galbana, C. calcitrans, C. gracilis*	*Balanus laevis*	Romero *et al.,* 2004
Thais clavigera	190			LD		300-320				NA	NT	Spight, 1976
Thais dubia				DD		1320				NA	NT	Spight, 1976
Thais haemastoma	107		500-900; 4000 eggs	PD	15 @ 24 °C	130-160				NT	NT	D'Asaro, 1966
Thais hippocastanеum				DD		> 700				NA	NT	Spight, 1976

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development (days)	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Thais hippocastaneum				DD		> 700				NA	NT	Spight, 1976
Thais rustica	80		1070 eggs, 400 embryos	LD						NA	NT	Spight, 1976
Thais tissoti	215		255-295 eggs, 19-32 embryos	PD	19	231				*Tetraselmis* sp.		D'Asaro, 1991
Trophon acanthodes	213-236		6000 eggs	DD						NA	NT	Pastorino & Penchaszadeh, 2009
Trophon geversianus		6-26 per oviposition event		DD						NA	NT	Cumplido *et al.,* 2010
Urosalpinx cinerea	240, 300-400		28-50 eggs	DD	60	800-1000				NA	NT	Spight, 1976

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Buccinidae												
Babylonia areolata			390-415 eggs	PD	5 to 6 days	422-433		23-30	850-956	*Dicrateria zhanjiangensis*, *Platymonas subcordiformis*	12mM KCl	Sangsawangchote *et al.,* 2010
Babylonia spirata	260-280	35	350-800 eggs	PD	7 to 8 days	17		26-28 °C		*Chaetoceros calcitrans, Tetraselmis gracilis, Nannochloropsis salina, Isochrysis galbana*		Sreejaya *et al.,* 2004, Ke *et al.,* 2000
Babyonia formosae habei				PD		415	10 to 20	24.5-25.5°C	880-1010	*Dicratelia zhanjiangensis*, *Platymonas subcordiformis*	12mM KCl	Zheng *et al.,* 2001, 2005, 2010, Ke *et al.,* 2000
Buccinum undatum		140	Ave. 2700, but 10% of caps had no or few eggs. Est 3700 hatchlings	DD	5-8 months					NA	NT	Martel *et al.,* 1986

Family/Species	Egg diam. (µm)	No. caps. female^{-1}	No. of eggs or embryos cap.$^{-1}$	Mode of development	Time for intracap. development (days)	Hatch. size (µm)	Time for post-hatching larval development	Post hatch. larval temp. (°C)	Size at settlement (µm)	Diet	Settlement cue	Source
Busycon carica				DD						NA	NT	Harding, 2006
Busycotypus canaliculatus				DD						NA	NT	Harding, 2006
Neptunea antiqua	180	14-84	5000 eggs	DD	6-7months	8.32			6-12 mm 1-3 individual hatch per cap.	NA	NT	Power & Keegan, 2001
Fasciolaridae												
Pleuroploca aurantiaca	240	26-34	296-412 eggs	DD	30		3884			NA	NT	Meirelles & Matthews-Cascon, 2005

APPENDIX C: **Reproductive conditioning of Dicathais orbita**

C.1 *Introduction*

A number of studies have shown that manipulating the temperature and food regime can affect the condition and timing of reproduction in whelks (Navarro *et al.,* 2002; Carrasco *et al.,* 2006; Nasution & Roberts, 2005). Controlling factors that mimic a more rapid seasonal change may allow animals to recover more rapidly after reproduction and lead to increased aquaculture production. The aim of this study was to determine the effect of manipulating light and temperature on the reproductive cycle of *D. orbita* and different treatments affects dietary choice.

C.2 *Methods*

Adult specimens of *Dicathais orbita* with shell length between 60 and 70 mm (n = 144) were collected from subtidal reef at O'Sullivans Beach (-35.118697°, 138.468128°), South Australia during May 2007 and transported to Flinders University where they were held in aquaria with sufficient food until the start of experiments in June 2007. Immediately prior to reproductive conditioning treatments, shell length of each specimen was measured 0.1 mm from spire to siphonal canal with vernier callipers and sex was determined by narcotizing animals with 0.5 M MgCl following the methods in Chapter 4. Specimens were tagged (Hallprint Pty Ltd, Victor Harbour, South Australia, Australia) for identification and observed hourly until recovered from sex identification.

C.2.1 Experimental design

The experimental reproductive conditioning system comprised of 4 independent recirculating systems (RAS), each with 6 replicate, 10 L insulated plastic aquaria. Each system was provisioned with their own filtration assemblage comprised of a protein skimmer, 60 L sump with bioballs (Polytech™) and UV purifier (Biologic®, BIO-3.0). Temperature was controlled using an immersed 50 W water heater (Eheim Jäger) in each aquarium or by an inline chiller system (CW1000) (Figure A.1). Flow rate to each aquarium was maintained at 1 L min^{-1}. Temperature was checked daily using floating thermometers in each aquarium. Dissolved oxygen, pH, salinity (YSI 556MPS) and ammonia/ammonium (Hach FF-1A) were measured every other day.

Three male and three female specimens were randomly allocated to each aquarium. Live mussels (*Mytilus galloprovincialis*) and cockles (*Donax deltoides*) were provided by commercial aquaculture and fishery respectively after size grading to ensure reasonably consistent shell length and provided *ad libitum* as food for *D. orbita*. Shells of prey consumed were recorded and removed daily or every other day and replaced with the same species. Faecal matter was siphoned from each aquarium when food was replaced and the system was topped up with FSW via the sump to avoid rapid temperature changes in the aquariums. At the beginning of the experiment, water temperature, salinity and photoperiod were controlled to match that occurring along the metropolitan coast where specimens had been collected from. One system (Natural) continued to be controlled to mimic the ambient coastal water temperatures and photoperiod throughout the course of the conditioning process. The second system (Rapid), used historical data (IMOS, 2011) to simulate progression of the seasonal cycle of water temperature and photoperiod in the approximately half the time as the Natural system (Table A.1).

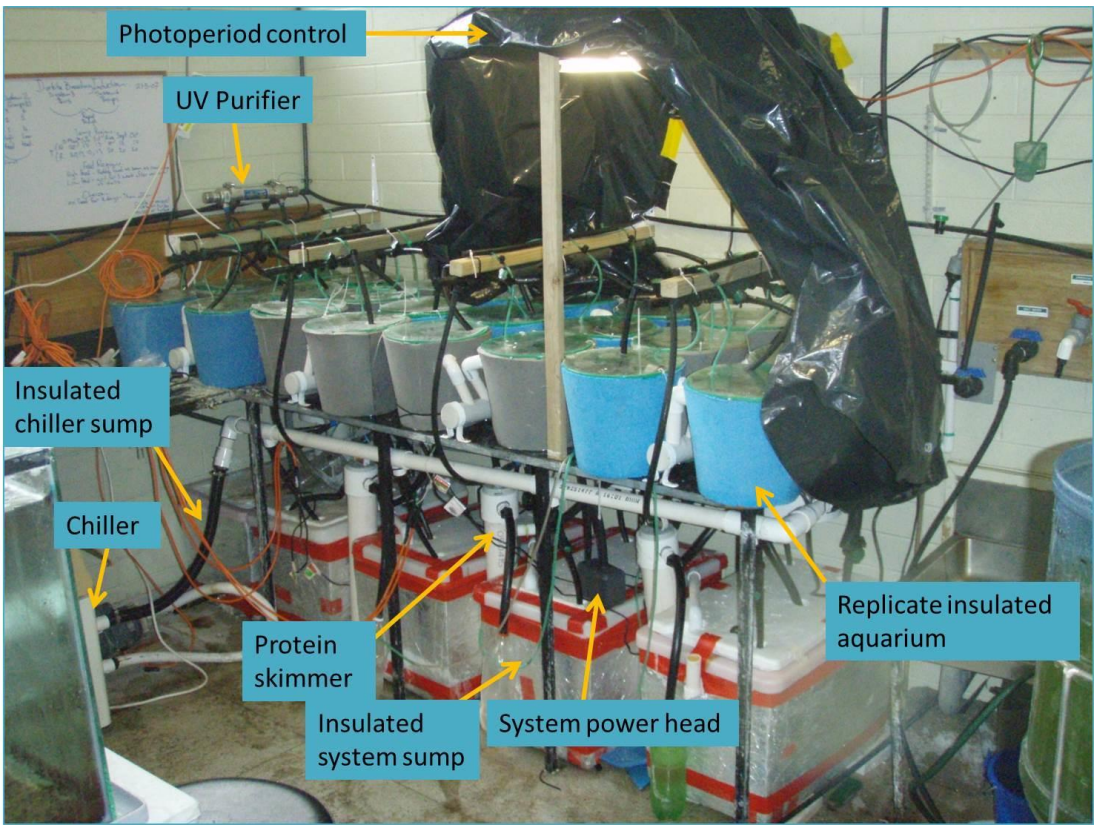

Figure A.1. Recirculating aquaculture system (RAS) used for broodstock conditioning.

Each month of the experimental period one male and one female specimen were chosen randomly from two replicate aquariums in each system. The shell length was measured using vernier callipers to the nearest 0.1 mm before being narcotized and prepared for histology. Two specimens were also collected each month from the wild population at O'Sullivans Beach reef for comparison. Preparation for histology consisted of removing the shell by cracking it with a vice at the junction of the body whorl and spire, and removing the soft body by severing the columellar muscle at the shell. The weight of the soft body was recorded to the nearest 0.01 g and then fixed in 10 % neutral buffered formalin and stored until dissection. Gonads were cut into 3 regions, posterior (1/3 from the distal end of the gonads), middle (2nd third from the distal end) and anterior (proximal 1/3 of gonad), dehydrated through an ethanol series, cleared in chloroform and embedded in paraffin. At least three serial transverse sections (5 µm) were obtained from each region from all specimens.

Sections were stained with modified Harris Haematoxylin and Eosin Y with Phloxine B (Thompson, 1966). Examination was carried out under a compound microscope and the stages of gametogenesis were evaluated according to those used by Vasconcelos *et al.* (2008c).

Table A.1. Temperature and photoperiod regime for broodstock conditioning of *Dicathais orbita*

Month	Natural		Rapid	
	Temperature (°C)	Photoperiod (L:D)	Temperature (°C)	Photoperiod (L:D)
June	20 -> 18	12:12	13	12:12
July	15	12:12	10	10:14
August	13	10:14	10 ->12	12:14
September	10	10:14	15 -> 18	14:10
October	10 -> 12	12:12		
November	12 -> 15	12:12		
December	15 -> 18	14:10		

C.3 *Results and conclusion*

No mating was observed during the experiment and there were no egg capsules deposited in any of the experimental tanks. On average *D. orbita* in the natural treatment consumed 13.3 ± 3.1 mussels and 10.5 ± 1.7 cockles per month per aquarium, while in the rapid treatment 14.2 ± 2.4 mussels and 11.4 ± 0.9 cockles were consumed per month. The mean increase in shell length was 2.3 ± 0.7 mm for female and 2.2 ± 0.81 mm for male *D. orbita* in the natural treatment and 1.8 ± 0.74 mm for female and 1.7 ± 0.55 mm for males in the rapid treatment (Figure A.2). The mean soft body weight (SBwt) increase was 2.08 ± 0.91 g for female *D. orbita* and 1.88 ± 0.89 g for males in the natural treatment. In the rapid treatment the mean SBwt was 1.35 ± 0.52 g for females and 1.2 ± 0.62 g for males (Figure A.2). At the cessation of the rapid treatment (October) both shell length and soft body weight were slightly greater than in the rapid treatment than the natural treatment, with the

exception of shell length increase for males in the rapid treatment (2.3 mm vs. 2.5 mm) (Figure A.2).

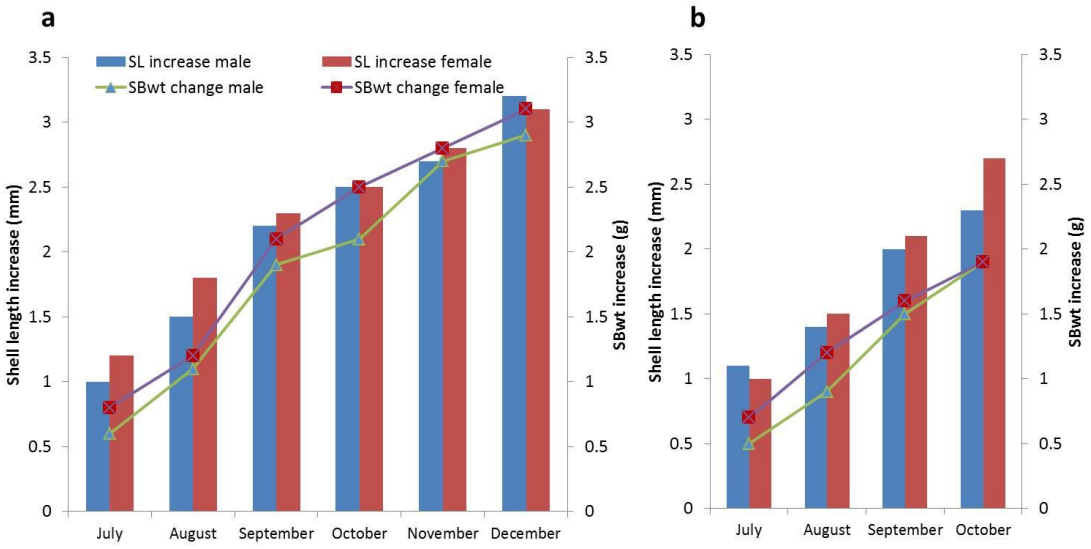

Figure A.2. Shell length and soft body weight (SBwt) increase of *Dicathais orbita* under natural (a) and rapid (b) temperature and photoperiod regimes. Data represents average of duplicates from replicate aquariums.

Samples were not suitable for histological analysis as fixatives did not penetrate deep enough into the tissues resulting in putrefaction of over half of the anterior and middle sections of the gonad and nearly one third of posterior sections. It is recommended that future studies of this type remove the gonad from the rest of the somatic tissue to ensure better penetration of fixative into the target tissue.

The recirculating system performed well in mimicking ambient coastal marine conditions and allowed for separate fine-scale control over photoperiod and temperature. It would also allow for control over salinity should this parameter be included as an experimental factor. All physicochemical parameters were maintained within narrow ranges throughout the experiment: salinity 36.5-38 psu, pH 8.02-8.42, DO 5.8-6.2 mg L^{-1}, ammonia/ammonium < 0.1 mg L^{-1}. Water temperature stabilised within half an hour of adjustment and controls allowed for adjustment of

approximately 0.5°C. Further work should consider the use of this system to investigate alternative combinations of treatments using temperature, salinity, photoperiod or photointensity and diet. It is recommended that more individuals be used in each aquarium, after appropriate pilot studies to ensure that ammonia levels can be maintained, to determine if sex ratio or the number of individuals affects breeding.

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