Reproduction, larval dispersal and population genetics of the sponge *Rhopaloeides odorabile*.

Thesis submitted by

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December, 2006

For the degree of Doctor of Philosophy

in the School of Marine and Tropical Biology, James Cook University,

Townsville, Queensland, Australia

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Research funding for this PhD study was provided through the Australian Institute of Marine Science, James Cook University, Research advancement program – Finfish and emerging aquaculture, CRC Reef, the Linnean Society of NSW. An Australian Postgraduate Award and James Cook University grants provided stipend support.

Acknowledgements

Looking back over the past four years it is all so clear. What seemed like an insurmountable cliff face was really a steady gradient, the summit of which was conquered slowly, one step a time. The pitfalls, frustration, and misguided delusions of failure all put into a sensible framework of perspective with the help of a mountain of people.

Most notably are my supervisors, the Professor, Rocky de Nys, Dean Jerry and Chris Battershill. Although there is no longer a student-mentor relationship I am sure it will be replaced with a colleague/friend relationship well into the future. Rocky "your door is always" open attitude is a commendable approach in supervising students particularly for those mad "I don't know what I am doing moments". Further, your generous support and encouragement to follow my own direction of research were much appreciated (particularly with a very important and relevant trip to France to study sponge research techniques). Dean, thanks for your mentoring over the last four years, without your help….well, I don't know where I would be. Thanks also for your help in problem solving when things were not working and inspiring confidence in me that something would eventually work. Chris, many thanks for your insight and help over the past 5-6 years, both in experimental design, and also with the world of sponge ecology.

Many volunteers helped in the extensive field components, providing valuable contributions in the collection of data, often undertaken in arduous conditions with low visibility diving and "surfable seas". Many thanks to my colleague Piers Ettinger-Epstein who accompanied me on all field trips and provided amusing moments of distraction, that were invaluable during times of frustration that come when the wind is

blowing 20 knots the seas are rough, and currents strong. A heart well thankyou also to Ray Bannister, Andy Cole, Darren Cocker, Martial Depcysnki, Matt Fraser, Michael Horne, Michelle Jonkiel, Dan Louden, Dommie Loong, Anne-Maree Lynch and the staff at OIRS, Anna , Ollie, Kylie, Rob and Pete.

Technical help in molecular methods was provided from Curtis Lind and Renate Kvingedal, and with a particularly big thank you to Carolyn Smith-Kuene and Brad Evans whose technical and practical instructions in molecular methods were invaluable. Also to the members of the de Nys lab who always provided a friendly work environment: Piers, Ray, Dan, Mike, Jana Guether, Nick Paul, and Andrew Scardino. Sue Reily and Jane Webb were always available for help in histology, sometimes at odd times during the weekend.

Thankyou to a core group of people from the AIMS sponge group; Libby Evans-Illidge, Cartsten Wolff, Jane Gioffre, and Alan Duckworth, provided friendly assistance on the many trips aboard the "Lady Basten" to collect samples. Also from AIMS, Beth Ballment provided valuable advice and assistance for the allozyme electrophoresis work. Pat Bergquist helped with the identification of sponge gametes.

Finally, thanks to my girls [redacted] both of who have travelled this long and often arduous road over the last eight years. Your continual love and support, in my early mid life crisis to change careers, was the only way I could have got here. Now,….. lets get our lives back.

Abstract

Reproductive strategies and larval dispersal behaviours are key factors in the distribution patterns of marine taxa. Furthermore, our understanding of population distributions, and the processes that influence and maintain them is fundamental to the conservation and management of marine benthic environments. Whilst reproductive and larval dispersal processes are well documented for a range of sessile marine invertebrates our understanding is predominantly related to corals. For other dominant sessile invertebrates, such as sponges, these processes are less well understood, even though they form an important component of the benthos.

Sponges are found from polar to tropical seas, in both marine and freshwater environments, with an estimated 15, 000 species worldwide. Their ecological roles are equally diverse with functional roles implicated in reef consolidation, and bio-erosion, habitat refuges, and benthic-pelagic coupling processes. Despite their presence and important functional roles, our understanding of processes that influence populations are still unclear. This study contributes to this understanding by quantitatively examining the reproductive processes, larval dispersal behaviours and population genetic structure of *Rhopaloeides odorabile* (Dictyoceratida; Demospongiae), a common sponge species of the Great Barrier Reef (GBR).

Sexual reproductive processes of *Rhopaloeides odorabile* were quantified using histological sections of samples from Pelorus Island collected over two reproductive seasons. Further sampling was undertaken to compare reproduction of populations from different locations including Pelorus Island (inner shelf reef), Rib Reef (mid shelf reef), and Pith Reef (outer shelf reef). *Rhopaloeides odorabile* is viviparous and gonochoristic. Commencement and cessation of gametogenesis coincides with rising

and falling sea surface temperatures (≈ 20-28^{o}C). Spermatogenesis occurs from September until December. Females initiate oogenesis in October with the asynchronous development of oocytes, embryos and larvae occurring within brood chambers. A larval release period of 5-6 weeks occurs during January and February. There is a clear gradient of female reproductive effort across the shelf reefs with reproductive output increasing with increasing distance from the coastline. Female reproductive effort for *R. odorabile* varied significantly across the shelf reefs on two levels. Firstly, sponges from the outer and mid reefs have a reproductive output, per unit volume, of up to 15 times more than inner reef sponges. Secondly, in conjunction (and co-related) to the reproductive output is investment into the size of oocytes. Mean oocyte diameters increased in size from inner to mid and outer reefs. Inner reef sponges recorded mean oocyte sizes of 156 μm (±9.4) compared to mean sizes of 203μm (±8.7) and 195μm (±8.9) for mid, and outer reef sponges respectively. Therefore, both increased egg size and egg numbers contribute to a much larger reproductive output. Reproductive output for inner shelf sponges may be compromised from exposure to terrigenous run-off resulting in sub-optimal habitat conditions.

The poor motility of many sessile marine invertebrate larvae makes active dispersal and habitat selection challenging. Manipulative experiments were undertaken using larvae from *R. odorabile* to quantify behaviours associated with vertical migration, phototaxis, swimming ability and settlement responses to cues associated with light, settlement surface topography, coral rubble and biofilms. Following an afternoon release larvae are cued by light and migrate vertically to the surface for 6 - 18 hours. From 24 hours larvae move from the surface to the bottom, maintaining this position for up to 54 hours before settling or dying. Larvae do not display gregarious settlement patterns or a preference for settlement surface topographies but do

preferentially settle to light exposed surfaces rather than shaded sites. Settlement to individual treatments of biofilms or coral rubble was higher when compared to controls with no cue. In addition, higher larval settlement to treatments containing non-sterile coral rubble compared to those containing sterile coral rubble demonstrates settlement is almost certainly a response to the biofilm, rather than the physicality associated with coral rubble. However, the transition from initial settlement to metamorphosis was higher when treatments comprised a combination of biofilm and coral rubble substrate suggesting a combination of cues is responsible for optimum recruitment. Therefore, vertical migration to surface waters is likely to facilitate passive dispersal over medium to large distances via wind driven surface currents. Larvae have the ability to swim and explore the substratum on their return to the benthos. The importance of settling to light exposed, biofilmed substrata facilitates settlement to microhabitats favourable to continued survival and growth (recruitment).

Population genetic results from allozyme and mtDNA data of *R. odorabile* support genetic differentiation at both the broadest (i.e. 10's kms), and smallest spatial scales (1 km) examined in this study. In addition, levels of genetic connectedness are also observed across the same spatial scale comparisons, providing an overall pattern of chaotic genetic structure. Whilst extensive larval dispersal would explain genetic homogeneity over broad spatial scales, fine scale population subdivision would also suggest larval dispersal is restricted between some of the sites examined. The behavioural strategies of vertical migration, coupled with pre-competency periods of up to 54 hours for *R. odorabile* larvae, provide a mechanism for passive dispersal via current systems throughout the sampled sites in this study. However, recognising that the GBR comprises a patchy mosaic of reef systems, many associated with self-entrained circular or eddy current systems, is of critical importance in explaining

patterns of genetic variation, and therefore larval dispersal patterns. It is proposed that the degree of larval dispersal is subject to reef specific hydrodynamics, and current patterns at the time of larval release, explaining the genetic patchiness observed in this species. Varying intensity of current regimes at spawning times will lead to localised recruitment at some sites or the occurrence of random or occasional dispersal over extensive distances, thereby explaining genetic homogeneity between more distant sites.

Although *R. odorabile* broods larvae, larval dispersal as inferred from population genetic data, and coupled with larval vertical migration behaviour, suggests larval dispersal can occur across moderate spatial scales beyond natal reefs. Overall, the variable patterns of reproductive output together with seasonality demonstrated to date for sponges, make generalisations of trends difficult, and illuminate the complexity in reproductive ecology for Porifera. Importantly, recognising the significance of reproductive processes for benthic invertebrates, will contribute to our understanding of the roles they play in the recruitment and maintenance of populations. This is particularly relevant for sponges, which are a major part of many benthic environments, but attract a comparatively minor focus of research effort to manage and conserve reefs.

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Chapter 1: General Introduction

Ecology and distribution patterns of benthic marine invertebrates

For sessile marine invertebrates a complex myriad of processes influence distributions patterns, arguably a result of a planktonic larval phase followed by a sessile adult habit. To fully understand population distributions of benthic marine invertebrates it is necessary to determine the effects of processes and strategies that occur at critical points along the life cycle continuum, for both the benthic adult and planktonic larval phases.

Processes contributing to marine benthic community structure have been investigated for well over one hundred years (review Morgan 2001). However, much of this has centred on two generalised proposals, the first advocating the importance of post settlement influences (predation and competition) (Connell 1961a, b, Paine 1966, 1969) and the second suggesting community assemblages are largely determined by levels of larval supply (review Underwood & Keogh 2001). Both proposals rely on a decoupling of the adult phase from the larval planktonic phase which, to a large degree, has polarised the scientific community over "false dichotomies rather than accepting multiple factors regulate complex life cycles and complex communities"(Morgan 2001).

The influence of competition and predation are clearly important influences to adult benthic community assemblages (Connell 1961a, b, Paine 1966, 1969). However, models developed in association with these post-recruitment processes are inherently reliant on levels of larval recruitment or supply-side ecology (Underwood & Keough 2001). Supply-side ecology, as a process influencing community structure, has been considered since the early-mid

twentieth century (Morgan 2001, Underwood & Keough 2001) but was popularised by (Lewin 1996) and later by numerous others (Young 1987, Underwood 2000, Underwood & Keough 2001). Supply-side ecology identifies variability in the supply of larvae available for recruitment and consequently has implications for population dynamics. To explain the patchy distribution in time and space that is commonly observed in marine communities, a plethora of work has been undertaken under the umbrella of "supply-side ecology" including, larval transport mechanisms (Metaxas 2001), larval pre-competency periods (Harii et al. 2002), planktonic survival/mortality (Morgan 1995), population genetics and connectivity patterns (Levin 2006), and larval settlement processes (Pawlik 1992).

For sessile marine invertebrates the planktonic larval dispersal phase has significant implications for population distributions, the importance of which can be encapsulated within the framework of two simplistic but effective questions as proposed by Levin (2006). Firstly, *how far do larvae disperse and what are the processes that mediate their dispersal distance?* Larval dispersal capabilities can be influenced by larval pre-settlement behaviours, pre-competency periods, vagility, larval release periods, and the mode of larval development (Levin 2006). Secondly, *where do recruits within a population originate?* The mode of recruitment (i.e. endogenous or exogenous) can influence patterns of connectivity and spatial structure of populations. In this thesis both of these questions, and the components they encompass, are considered for populations of the sponge *Rhopaloeides odorabile*.

Sponges

The Porifera encompasses a large conspicuous group of organisms that inhabit aquatic ecosystems with a biodiversity in excess of 15,000 species

(Hooper and Levi 1994). In comparison to most metazoans they function without the structural complexity of organs or tissue and rely on the complex functioning and flexibility of specialised cells (Knox et al 1994). Paradoxically this simple level of structural organisation has not constrained the range of life history traits or presence and diversity within ecosystems of this phylum. Indeed, in many of these ecosystems sponges play pivotal functional roles (Wulff 2001). Despite the importance and presence of sponges, the influence of larval dispersal and recruitment on population dynamics remains largely overlooked. Moreover, whilst aspects of reproduction, larval dispersal, and larval settlement are documented, quantitative studies detailing the larval production, release and settlement of sponges *in situ* are limited, resulting in a fragmented picture of key components influencing population dynamics. The challenge now is to integrate data on reproductive output and larval behaviours with broader population spatial patterns.

Reproduction

Because sexual reproductive processes influence levels of larval recruitment, and therefore adult distributions, developing an understanding of the reproductive ecology for dominant reef taxa is a fundamental step in the management and conservation of these habitats. The cumulative body of evidence to date highlights the remarkable plasticity of sexual reproductive strategies present in sponges. For example, in terms of larval development both viviparity and oviparity is recorded in sponges (Mariani et al. 2000, Whalan et al. 2005). Sponges can be hermaphroditic (Fell 1983, Fromont 1999), and gonochoristic (Fell 1983, Witte et al. 1994). Clonal reproduction has also been demonstrated in sponges (Zilberberg et al. 2006).

Although aspects of sexual reproduction in sponges are well documented, our specific understanding of reproductive biology for GBR sponges relies on a handful of species (Fromont 1994a, b, Fromont & Bergquist 1994). Furthermore, reports on sponge reproduction, whilst informative are often descriptive accounts of fundamental reproductive biology, with few studies examining the implications of key inter-relationships between reproductive processes and population dynamics (Ayling 1980, Fromont 1994b, Fromont & Bergquist 1994, Uriz et al. 1995). In addition, there are also limited reports that have attempted to bring these threads together to provide a comprehensive overview of the processes affecting the population biology of a sponge (Maldonado & Young 1996, Uriz et al. 1998). Whilst knowledge of reproductive processes that determine larval supply is critical to developing our understanding of population dynamics it is also key to link this knowledge with other related data that influence population structures, including larval dispersal behaviours.

Larval Dispersal and Settlement

The small size of many marine invertebrate larvae complicates assessments of larval dispersal and therefore the effects of dispersal on adult population structures. Directly tracking or tagging larvae has been successful for few sessile marine invertebrate taxa and only two studies have been successful in tracking sponge larval dispersal in the field (Lindquist et al 1997; Uriz et al 1998). Although both rely on low numbers of observations, two out of the three species followed showed restrictive dispersal behaviours and preferring a demersal habit, with the remaining species dispersing away from the substratum (Lindquist et al. 1997, Uriz et al. 1998). To overcome the difficulties of following larvae, Mariani

et al (2006) used a novel combination of plankton tows and hydrodynamics to examine the dispersal abilities of five species of sponge larvae. The findings in this study, combined with data on dispersal cues and swimming ability, indicate swimming abilities in some sponge larvae can overcome fine scale habitat hydrodynamics and supporting the importance of larval behaviours to dispersal (Mariani et al. 2006).

The small size and fragility of sponge larvae accentuates the logistical difficulties in directly tracking or tagging larvae to gauge dispersal capabilities. As a consequence, most of our understanding is related to indirect measures associated with larval behaviours, competency periods, population genetic data, or modelled data of passive particles and hydrodynamics.

In a recent review Maldonado et al (2006) consolidated the current state of knowledge on sponge larval ecology determining that for the eight types of sponge larvae known to date, all are lecithotrophic with short competency periods ranging from a few minutes to two weeks. With few exceptions sponge larvae use cilia for orientation (Maldonado et al. 2003, Maldonado 2006) and movement (Maldonado et al. 2003). Sponge larvae have swimming speeds ranging 0.1–1cm sec^{-1} (Maldonado 2006), and as for many other weak swimming marine invertebrate larvae, active horizontal dispersal is considered unlikely (see reviews; Pawlik 1992 Metaxas 2001, Maldonado 2006). It is proposed that they disperse passively, in accordance with hydrodynamics, over broad spatial scales and use their swimming abilities to determine active substratum exploration for settlement at microhabitat scales (Pawlik 1992, Maldonado 2006). For sponges, empirical evidence demonstrating that vertical migration facilitates passive dispersal is limited, resulting in an unclear picture as to the importance of processes such as

vertical migration for larval dispersal. Moreover, for the few species that vertical migration has been shown, the mechanisms driving directional movement are still unclear (Uriz et al 1998). For other sponges, directional movement of larvae to the benthos following release occurs through a range of mechanisms including negative phototaxis and larval buoyancy (Woollacott 1990, Maldonado & Young 1996, Maldonado et al. 1997)

Our understanding of larval settlement processes in sponges is limited. Light can be an important cue at small spatial scales aiding habitat selection in sponge larvae with negative phototaxis and facilitating settlement to cryptic habitats (Maldonado & Uriz 1998). Micro-refuges are also important for some sponge larvae with grooves and crevices providing a microhabitat that offers refuge from grazing herbivores (Maldonado & Uriz 1998). Settlement in response to treatments of biofilms (Keough & Raimondi 1995, Woollacott & Hadfield 1996), coral rubble and coralline algae (Jackson et al. 2002) have been demonstrated, but it is still unclear whether settlement in these studies are a response to chemical cues or to the micro-topography of the substrata tested. Although many other observations of larval settlement have been recorded these are often without experimental manipulations to establish if cues are implicated in settlement. Interestingly, some sponge larvae settle to experimental dishes such as glass or plastic raising questions concerning the role cues play for these species in sponge larval settlement (see review Maldonado 2006). Clearly, more rigorous testing offering choices of substrata with different cues are required before we can better understand settlement behaviours for sponge larvae.

Whilst information on the role sponge larval behaviours play in dispersal is compelling, these data on its own only provides a fragmented picture of the

influence of dispersal on population structure. However, information on larval dispersal behaviours in addition to population genetic data can be fundamental to how we interpret geographical structuring of populations. Population genetic data reflects a combination of historical, ecological, behavioural and environmental processes that determine rates and patterns of dispersal amongst populations (Grosberg & Cunningham 2001). Population genetic data for sponges in some cases show population subdivision amongst sites separated by just a few kilometres (Whalan et al 2005), but more commonly at scales of tens to hundreds of kilometres (Benzie et al. 1994, Davis et al. 1996, Sole-Cava & Boury-Esnault 1999, Duran et al. 2002, Duran et al. 2004a, b). These levels of population subdivision suggest that larval dispersal is restricted over relatively small spatial scales for most sponges, although occasional panmictic population genetic structures for some species also indicate widespread larval dispersal in the order of thousands of kilometres (Lazsoski et al 2001). In addition, hydrodynamic modelling of sponge larvae as passive particles also predicts restricted dispersal (Cropper et al. 2001).

To understand the factors governing population spatial patterns requires an integration of the reproductive processes that determine larval supply coupled with key information that influence larval dispersal and settlement. Population genetic and modelled data demonstrate restricted larval dispersal and corroborate the restrictive dispersal patterns associated with larval behaviours, mobility and short competency periods observed in many sponge larvae. However, there is still further work to be undertaken before our understanding of the influences of reproductive processes, larval dispersal and settlement behaviours on population spatial patterns is complete.

Thesis Aims

This thesis addresses key processes associated with reproduction and larval behaviours on the population genetic structure of the sponge *Rhopaloeides odorabile. Rhopaloeides odorabile* (Dictyoceratida; Demospongiae) (Thompson et al. 1987) is an amorphous, olive green, dictyoceratid sponge, which commonly occurs throughout the central Great Barrier Reef (GBR) (Wilkinson & Cheshire 1989) (Fig 1.1). There is little information detailing the ecology of this sponge although a comprehensive assessment of its symbiotic microbial ecology has been undertaken (Webster et al. 2001a, b, Webster et al. 2002).

Fig. 1.1. The study sponge *Rhopaloeides odorabile*.

Manipulative experiments were undertaken to quantify key aspects of reproduction and larval dispersal, and settlement of *R. odorabile,* while molecular techniques were used to elucidate population genetic structure of *R.odorabile* in the central GBR. The combination of these data will increase our understanding of the role reproduction and larval behaviour play in explaining spatial patterns of populations. In doing so this thesis is presented in four data chapters that address the reproduction, larval dispersal and settlement, and spatial scale of genetic variation for populations of *R. odorabile* on the central GBR.

Chapter 2 provides a fundamental outline of sexual reproduction in *R.odorabile* from one location over two reproductive seasons using data quantified from histological slide sections. Key information on reproduction provides the first step towards developing an understanding of parameters that influence levels of larval supply. Data are provided on the timing of reproduction, the mode of larval development and sexuality, sex ratios, size at sexual maturity and reproductive output.

Chapter 3 expands upon the fundamental reproductive biology described in Chapter 2, by presenting comparative reproductive data for *R.odorabile* from several locations across an environmental gradient (i.e. water quality). Reproductive output, sizes at sexual maturity and sex ratios are compared amongst sponges from inner (coastal) reefs exposed to turbid waters influenced by terrigenous run-off, and compared to sponges that occur in the clearer oceanic waters of the mid and outer shelf reefs of the GBR. Comparisons are drawn between levels of reproductive output, size at sexual maturity and sex ratios, and highlights the effects of terrigenous run-off on reproduction for this species.

Chapter 4 examines the behaviour of larvae from release until settlement by using as series of manipulative laboratory experiments that quantify pre-settlement larval dispersal and settlement behaviours. This chapter provides a comprehensive picture of larval behaviour from release to settlement, including swimming ability, patterns of vertical migration and a thorough presentation of data detailing the multiple cues implicated in settlement and metamorphosis for *R.odorabile* larvae.

Chapter 5 elucidates the population genetic structure of *R.odorabile* using allozymes and mitochondrial DNA (CO1) markers that provide data on the spatial

scale of genetic variation and levels of connectivity over distances of 1-140 kms within the central GBR. The genetic patterns demonstrated in this chapter are discussed in terms of larval dispersal behaviours identified in Chapter 4 and also in the context of historical patterns of gene flow.

Chapter 6 provides a synthesis of the data presented throughout the thesis, and provides a broad overview for the implications of reproduction, larval dispersal and settlement to the population genetic structure of *R. odorabile*.

Chapter 2: Characterising Sexual Reproduction in *R. odorabile*.[1]

2.1 Introduction

Sexual reproduction comprises a schedule of interrelated events that culminate with a redistribution of genes invested into offspring (Knox et al. 1994). In marine benthic invertebrates energy is diverted into sexual reproduction to facilitate gametogenesis, fertilisation, embryogenesis and spawning of propagules. Coupled with this are additional factors that influence levels of reproductive success, including timing, frequency, duration and intensity of reproductive processes (Szmant 1986). How this spectrum of reproductive processes are employed can influence the recruitment of offspring (Szmant 1986). In addition, reproductive events have genetic consequences, and therefore information on reproductive processes constitute a fundamental first step to developing an understanding of both ecological genetics and evolutionary biology.

Our comprehension of population dynamics is also guided by information on key sexual reproductive parameters including reproductive seasonality (Vargas-Angel et al. 2006), mode of sexuality and sex ratios (Szmant 1986), size at sexual maturity (Kapela & Lasker 1999), degree of parental care (few well developed brooded larvae vs numerous broadcast small gametes) (McEdward 1997), and size to fecundity relationships (Babcock 1991). Whilst reproductive processes and their evolutionary/ecological implications are well documented for many tropical sessile marine invertebrates, our understanding is predominantly related to corals (Harrison & Wallace 1990; Hall & Hughes 1996; McFadden et al. 2001).

[1]Chapter 2 adapted from Whalan S., Battershill C. & de Nys R. Sexual Reproduction of the brooding sponge *Rhopaloeides odorabile*. *Coral Reefs*. In press.

For a number of other dominant benthic invertebrates, such as sponges, reproductive processes are less well understood.

Sexual reproduction in sponges has been well documented most notably with comprehensive descriptions of gametogenesis and embryogenesis (Fell 1983). There are fewer investigations of the role reproductive processes play in population dynamics. For example, it is well established that sponges display a panoply of reproductive strategies, with examples of viviparity (Ilan & Loya 1990, Kaye 1991, Ilan 1995, Ereskovsky et al. 2005), oviparity (Hoppe & Reichert 1987, Corriero et al. 1996, Mariani et al. 2000, Mariani et al. 2001), gonochorism (Fell 1983, Kaye & Reiswig 1991a, Witte et al. 1994), and variant forms of hermaphroditism (Fell 1983, Fromont 1999). Indeed, some species change sexuality between seasons (Gilbert & Simpson 1976, Fell 1983). The environmental factors regulating sponge reproductive cycles including sea temperatures (Fromont & Bergquist 1994, Witte et al. 1994, Ereskovsky 2000), and photoperiods and lunar phases (Fromont & Bergquist 1994), are also documented. Given the contributing role reproductive output plays in recruitment, and therefore adult distribution, there is a surprising lack of information. However, this is partly due to difficulties of estimating fecundity for an organism lacking designated gonads (Bergquist 1978). A comparison of reproductive output among taxa to establish general trends is also hindered by the disparate methods to estimate reproductive output in sponges (Table 2.1). Key processes that influence larval supply, and therefore population dynamics, in particular the relationship between body size, sexual maturity and reproductive output, is not well understood for the Porifera (Ayling 1980, Fromont 1994b, Uriz et al. 1995).

Quantifying the reproductive ecology for dominant reef taxa is fundamental to the management and conservation of these habitats. Dictyoceratid sponges are an important group of sponges that occur throughout the GBR. This study aims to provide detailed reproductive information on *Rhopaloeides odorabile*, a common dictyoceratid sponge occurring throughout the central GBR. Specifically, a range of sexual reproductive processes for this species are determined, including seasonality, mode of reproduction and sexuality, sex ratios, gametogenesis, size of sexual maturity, and relationships between size and levels of reproductive output.

2.2 Materials and Methods

2.21 Study site

Samples were collected from sponges inhabiting the fringing reefs of the southern and northern shorelines of Pelorus Island (18° 33.44′ S, 146° 29.33′ E). Pelorus Island is part of the Palm Island group, a series of islands situated within the inner shelf reefal systems of the GBR. This site was chosen because of the relatively high abundance of *R. odorabile* that inhabit the shallow waters (5-9 metres) of the reef flat.

2.22 Collection and preservation of samples

To describe the reproductive biology and quantify reproductive output of *R. odorabile,* samples were collected monthly from August 2003 to March 2005. A total of 50 samples were collected each month using SCUBA. This included the repeated collection of samples from 11 individuals, tagged at the commencement of the study, to determine mode of sexuality (i.e. hermaphroditism or gonochorism). To develop an understanding of seasonality in mode of reproduction, gametogenesis, sex ratios and reproductive output, samples were

collected from a further 39 haphazardly chosen sponges. To avoid repeated sampling from the same individual, wedge shaped samples (~3 x 3 x 3cm) were cut and collected from each sponge. Sponges with wedge shaped cuts then represented previously sampled sponges and were avoided.

Because sponges do not have designated gonads, and gametes and larvae occur throughout the mesohyl (Bergquist 1978) samples were haphazardly collected from each sponge. A pilot study was undertaken to validate this sampling strategy. One section was taken from the surface (i.e. the first 5cms) and another from the remainder of the sponge (i.e. central or basal layers) of several reproductive individuals (n=30) to establish whether reproduction varied between different regions of the sponge. There was no significant difference of propagule numbers between different regions of sponge (Paired sample t–test $p > 0.05$ - both male and female). Removing small parts of sponge did not appear to damage or interfere with sponge survival with evidence of regeneration of the cut surface taking place within a few days. To enable comparisons between size and various reproductive measures, each sampled sponge was measured along the longest axis of length, height, and width to determine size.

Following the field collection of samples sub-samples 4-5 mm thick were excised and placed directly into histology cassettes and then fixed in the gonad fixative, FAACC [1 litre = 100 ml formaldehyde (40%), 50 ml glacial acetic acid, 13 gm calcium chloride dihydrate and 850 ml of tap water (Fromont 1999)] pending histology. Sea surface temperature data comprising single measurements taken once a month were provided by the Australian Institute of Marine Science Remote Sensing and Data Centre.

Table 2.1. Summary of published accounts detailing larval development, mode of sexuality, and quantification of gametes, zygotes and larvae in sponges. Reproductive output is expressed, where data are available, both in proportions (%), and numbers of propagules, in choanoderm. Larval development; V is viviparous, O is oviparous. Sexuality; G is gonochoristic, H is hermaphroditic. ♀ Product; O is oocyte E is embryo, L is larvae. Sample sizes are denoted by n and indicate maximum sample size for those studies reporting different sampling sizes over more than one season.

Species	**Larval-devel**	**Sexuality**	**♀ Product**	**n**	**Fecundity**				**Ref**
					♀ #/mm²	**♀ %**	**♂ #/mm²**	**♂ %**	
Poecilosclerida									
Crambe crambe	V	-	E	-	0.76*	-	-	-	1
Mycale contarenii	V	-	E, L	27	0.07-0.2*	1.3-1.5	-	-	2
Mycale sp.	-	-	L	1	-	2-3	-	5-10	5
Mxyilla incrustans	V	H			-	7.3			10
Chondropsis sp.	V	H	E	30	0.1*	-	70*	-	6
Chondropsis sp.	V	H	L	-	0.002- 0.03*	-	-	-	6
Stylopus sp. orange	V	H	E	70	0.2*	-	100*	-	6
Stylopus sp. orange	V	H	L	-	0.01-0.04*	-	-	-	6
Stylopus sp. pink	V	H	E	70	0.2*	-	100*	-	6
Stylopus sp. pink	V	H	L	-	0.006-0.02*	-	-	-	6
Anchinoe sp.	V	H	E	15	0.2*	-	100*	-	6
Anchinoe sp.	V	H	L	-	0.05-0.2*	-	-	-	6
Raspalilia topsenti	-	-	O	35	320*	-	-	-	6
Iophon piceus	V	H			-	12			10
Haplosclerida									
Xestospongia bergquista	O	G	-	3	1.8 – 3.1*	-	5.9*	-	3
Xestospongia exigua	O	G	-	3	1*	-	7.2*	-	3
Xestospongia testudinaria	O	G	-	3	1.5-1.9*	-	9.7*	-	3
Haliclona amboinensis	V	G	-	3	1.3-2.4*	-	0.7-1.1*	-	3
Haliclona cymiformis	V	G	-	3	0.46-0.78*	-	0.84-1.6*	-	3
Haliclona loosanoffi	V	G	O, E, L	-	0.04-10.6*	-	-	-	8
Niphates nitida	V	G	-	3	1.6-3.2*	-	0.6-2.4*	-	3
Hadromerida									
Aaptos aaptos	O	G	O	92	150*	-	170*	-	6
Polymastia sp.	O	G	O	170	130*	-	60*	-	6
Tethya crypta	-	-	-	-	-	1	-	-	5
Tethya aurantium	O	G	O	19	47-689*	1	-	-	7

Dictyoceratida									
Ircinia strobilina	V	G	-	10	-	1-10**	-	1-10**	4
Rhopaloeides odorabile	V	G	O, E, L	181	0.01-0.13	0.02-1.03	4.6-14.	0.72-2.97	11
Verongida									
Verongia gigantea	-	-	-	2	-	<1	-	10-20	5
Astrophorida									
Ancorina alata	O	G	O	60	50*	-	1000*	-	6
Spirophorida									
Tetilla sp.		G	O	10	19.3-88.2 *1 155 *2				9
Halisarcida									
Halisarca dujardini	V	G	O, L		-	49- 69.5		65	10
Halichondrida									
Scopalina lophyropoda	V	-	E	-	0.14*	-	-	-	1

Table 1

Notes: * data adjusted to a uniform measure (i.e. mm^2). ** distinction between sexes not stated. *[1] samples from shallow habitats, *[2] sponges from deep habitats.

Source: 1 (Uriz et al. 1998); 2 (Corriero et al. 1998); 3 (Fromont 1994a); 4 (Hoppe 1988); 5 (Reiswig 1973); 6 (Ayling 1980); 7 (Corriero et al. 1996); 8 (Fell 1976b); 9 (Meroz-Fine et al. 2005); 10 (Ereskovsky 2000); 11 Whalan this study.

2.23 Histology and visualisation of reproductive propagules

To visualise reproductive propagules with light microscopy samples were processed using standard histological techniques. Each sample was processed with an automated tissue processor using an alcohol dehydration and xylene clearing system followed by paraffin impregnation under vacuum. Samples were then blocked into paraffin and cut with a microtone at 5μm. To facilitate cutting the tough skeletal fibres, and to provide intact sections, the surface to be cut was soaked in a solution of 10% ammonia on ice for 10-15 minutes prior to cutting. Tissue sections were stained with haematoxylin and eosin.

2.24 Quantifying reproduction

Each sample was observed with a light microscope to identify reproductive propagules. This provided fundamental information on the reproductive season, gametogenesis, mode of larval development, sex ratios, mode of sexuality (using the repeated data from tagged individuals) and reproductive output.

Because the development of gametes and embryos can occur throughout the sponge (Bergquist 1978), it is difficult to use conventional measures of reproductive output associated with gonad structures. To establish reproductive output in *R. odorabile* a relative index of reproductive output (ROI) was used, modified from Corriero et al. (1998). A Leica DC image acquisition camera, mounted onto a light microscope, was used to capture digital images of reproductive propagules within slide tissue sections. Leica IM50 software was then used to calculate diameters and surface area measurements of reproductive propagules. The surface area of the entire slide tissue section was also determined.

The ROI was then calculated as the combined percentage of reproductive propagules occupied within the whole tissue sample area. Reproductive propagules were classified as oocytes, embryos, or larvae in females, and spermatic cysts in males. The comparatively low numbers of propagules in females enabled measurement of all propagules within the slide section. In males however, the number of gametes within each tissue section were substantially higher. Therefore the surface areas of spermatic cysts from three separate microscope fields of view were calculated and a mean value determined. The field of view is equivalent to $1mm^2$. Importantly, for both sexes, ROI is a per unit estimate of the proportion of tissue comprising propagules (% propagules mm^{-2}). Total reproductive output for each entire sponge was then calculated by converting ROI to a volume (ROI x slide section thickness of 5 μm) and multiplying the ROI (volume) by total sponge volume.

Because some sections resulted in propagules not being sectioned medially, ROI potentially underestimates reproductive output. Therefore, total numbers of propagules were also recorded and plotted against their respective ROI to assess the correlation between the two estimates. Correlation co-efficients were then tested for significance. Significant relationships between ROI and numbers of propagules ($r^2 > 0.8$, $P<0.01$, for both sexes) show that ROI is a valid and reproducible measure of reproductive output. This study focuses on ROI, however the use of both numbers, and proportions of propagules (ROI), enables comparisons to be made to previously reported measures of sponge reproductive output.

2.25 Statistical Analysis

One-way ANOVA was used to detect differences between reproductive output and seasons, and gamete sizes over time. Data was log transformed (Ln+1) to validate the assumptions of normality and homoscedasticity before ANOVA was executed. Tukey's (HSD) post hoc tests were used to establish where significant differences occurred. To assess relationships between levels of reproduction and sponge size two series of scatter plots and correlation coefficients were constructed. The first assessed relationships between size and the ROI (proportional rate of propagules mm $^{-2}$), and the second for data comprising the total reproductive output per whole sponge. This was done for both male and females. To test for departures from unity sex ratios Chi squared goodness of fit tests (Pearson's) were performed. Significance of correlation coefficients (Spearman's Rho) was determined to detect for significant patterns between adult size and levels of reproductive output. The measure of variance associated with the reporting of all mean values, is one standard error.

2.3 Results

2.31 Reproductive cycle and development

Reproduction was quantified for 181 individuals over the entire study period (77 in 2003/04, 104 in 2004/05). Sexual reproduction in *R. odorabile* occurs during a very distinct season, with female gametogenesis commencing in September and continuing until February. Male gametogenesis commences in October and continues until January (Fig 2.1). Sea surface temperatures (SST) ranged from 20.1 ^{o}C in August to 29 ^{o}C in December (Fig 2.1). The gradual rise in temperatures coincided with reproduction in *R. odorabile* with oogenesis

commencing in September when SST are increasing. The release of larvae occurs in December and January when SST peak. SST decline coincide with the cessation of reproduction in late February.

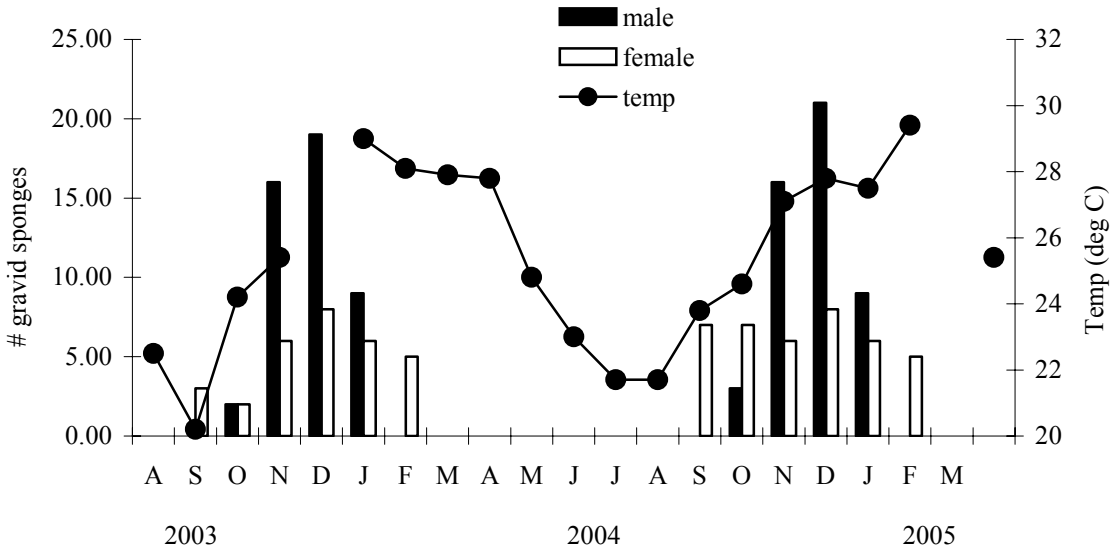


Fig. 2.1. Timing of gametogenesis, showing numbers of reproductive individuals for both sexes in relation to sea surface temperatures. Breaks in SST data indicate missing data points (i.e. Nov 2003 & Feb 2005)

2.32 Gametogenesis

Spermatogenesis

Spermatogenesis occurs asynchronously throughout the mesohyl, but is synchronous within any one spermatic cyst, commencing in October and continuing until January, again coinciding with rising and falling SST. Spermatic cysts are round and initially typified with the presence of a few large spermtocytes, developing to form dense spermatic cysts containing numerous spermatids (Fig 2.2 a-e). Spermatic cysts showed a mean size of 45.24 μm (±0.26 μm, range 11-90 μm).

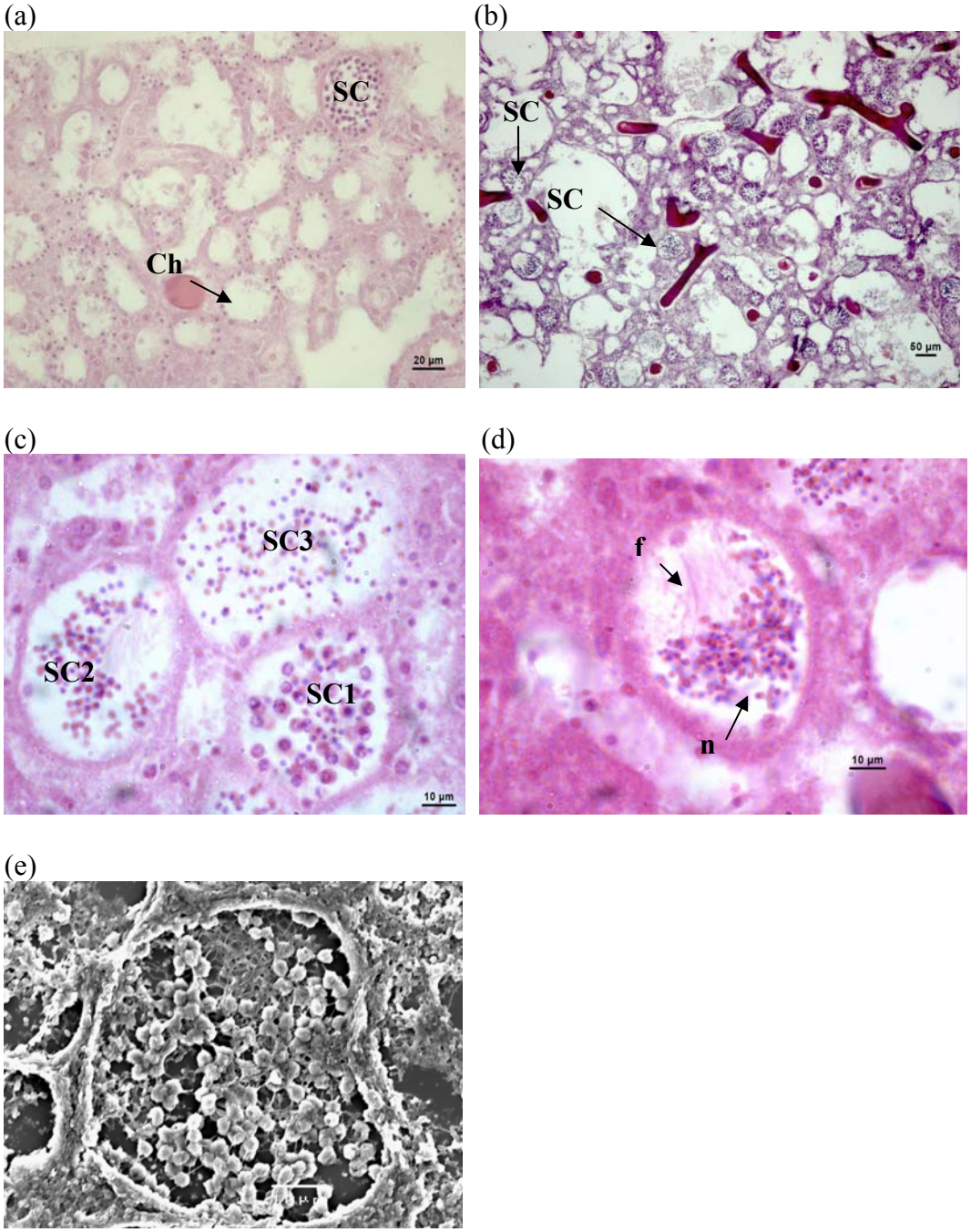


Fig. 2.2 (a-e). Series of photo-micrographs detailing male gametogenesis in *R.odorabile*. 2.2 (a) spermatic cysts (SC) surrounded by choanocytes (Ch); 2.2 (b) distribution of spermatic cysts showing asynchronous development of spermatic cysts; 2.2 (c) asynchronous development of spermatic cysts, (SC1-SC3), showing the different stages of sperm development occurring among spermatic cysts. Development of spermatids within each cyst however is synchronous; 2.2 (d) details of developing spermatids showing nucleus (n) and flagellum (f) of sperm. This is also seen in 2.2 (e), a scanning electron microscope image of developing spermatids within a spermatic cyst.

Oogenesis, embryogenesis and larval development

Rhopaloeides odorabile is viviparous with consistent patterns of oogenesis, embryogenesis, and larval development occurring from September until February over both seasons (Fig 2.3). Oogenesis commences in September with the formation of oocytes and continues asynchronously throughout the choanoderm until January when eggs are still present. Oocytes progressively increase in size over the reproductive season. At the beginning of the season oocytes show a mean diameter of 50.28 μm (±12.6), achieving a mean diameter of 109.9 μm (±13.22) at the end of the season in January. There are significant increases in oocyte size over the season (Fig 2.4, ANOVA; $F_{4, 427} = 14.32$, $p < 0.01$) which occurs over the first two months (i.e. September-October) and with no further increase in oocyte size for the remainder of the season (Fig 2.4, Tukey's HSD). Asynchronous development of both gametes and zygotes occurs, with oocytes, embryos and larvae often developing within the same brood chamber (Fig 2.5 a). The earliest oocytes detected in this study were spherical with a nucleus and nucleolus (Fig 2.5 b & c). They develop a follicular epithelium, consisting of several layers of follicular cells, with maturation (Fig 2.5 d). Some oocytes remain unfertilised and are resorbed (Fig 2.5e). Fertilisation presumably starts in early September resulting in the development of embryos until January. Embryogenesis also occurs asynchronously with several stages of cleavage often seen within one individual (Fig 2.5 b-d). The presence of early stage larvae was seen in December until February. Larvae are typically parenchymellae with a mean length of 270 μm (±4.16) and distinguished from

embryos by the presence of elongated peripheral cells surrounding a dense accumulation of interior cells (Fig 2.5f).

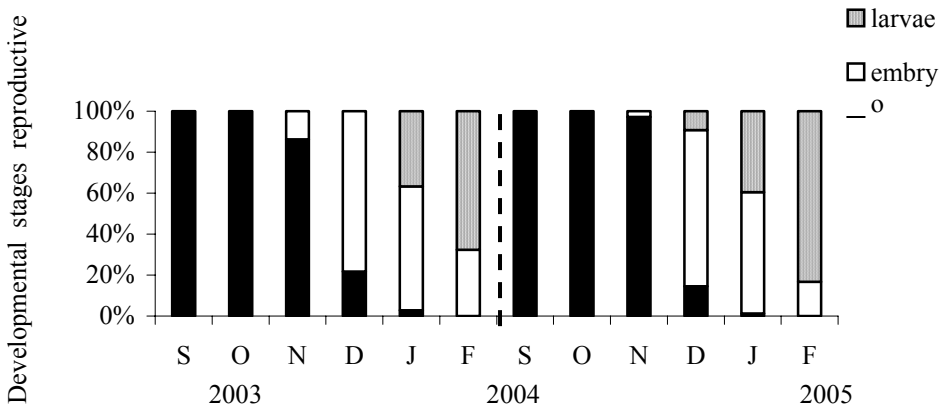


Fig. 2.3. Proportions of reproductive development stage in female *R.odorabile* (egg, embryo or larva) for each month of the study. Dashed line demarcates reproductive seasons.

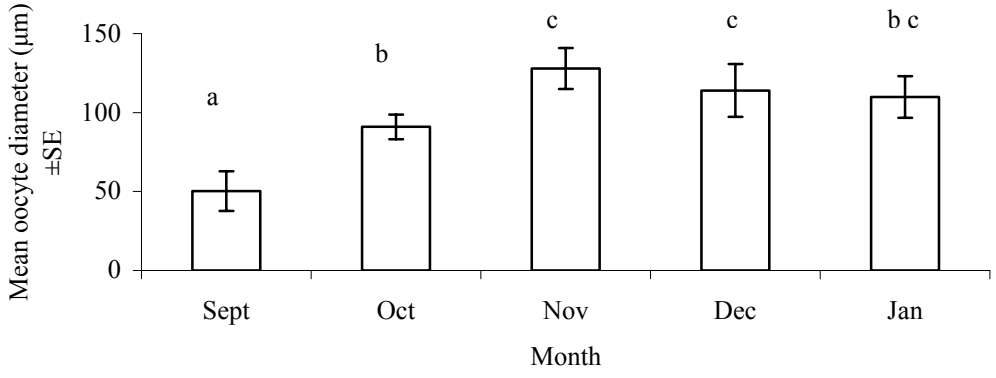


Fig. 2.4. Mean oocyte diameters (±SE) from all reproductive *R. odorabile* females over both reproductive seasons (combined data). Letters denote significant differences among times (Tukey's HSD).

(a) (b)

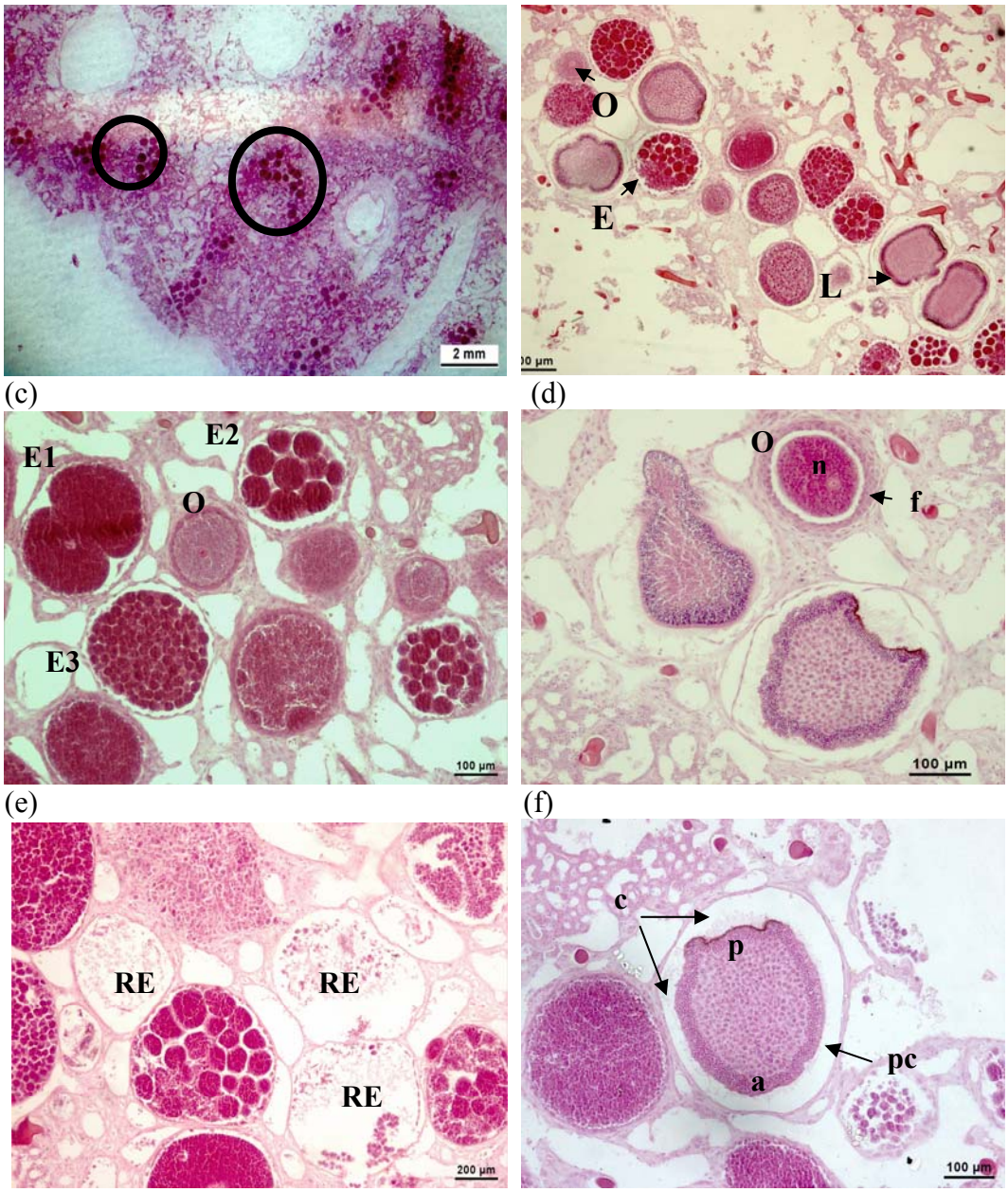


Fig. 2.5 (a-f). Series of histological sections detailing gametogenesis in *R.odorabile* females. 2.5 (a) Reproductive section viewed under a stereo dissecting microscope showing regionalisation of propagules in brood chambers (stained red and circled); 2.5 (b) asynchronous development of oocytes (O) embryos (E) and larvae (L) all occurring within the one brood chamber; 2.5 (c) Depiction of oocyte (O) and embryonic development with early (two stage division) (E1) and more advanced embryos (E2 & E3); 2.5 (d) Further details of oocyte (O) showing nucleus (n) and follicular cellular layer (f) that typically surrounds oocytes; 2.5 (e) Egg degeneration following unsuccessful fertilisation. Resorption of yolk vitelline plates has left a vacuous egg (RE); 2.5 (f) longitudinal section of a developed larva, showing interior cellular mass, distinct peripheral cellular layer (pc), anterior (a) and posterior (p) pole. Larva has a ciliated surface surrounding the body and a cillia clump (c) at the posterior pole.

2.33 Mode of sexuality and sex ratios

The repeated sampling of 11 tagged sponges over two reproductive seasons revealed that individuals developed male and female gametes separately, clearly showing this species to be gonochoristic (Table 2.2). Furthermore, female and male gametes never occurred together in the same individuals in all 181 reproductive sponges examined during the study period. Although females were reproductive for 6 months compared to 4 months for males, for those months both are reproductive males dominated samples with a mean 66% (±1.22) compared to 33% (±0.58) for females (Fig 2.6). Male to female sex ratios calculated when both sexes show optimal output (i.e. November and December) equate to 2.5: 1 which is a significant departure from a sex ratio of unity (Pearson's χ^2, 1 df, $p < 0.01$).

Table 2.2. Reproduction in tagged sponges repeatedly sampled over a two-year period to assign mode of sexuality in *R. odorabile.* F = Female reproductive propagules; M = male gametes. No reproduction was observed from March to September in both years and in February 2004.

	2003			2004				2005	
SPONGE	Oct	Nov	Dec	Jan	Oct	Nov	Dec	Jan	Feb
#1	-	-	F	F	-	-	-	F	F
#2	F	F	-	-	-	-	-	F	F
#3	F	F	-	-	-	F	-	F	F
#4	-	-	-	-	F	F	-	F	-
#5	-	-	-	-	-	F	-	F	-
#6	-	-	-	-	F	F	-	F	-
#7	-	F	F	-	F	F	F	F	-
#8	-	-	-	-	M	-	M	-	-
#9	-	-	-	-	M	-	M	-	-
#10	-	-	-	-	-	M	M	-	-
#11	M	M	M	-	-	M	M	-	-

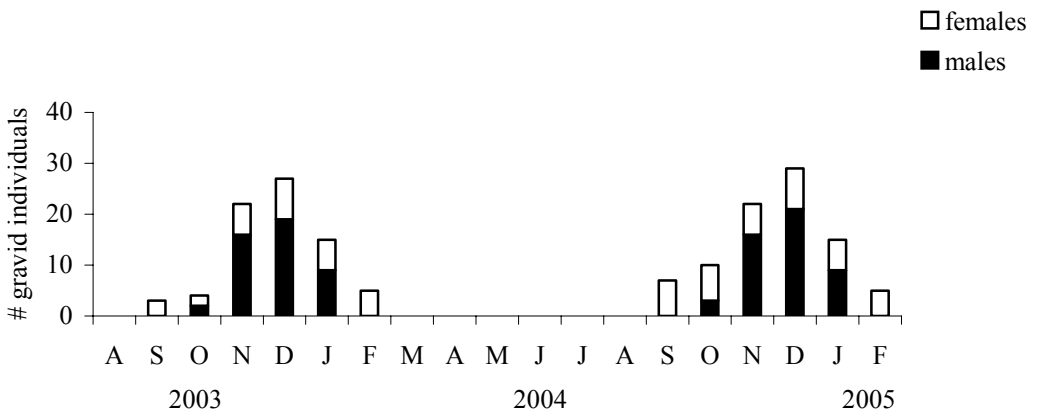


Fig. 2.6. Total numbers of gravid male and female *R. odorabile* sponges for each month of the study. Sex ratios are shown over for the entire period but statistical analysis is related to data for November and December when both sexes are considered to be optimally reproductive.

2.34 Reproductive output

Mean levels of reproductive output (ROI) were highest in males with 1.77% (±0.32) of choanoderm dedicated to gametes. In comparison mean levels of reproductive output for females was 0.30% (±0.09). For males approximately 1.47% and 2.77% of choanoderm was occupied with spermatic cysts in October 2003 and October 2004 respectively, peaking at 2.60% and 2.97% in December 2003 and November 2004 respectively (Fig 2.1 & Table 2.3). Reproductive output was lower for females with reproductive propagules comprising less than 1.03% of choanoderm (range 0.001-1.03%). Mean numbers of propagules followed similar trends with higher levels of propagules present in males (10.36 mm^{-2} ±1.47, range 4.60 - 14.4) than females (0.08 mm^{-2} ±0.01, range 0.01 - 0.18) (Table 2.3). Furthermore, there are clear correlations between the estimates of propagules per mm^{-2} and ROI for both sexes (Pearson: males - 0.94, $r^2 = 0.89$, $p < 0.01$; females - 0.92, $r^2 = 0.85$, $p < 0.01$), and determining that ROI is a valid measure of reproductive output. Reproductive activity for both sexes was consistent over

both seasons (ROI - Fig 2.7. ANOVA: males, $F_{1, 82} = 1.19$, $p = 0.27$; females $F_{1, 71} = 0.09$, $p = 0.75$), peaking during November and December. This time period is when SST are also peaking, reaching temperatures of approximately 29 ^{o}C.

Table 2.3. Monthly proportions of propagules (ROI) and numbers of propagules mm^{-2}, of choanoderm (mean ± SE). Propagules for females include oocytes, embryos and larvae. Male propagules are spermatic cysts. Overall mean is the mean of means for combined reproductive months. Dashes indicate no reproductive samples present.

Month	Female ROI	#/mm^2	Male ROI	#/mm^2
Sept 03	0.003 (±0.01)	0.02 (±0.01)	-	-
Oct 03	0.02 (± 0.01)	0.03 (±0.01)	0.72 (±0.32)	5.80 (±2.00)
Nov 03	0.09 (± 0.03)	0.07 (± 0.02)	2.27 (±0.43)	14.40 (±1.73)
Dec 03	0.49 (± 0.19)	0.12 (± 0.04)	2.60 (±0.39)	13.90 (±1.91)
Jan 04	0.42 (± 0.15)	0.09 (± 0.02)	1.43 (± 0.26)	10.80 (±2.47)
Feb 04	0.07 (± 0.03)	0.02 (± 0.01)	-	-
Sept 04	0.001 (±0.00)	0.01 (± 0.01)	-	-
Oct 04	0.09 (±0.05)	0.10 (± 0.04)	1.36 (± 0.48)	8.00 (±2.31)
Nov 04	0.18 (± 0.06)	0.08 (± 0.02)	2.97 (± 0.37)	14.40 (±1.43)
Dec 04	1.03 (± 0.34)	0.18 (± 0.05)	2.37 (± 0.20)	11.00 (±0.93)
Jan 05	0.57 (± 0.20)	0.13 (± 0.04)	0.46 (± 0.22)	4.60 (±0.63)
Feb 05	0.16 (± 0.31)	0.10 (± 0.05)	-	-
MEAN (overall)	**0.30** (± 0.09)	**0.08** (± 0.01)	**1.77** (± 0.32)	**10.36** (±1.47)

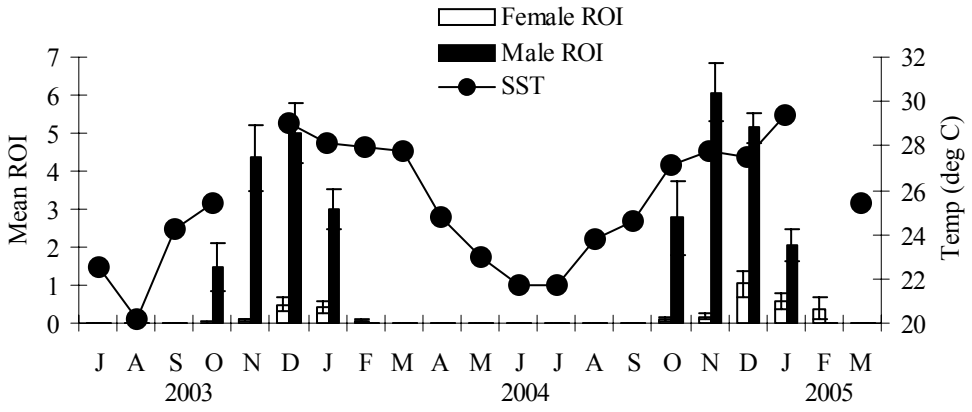


Fig. 2.7. Mean reproductive output index (ROI) (± 1SE) for both sexes of *R .odorabile* and corresponding sea surface temperatures during the study. Reproductive output index is equivalent to the proportions of propagules within 1 mm^2 of choanoderm. Breaks in SST data indicate missing data points (i.e. Nov 2003 & Feb 2005).

2.35 Size at sexual maturity

The size range for all sponges sampled (both reproductive and non reproductive) was 8 – 31,250 cm ^{3} (Fig 2.8). The smallest reproductive individuals were 176 cm^3 (11 x 8 x 2 cm) & 192 cm^3 (12 x 4 x 4 cm) for females and males respectively. The mean size of female reproductive sponges was 4,639 cm^3 (±850) (range 176 – 31,250). Reproductive males showed a mean size of 2,673 cm^3 (±417.9) (range 192 – 12,920).

For both female and male reproductive sponges there is no clear relationship between adult size and the reproductive output index (i.e. % propagules per mm^2) (Spearman's Rho - males 0.22 $p > 0.01$ females 0.38 $p > 0.01$). For example, there are clear examples of the smallest sampled males and females being as gravid, per mm^2, as the largest sampled sponges. In addition there is no significant relationship between size of oocytes in females and body size (Spearman's Rho – 0.06 $p > 0.01$). However, when total reproductive output is calculated for each reproductive sponge, to account for total body size and therefore total propagule production, there are predictable significant positive correlations of adult size and total reproductive output (Figs 2.9 and 2.10. Spearman's Rho – males = 0.81, $p < 0.01$; females = 0.64, $p < 0.01$).

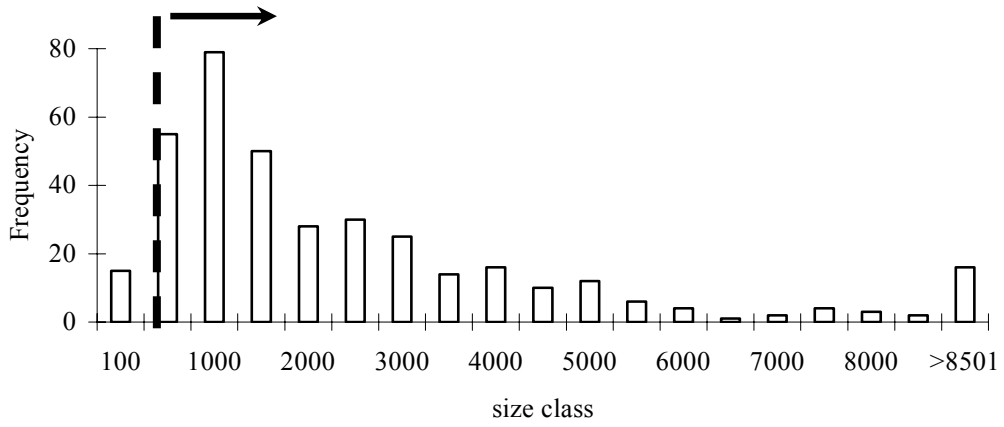


Fig. 2.8. Frequency histogram of sizes (cm^3) for all sampled *R.odorabile* individuals during September 2003 - March 2005. The smallest reproductive sponge was recorded at 176 cm^3. The dashed line and direction of arrow therefore indicates the threshold size class (i.e. > 500 cm^3) for reproductive individuals.

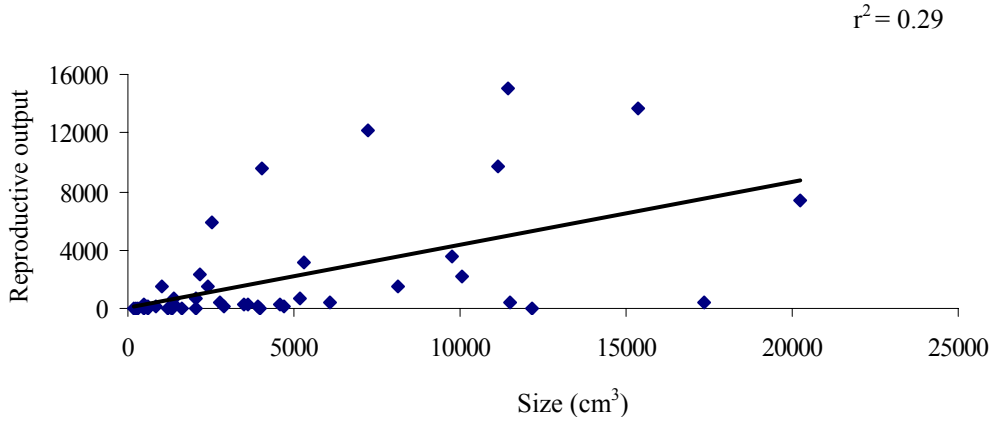


Fig. 2.9. *R.odorabile* females. Scatter plot of reproductive output versus sponge size. Reproductive output calculated from the ROI (% propagules mm^{-2}) and represents the density of propagules within the total sponge choanoderm.

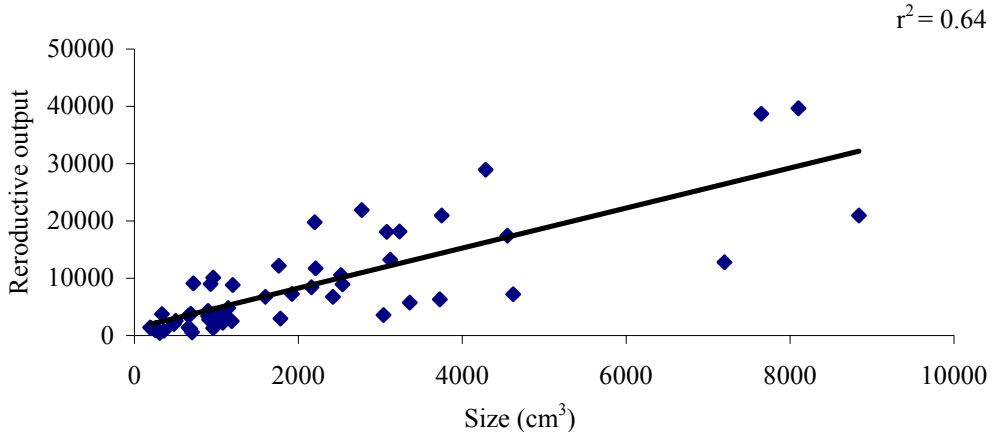


Fig. 2.10. *R.odorabile* males. Scatter plot of reproductive output versus sponge size. Reproductive output calculated from the ROI (% propagules mm^{-2}) and represents the density of propagules within the total sponge choanoderm.

2.4 Discussion

Seasonality and gametogenesis

Rhopaloeides odorabile is a gonochoristic, viviparous species with a distinct annual reproductive cycle. Females are reproductive for six months and males for four months. Gametogenesis coincides with rising SST during September (females) and October (males). Fertilisation and embryogenesis progress asynchronously from October until January the warmest months. The fate of unfertilised oocytes was also established with low numbers of unfertilised eggs being resorbed. Resorption of oocytes is previously reported in sponges (Gilbert 1974, Fell 1983), however this is the first record of oosorption in a Great Barrier Reef sponge. The degeneration of unfertilised oocytes late in the season suggests a threshold time period exists for the sponge to retain unfertilised eggs. Beyond this point the process of oocyte degeneration occurs. Whilst the trigger to resorb eggs is unknown the process of degeneration would suggest that the energetically valuable "yolk" is resorbed by the sponge presumably for use in

other metabolic processes. Larval release commences in January and finishes in late February, when SST begin to decline. Patterns of reproduction in marine invertebrates correspond well to environmental trends, with examples of regulation from sea temperatures, photoperiods, salinity, food resources, and lunar cycles (Harrison & Wallace 1990). For sponges, gametogenesis and spawning is reported to be regulated by SST in temperate (Usher et al. 2004), polar (Witte & Barthel 1994) and tropical environments (Fromont 1994b). However, few studies have documented whether reproduction is (co) regulated by other environmental effects. Fluctuations in photoperiod and rainfall (for species subject to the potential salinity fluctuations associated with riverine inputs) have been suggested as influential factors in determining reproductive cycles in sponges (Fromont 1994b). However, there is no rigorous experimentation that has clearly demonstrated whether one or a combination of effects is responsible for reproduction. Indeed it is still unclear whether there are also non-environmental regulatory effects initiated via endogenous pathways (review Fell 1983). For example, the finding that some sponge species are reproductive year round (Hoppe 1988), raises questions of whether non-environmental influences contribute to reproductive cycles. The seasonal trend of reproduction for *R. odorabile* clearly coincides with the warmer summer months with very clear boundaries of commencement and cessation of reproduction corresponding with rising and declining SST. It would be of interest to investigate if endogenous influences were contributing to reproductive cycles for *R. odorabile*, but with reproduction only occurring during the summer months it is more plausible that reproduction is environmentally determined. Whilst there is compelling evidence

of reproduction being regulated by SST in *R. odorabile* the effects of co-factors such as increased rainfall and photoperiods, also experienced during this time period, cannot be ruled out.

Sexuality and sex ratios

The results from 11 tagged sponges, coupled with the observation of gametes in the 181 reproductive individuals in this study, determine separate sexes in this species. Notably, single sex gametes were observed in tagged individuals during successive reproductive months for both seasons, ruling out either temporal or sequential hermaphroditism. In the present study we note that reproduction was not detected in all tagged sponges, an artefact of the random allocation of gametes in this sponge and the sampling strategy.

Rhopaloeides odorabile is gonochoristic and sex ratios in *R. odorabile* populations show a male biased ratio of 2.5:1. Sex ratios for sponges range from female biased to 1:1 (Fell 1976a; Ayling 1980), to male biased sex ratios (Fell 1976a). Unbiased sex ratios are the evolutionary stable allocation in randomly mating populations (Fisher 1930; Maynard Smith 1978). However, departures from sex ratio unity are reported for sessile marine invertebrates. For example, (Szmant 1986) suggests that female skewed sex ratios may be an evolutionary adaptation for brooding corals to overcome the physical constraints of brood space within a polyp. Skewed sex ratios may also be a response to overcome the challenges of fertilisation success faced by sessile, or slow moving, marine invertebrates, including proximity to mates (Sewell and Levitan 1992; Babcock and Keesing 1999), sperm limitation (Brazeau and Lasker 1992) and dilution of gametes (Oliver and Babcock 1992).

Reproductive output.

Female reproductive output in *R.odorabile* was consistent over both seasons studied showing densities of less than 1 propagule mm^{-2}. With the exception of *Haliclona ambionenesis*, *Niphates nitida* and *Haliclona loosanoffi* all reported fecundities for viviparous sponges show densities below 2 propagules mm^{-2}, with several recording densities below 1 propagule per mm^{-2} (Table 2.1). Not surprisingly, most sponges with higher levels of reproductive output are oviparous with some species investing up to 320 oocytes per mm^{-2} (Table 2.1). The comparative differences in reproductive output between viviparous and oviparous species presumably relate to parental care and consequential energetic costs associated with each reproductive mode.

Although the levels of reproductive output in *Rhopaloeides odorabile* are consistent with several other viviparous species, reproductive output can be influenced by strategies to accommodate investment for clonal reproduction, or as a response to environmental limitations. Sexual reproduction output can be compromised by investment into propagules via clonal reproduction (Corriero et al. 1998). For *R. odorabile* populations, clonal reproduction is considered unlikely, with genetic data suggesting no evidence of significant asexual reproduction (Whalan unpub data).

Reproductive investment for *R. odorabile* occurs for six months, commencing with oocyte production in September and larvae still being released in late February. Similar values of both reproductive output and length of time reproductive have been reported in other viviparous sponges (Ayling 1980, Hoppe 1988, Corriero et al. 1998). Species adopting extended or multiple spawning

periods reduce the risk of liberating larvae into unfavourable conditions (Ilan & Loya 1990). For *R. odorabile* the spawning window comprises approximately 6 weeks, greatly reducing the risks of releasing larvae into potential adverse conditions via one synchronised event.

Reproductive output in males increased gradually from October, peaking in December, which coincides with the time of maximum egg production in females. Reproductive output in *R. odorabile* showed a range of 4.6–14.4 spermatic cysts per mm^2, which compares well with reports for both viviparous and oviparous sponges (See Table 2.1). The asynchronous development of spermatic cysts may indicate multiple sperm-casting events for *R. odorabile* and is consistent with the asynchronous development of zygotes and larvae in females.

Size and sexual maturity

Although there is limited empirical data for growth rates in this species *R. odorabile* grows rapidly (Louden 2006) and sponges below 200 cm^3 have been observed between recruitment seasons (pers ob). The mean size of reproductive female sponges was 4,639 cm^3 so the finding that sponges as small as 176 cm^3 were reproductive suggests that sexual maturity is reached rapidly. Similar results were found in males. The smallest reproductive individuals in this study may therefore involve one-year-old recruits, and if this is the case, initial investment into biomass may not delay first reproduction. Investing in growth to reach a refuge size and delaying sexual maturity is a common strategy for sessile marine invertebrates and reduces mortality risks presumably associated with predation and competition (Babcock 1991). The small size of sexual maturation in *R. odorabile* therefore suggests a sufficient refuge size to combat mortality risks

associated with predation or overgrowth. Here, the strategy may be to achieve a sufficient refuge size to overcome juvenile mortality from incidental grazing, a process demonstrated for other sponge recruits (Maldonado & Uriz 1998). Alternatively this refuge may relate to a chemical defence. *Rhopaloeides odorabile* is rich in secondary metabolites with a proposed defensive role (Thompson et al. 1987). Whilst explanations have been provided as to the small threshold sizes of sexual maturity for this species, this study was not designed to address questions related to wider demographic issues. The complex dynamics between reproduction, growth and mortality is clearly an area for further study.

Interestingly, both males and females show no clear relationship for unit rates of fecundity to body size (i.e. propagules mm^{-2}). However, when total reproductive output is calculated (in accordance to total body volume) there are predictable, significant positive correlations for both sexes. This indicates that the smallest sponges produce equivalent rates of propagules per unit area as the largest individuals, but total reproductive output clearly increases with increasing body size. The positive increase of reproductive output with increasing body size supports the generalised paradigm that "fecundity" is proportional to body size (Williams 1975) as observed in other marine sessile invertebrates (Babcock 1991, Hall & Hughes 1996). For sponges there are conflicting patterns for the few reported studies available. For example, Uriz et al. (1995) found reproductive investment was positively correlated to size for *Crambe crambe* whilst other studies, have found no pattern, (Ayling 1980; Fromont 1994b).

In conclusion, this study has provided fundamental and timely data, on the reproductive biology, seasonality, mode of larval development and sexuality for a

common dictyoceratid Great Barrier Reef sponge. Although reproductive information for *R. odorabile* is consistent with many sponge species, there are also further questions which require investigation before our understanding of reproduction at the species level and within this phylum is complete. In particular, questions addressing size at sexual reproduction and trade offs between somatic and reproductive traits need more rigorous investigations. The variable patterns of reproductive output and seasonality demonstrated to date for sponges make generalisations of trends difficult and illuminate the complexity in reproductive ecology for Porifera.

Importantly, recognising the significance of reproductive strategies for benthic invertebrates will contribute to our understanding of the roles they play in larval dispersal, recruitment, and therefore adult distributions. The findings from this study constitute a fundamental first step in assessing aspects that determine larval supply and therefore adult distributions of *R. odorabile* populations. However, to fully address this question requires additional information on the role larval dispersal behaviour and subsequent recruitment processes play in determining adult distributions and is better inferred from investigations quantifying the larval ecology of *R. odorabile*.

Chapter 3: Reproductive Output Across a Water Quality Gradient

3.1 Introduction

"The life history favoured by natural selection maximises fitness, and this implies maximisation of fecundity and survival at all ages. The observed diversity in life histories suggests that there are constraints on what can be achieved in practice. Functional constraints occur if only certain combinations of age-specific fertility and survival are possible, either because of the physiology of the organism or because of the ***ecological impact of its environment***" (Partridge et al. 1991).

Life history theory is defined by a schedule of survival and reproduction with both components intuitively linked to fitness of individuals and populations (Bell 1980, Brommer 2000). A key limiting factor to organismal survival and reproduction is the availability and capture of resources to divert into growth and reproduction (Lechowisz & Blais 1988). As resources are limited there is differential allocation of resources to fundamental components (reproduction and growth) that ultimately determine fitness and this forms the conceptual framework of life history trait trade offs (Gadgil & Bossert 1970).

Sessile marine invertebrates can be adversely affected by habitats exposed to excess terrigenous derived sediment, nutrients, and pollutants (Rogers 1990, Fabricius & Wolanski 2000). Indeed, taxa exposed to the effects of excessive sediment often incur additional metabolic costs (Gerodette & Fleshig, 1979, Telesnicki & Goldberg 1995, Anthony & Fabricius 2000) that implicitly suggest a diversion of energy into survival or maintenance at the expense of other life history schedules such as reproduction and growth. Reduced levels of fecundity, fertilisation, settlement and recruitment have been reported for several coral species exposed to excessive sedimentation and nutrients associated with

terrigenous run-off (Fabricius 2005). Suspended particulate matter associated with sewage is also reported to impact reproduction in sponges (Roberts et al. 2006).

The sponge *Rhopaloeides odorabile* is found throughout the central GBR occurring on outer, mid and inner shelf reefs (Wilkinson & Cheshire 1989). The widespread occurrence of *R. odorabile* throughout the GBR exposes this species to variable environmental conditions, most notably water quality. The Great Barrier Reef is arbitrarily divided into the regions of inner, mid, and outer shelfs. Inner shelf reefs comprise a region adjacent to the coastline, with most reefs (and reef communities) exposed to the effects of terrigenous run-off (Devlin & Brodie 2005). Middle shelf reefs are sediment starved while outer shelf reefs are considered to be carbonate rich (Devlin & Brodie 2005).

Historically, inshore reefs of the GBR have been exposed to terrigenous run-off via river discharges, however increased agricultural development following European settlement has resulted in significant increases in levels of terrigenous run-off entering inshore waters (McCulloch et al. 2003). Furthermore, in association with increased levels of erosion of the fluvial flood plain are inputs of fertilisers (eg particulate nitrogen and phosphorus Devlin & Brodie 2005), herbicides pesticides and heavy metals (Haynes et al. 2000, Haynes et al. 2005, McMahon et al. 2005), which also contribute to the declining water quality of inshore waters. Importantly, the influence of terrigenous run-off and associated sediment, nutrients, and pollutants results in a water quality gradient across the shelf reef platform (Fabricius & De'ath 2001, Fabricius et al. 2005). The proximity of inner shelf reefs to the coastline exposes these reefs to terrigenous run-off, particularly during flood events in the Austral summer, with the

increasing distances of mid and outer shelf reefs from the coastline resulting in less exposure to the effects of terrigenous run-off (Devlin & Brodie 2005). Evidence of the deleterious effects of increased turbidity on coral reef organisms is compelling (review Fabricuis 2005). Reduced levels of coral biodiversity (Fabricius et al. 2005), reproduction and recruitment for hard corals (Gilmour 1999), and taxonomic phase shifts from hard coral to macroalgae communities is associated with increased turbidity (Hunter & Evans 1995, McCook 1999). The effects of increased turbidity on sponge biodiversity (Carballo 2006), growth and reproduction (Roberts et al. 2006) have been documented in a few limited studies. However, there is no data detailing the effects of increased turbidity on reproduction for GBR sponges.

Although the level of reproductive output for *R. odorabile* falls within the range reported for other viviparous species (Chapter 2) this is exclusively drawn from *R. odorabile* inhabiting inner shelf reefs. The inner shelf reefs at Pelorus Island are less than 20 kilometres from the coastline and are exposed to higher levels of turbidity associated with terrigenous run-off than mid and outer shelf reefs. This provides the opportunity to determine if the levels of reproductive effort of inner reef sponges are representative of the species, or an indication of populations exposed to environmental (water quality) conditions that characterise inner shelf reefs. In addition the variable habitat range of this sponge provides an opportunity to determine if inner shelf reefs provide suboptimal habitats, and indeed whether offshore populations are able to invest more in reproduction, and therefore exhibit reproductive fitness advantages. Using the fundamental understanding of reproductive biology of *R. odorabile* gained from Chapter 2, this

study used histological samples of reproductive sponges collected from habitats subject to different levels of water quality, at inner, mid and outer shelf reefs, to compare levels of reproductive output, sex ratios, proportions of sponges that are reproductive, and size at sexual maturity.

3.2 Materials and Methods

3.21 Study site

Terrigenous input from coastal river discharge results in a water quality gradient that diminishes with distance from the coastline. To determine reproductive output for *R. odorabile,* along this water quality gradient, sponges were sampled from locations at increasing distances from the coast. Specifically, samples were collected from sponges within the central GBR from (1) the inner (coastal) fringing reefs of Pelorus Island, (2) mid shelf reefs at Rib Reef and (3) outer shelf reefs at Pith Reef (Fig 3.1). Pelorus Island is approximately 17 kms from the coastline, Rib Reef 35 kms and Pith Reef 104 kms.

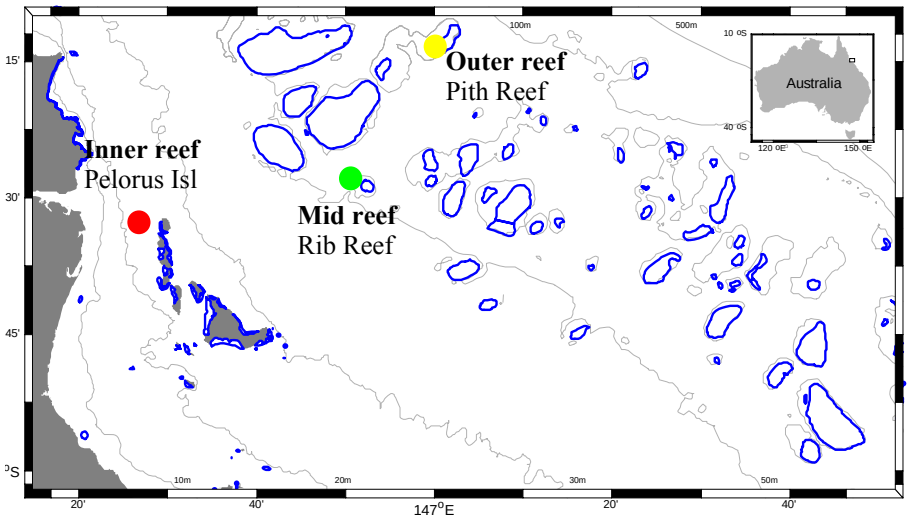


Fig. 3.1. Location map showing collection sites of *R. odorabile* for reproductive ecology comparisons. Pelorus Island represents the inner shelf, Rib Reef represents the mid shelf, and Pith Reef indicates outer shelf collections.

3.22 Collection and preservation of samples

To describe the reproductive biology and to quantify reproductive output of *R. odorabile,* samples were collected on three separate occasions (November 2005 December 2005 and January 2006) which coincides with the months *R. odorabile* is most reproductive (Chapter 2). To quantify reproductive output, numbers of reproductive sponges and sex ratios, samples were collected from 30 haphazardly chosen sponges each collection period using SCUBA. This provided a total sample size of 90 for each location. To avoid repeated sampling from the same individual wedge shaped samples were cut and collected from each sponge. Sponges with wedge shaped cuts then represented previously sampled sponges and were avoided.

Because sponges do not have designated gonads, and gametes and larvae occur throughout the mesohyl (Bergquist 1978), samples were randomly collected from each sponge. A pilot study (Chapter 2) was undertaken to validate this sampling strategy. One section was taken from the surface (i.e. the first 5 cms) and another from the remainder of the sponge (i.e. central or basal layers) of several reproductive individuals (n = 30) to establish whether reproduction varied between different regions of the sponge. There was no significant difference of propagule numbers between different regions of sponge (Paired sample t –test $p > 0.05$ - both male and female). Removing small parts of sponge did not damage or interfere with sponge survival with evidence of regeneration of the cut surface taking place within a few days, and a new pinacoderm established within one month. To enable comparisons between size and various reproductive measures, each sampled sponge was measured along the longest axis of length, height and width to determine size.

Following the field collection of samples, sub samples 4-5 mm thick were excised and placed directly into histology cassettes and then fixed in the gonad fixative, FAACC [1 litre = 100 ml formaldehyde (40%), 50ml glacial acetic acid, 13 gm calcium chloride dihydrate and 850 ml of tap water (Fromont 1999)] pending histology.

3.23 Histology and visualisation of reproductive propagules

To visualise reproductive propagules with light microscopy samples were processed using standard histological techniques. Each sample was processed with an automated tissue processor using an alcohol dehydration and xylene clearing system followed by paraffin impregnation under vacuum. Samples were then blocked into paraffin and cut with a microtone at 5μm. To facilitate cutting the tough skeletal fibres, and to provide intact sections, the surface to be cut was soaked in a solution of 10 % ammonia on ice for 10-15 minutes prior to cutting. Tissue sections were stained with haematoxylin and eosin.

3.24 Quantifying reproduction

Each sample was observed with a light microscope to identify reproductive propagules. Because there are no designated gonads in sponges, and the development of gametes and embryos can occur throughout the sponge, it is difficult to use conventional measures of reproductive output associated with gonad structures. To establish reproductive output in *R.odorabile* a relative index of reproductive output (ROI) was used. A Leica DC image acquisition camera, mounted onto a light microscope, was used to capture digital images of reproductive propagules within slide tissue sections. Leica IM50 software was then used to calculate diameters and surface area measurements of reproductive

propagules. The surface area of the entire slide tissue section was also determined. The ROI was then calculated as the combined percentages of reproductive propagules occupied within the whole tissue sample area. Reproductive propagules were classified as oocytes, embryos or larvae in females, and spermatic cysts in males. The comparatively low numbers of propagules in females enabled measurement of all propagules within the slide section. In males however, the number of gametes within each tissue section were substantially higher. Therefore, the surface areas of spermatic cysts from three separate microscope fields of view were calculated, and a mean value determined. The field of view is equivalent to $1mm^2$. Importantly, for both sexes, ROI is a per unit rate estimate of the proportion of tissue comprising propagules (% propagules per mm^{-2}).

Because some sections resulted in propagules not being sectioned medially ROI could potentially underestimate reproductive output. Therefore, total numbers of propagules were also recorded and plotted against their respective ROI to assess the correlation between the two estimates. This is an approximate measure of the confidence of the ROI estimate, and as demonstrated in Chapter 2 provides an informative and valid measure of reproductive output. Both ROI and numbers of gametes were recorded in this study and both show similar results in estimating reproductive output for sponges from the three locations examined (see results). Given the accuracy of ROI, as validated by propagule numbers, the discussion of reproduction focuses on ROI.

For each collection period (November, December and January) the diameters of 50 randomly selected oocytes and spermatic cysts from reproductive

sponges at inner, mid, and outer reef sites were measured to establish if there were differential rates of gametes size production.

3.25 Statistical analysis

Statistical analysis was carried out using reproductive individuals only. Differences between numbers of reproductive sponges among sites (inner, mid and outer reefs), and months (Nov, Dec, and Jan) were determined using Chi-square (contingency table) analysis. Two-way ANOVA (location and time, both fixed factors) was used to detect differences in reproductive output between sponges on the inner, mid and outer reefs and time of collection for ROI, numbers of propagules and, sizes of oocytes and spermatic cysts. Tukey's (HSD) post hoc tests were used to establish where significant differences occurred. Tests for homoscedascity and normality of data were validated prior to performing ANOVA. Data were log transformed for sizes of gamete data, for each sex, to satisfy the assumptions of homoscedascity. To test for departures from unity sex ratios, Chi-square goodness of fit tests (Pearson's) were performed. The measure of variance associated with the reporting of mean values is 1 standard error.

3.3 Results

3.31 Numbers of reproductive individuals

Of the 270 sponges sampled 151 individuals were reproductive, equating to 62, 53 and 36 sponges at Pith, Rib and Pelorus Island respectively (Fig 3.2). There was no significant interaction between month and sites in determining numbers of reproductive male or female sponges (χ^2 = 1.12, df = 4, $p > 0.05$). However, numbers of reproductive sponges (males and females combined) did vary in accordance to both of the main effects of site and month (site: χ^2 = 6.93, df

= 2, p = 0.03. month: $\chi^2 = 10.82$, df = 2, p < 0.01). Furthermore, when each sex is considered separately, numbers of reproductive females were consistent across sites ($\chi^2 = 0.94$, df = 2, p = 0.63) but varied among months ($\chi^2 = 15.98$, df = 2, p < 0.01), whilst numbers of reproductive males varied among sites ($\chi^2 = 8.08$, df = 2, p = 0.02) but were similar among months ($\chi^2 = 1.75$, df = 2, p = 0.42).

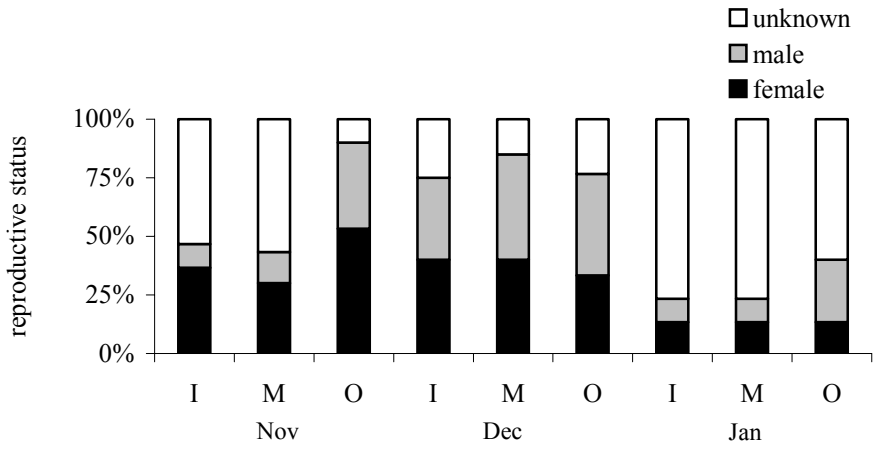


Fig. 3.2: Proportions of female, male, (and unknown) reproductive sponges at each site sampled on the central sector of the GBR. I is Inner shelf reef (Pelorus Island), M is Mid shelf reef (Rib Reef) and O is Outer shelf reef (Pith Reef).

3.32 Gamete size comparisons across locations

There was significant variation in oocyte diameters among locations (Table 3.1, Fig 3.3). For example, in December mean oocyte diameters were 156.92 μm (±9.4) for inner reef sponges, 203.79 μm (±8.71) for mid reef sponges, and 195.6 μm (±8.9), for outer reef sponges. Tukey's post-hoc test show significantly smaller oocytes for inner reef sponges than both mid (p = 0.04) and outer reef sponges (p = 0.03). In December mean diameters of spermatic cysts did not vary among locations showing 43.4 μm (±1.06), 40.5 μm (±0.94), and 42.7 μm (±1.24), for inner, mid and outer shelf reefs respectively.

Table 3.1. Summary results of two-way ANOVA to detect differences between sizes of gametes for both sexes among inner, mid and outer reef sponges and time of sampling.

	Oocytes				Spermatocytes			
Source	df	MS	F	p	df	MS	F	p
Site	2	19781	4.4	0.01	2	19.01	0.31	0.73
Time	1	7832	1.7	0.19	1	48.36	0.80	0.37
Site x Time	2	13942	3.6	0.05	2	233.82	3.85	0.02
Error	296	4547			294	60.68		

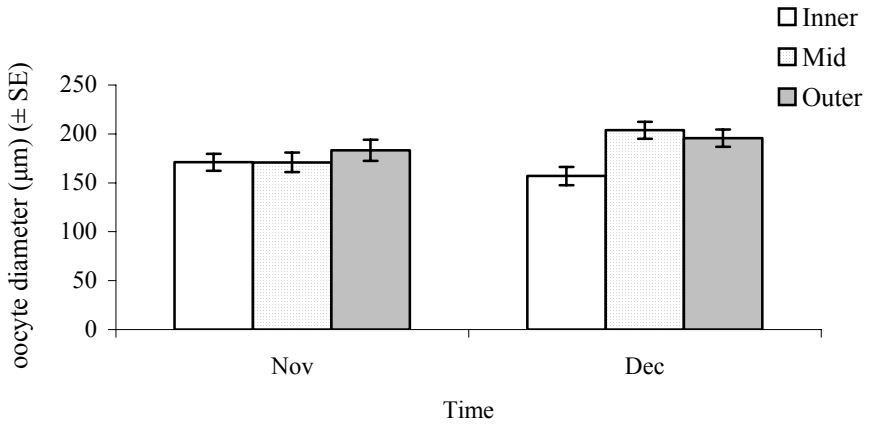


Fig. 3.3. *R.odorabile*. Mean oocyte diameter (± 1SE, n = 50 per site/month) for reproductive female sponges from inner, mid and outer reef sponges of the GBR in November and December. Inner is Pelorus Island, Mid is Rib Reef and Outer is Pith Reef.

3.32 Reproductive effort

Similar trends of reproductive output among locations were quantified for female ROI and number of propagules mm^{-2} (Fig 3.4 a & b). Consistent with the findings in Chapter 2 strong and significant correlations are apparent when both measures of reproductive output are compared (Females: inner $r^2 = 0.85$, $p < 0.01$; mid $r^2 = 0.96$, $p < 0.01$; outer $r^2 = 0.96$, $p < 0.01$. Males: inner $r^2 = 0.94$, $p < 0.01$; mid $r^2 = 0.97$, $p < 0.01$; outer $r^2 = 0.99$, $p < 0.01$), confirming ROI as valid measure of reproductive output. Whilst results are presented for both measures of reproductive output, ROI will form the focus of discussion in this study.

Female reproductive output both as a measure of ROI and number of propagules mm^{-2} varied significantly among locations (Fig 3.4 a & b, Table 3.2). Specifically, a gradient increase of female reproductive effort occurs across the shelf reefs with mid and outer reef sponges being up to 15 times more reproductive (e.g. Dec mean ROI; mid = 2.4 ±0.5, outer = 2.5 ±0.4) than sponges on inner reefs (Dec mean ROI = 0.16 ±0.1). Tukey's post-hoc test demonstrate ROI is significantly lower in sponges on inner reefs than sponges on mid ($p = 0.01$) or outer reefs ($p < 0.01$), although both mid and outer reef sponges show similar levels of ROI. In addition, higher levels of mean female ROI occur between December and January at all locations which is likely to be a reflection of size increases associated with developing embryos and larvae during this period.

Male reproductive output also showed similar results for both ROI and number of propagules mm^{-2}, although there were contrasting results for (time x site) interactions between the two measures (see Table 3.3). In contrast to female reproductive effort the level of male investment into reproduction (ROI) did not vary according to location (Fig 3.5 a & b, Table 3.3).

Table 3.2. Summary results of two-way ANOVA to detect differences between female ROI and, propagules per mm^{-2} among inner, mid and outer reef sponges and time of sampling.

		ROI				Propagule mm^{-2}		
Source	df	MS	F	p	df	MS	F	p
Site	2	8.99	7.06	0.002	2	0.05	2.24	< 0.01
Time	2	4.44	3.49	0.04	2	0.31	12.34	0.12
Site x Time	4	1.27	1.00	0.42	4	0.05	2.13	0.80
Error	61	1.27			61	0.03		

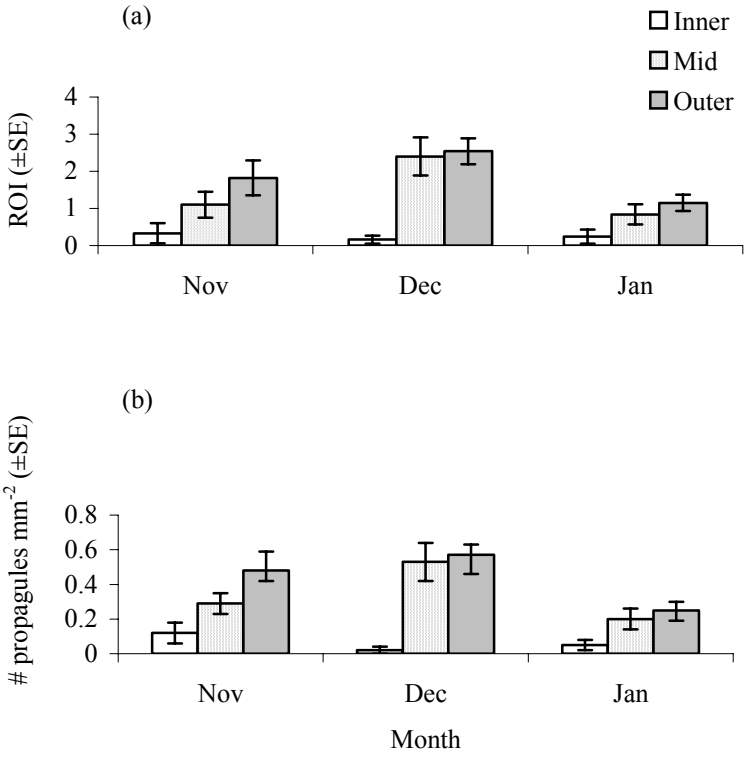


Fig. 3.4 (a & b). *R.odorabile* Female reproductive output. (a) Mean reproductive output index (ROI) for sponges during Nov-Dec 2005/06 and (b) mean number of reproductive propagules mm^{-2} for each of the locations sampled on the central section of the GBR. Inner is Pelorus Island, Mid Shelf is Rib Reef and Outer is Pith Reef.

Table 3.3. Summary results of two- way ANOVA to detect differences between male ROI and propagules (spermatic cysts) per mm^{-2} among inner, mid and outer reef sponges and time of sampling.

		ROI				Propagules mm^{-2}		
Source	df	MS	F	p	df	MS	F	p
Site	2	3.09	15.42	0.47	2	3.88	16.67	0.71
Time	2	0.15	0.77	<0.01	2	0.08	0.35	<0.01
Site x Time	4	0.34	1.71	0.16	4	0.79	3.41	0.01
Error	68	0.2			70			

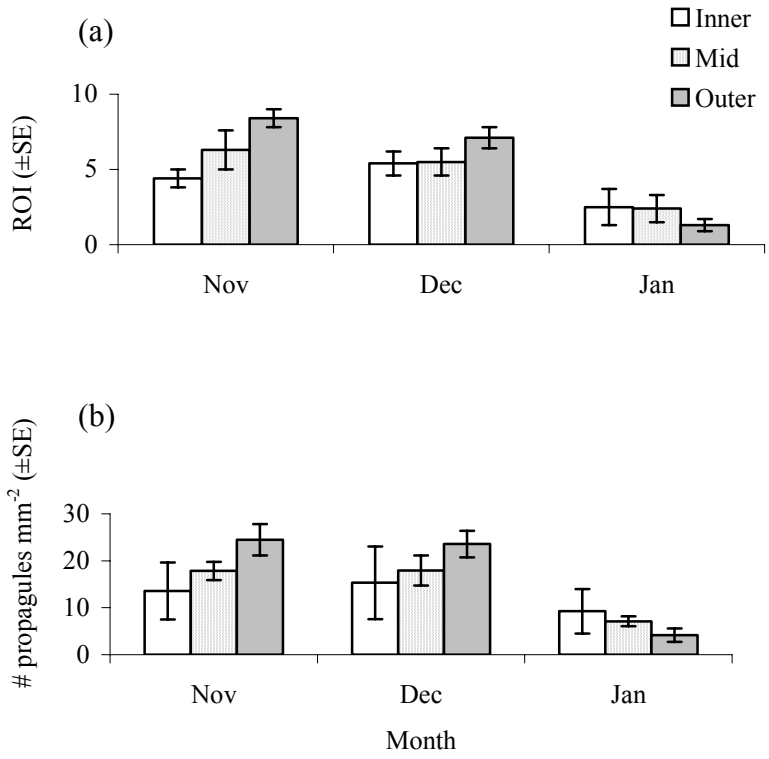


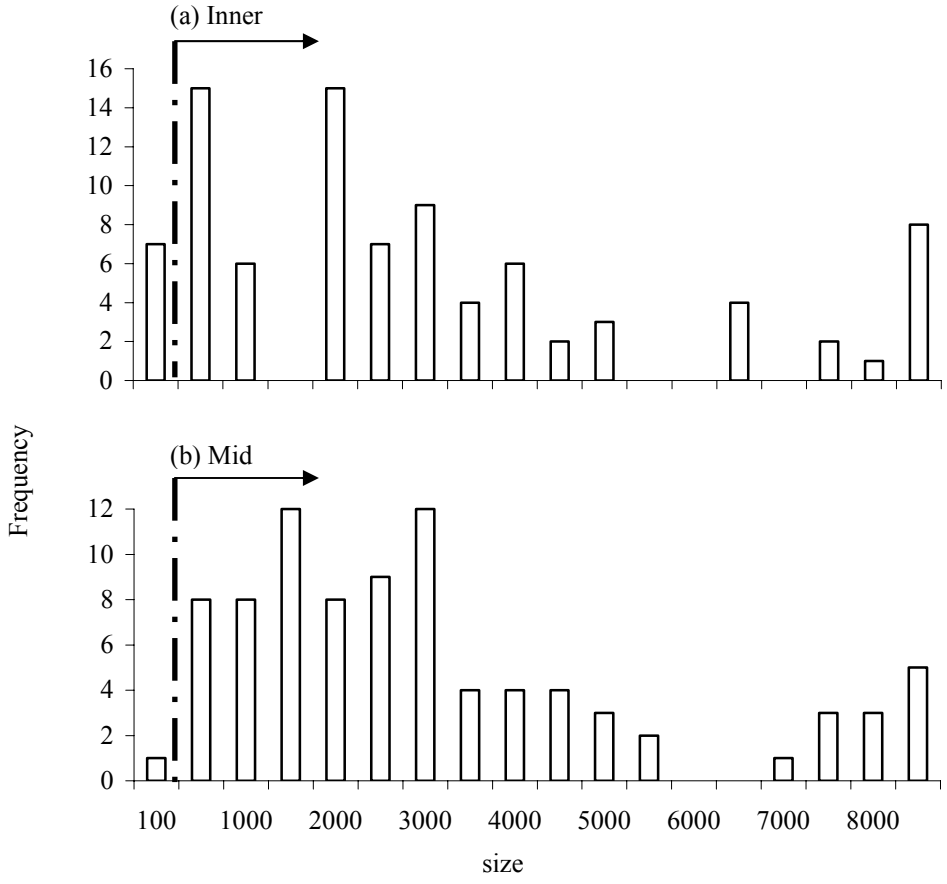
Fig. 3.5 (a & b). *R.odorabile*. Male reproductive output (a) Mean reproductive output index (ROI) for sponges during Nov-Jan 2005/06 and (b) mean number of propagules (spermatic cysts) mm^{-2} for each of the locations sampled on the central section of the GBR. Inner is Pelorus Island, Mid is Rib Reef and Outer is Pith Reef.

3.34 Size at sexual maturity and sex ratios

The size range for all sponges sampled (both reproductive and non reproductive) was 18-17,112 cm^3 for inner reefs sponges, 32-19,840 cm^3 for mid reef sponges and 54-16,576 cm^3 for outer reef sponges (Fig 3.6 a-c). The smallest female reproductive individuals were 18cm^3, 32cm^3, and 54cm^3 for inner, mid and outer reef sponges respectively. The smallest males were 18 cm^3 (inner reef), 32 cm^3 (mid reef) and 180 cm^3 (outer reef). The mean size of female reproductive sponges was 3,392 cm^3 (±1,466) for inner reef sponges (range 200-13,865 cm^3), 4,521 cm^3 (±1,053) for mid reef sponges (range 576-27,404 cm^3) and 3,981 cm^3

(±813) for outer reef sponges (range 54-11,286). Reproductive males measured 4,251 cm^3 (±1,171) for inner reef sponges (range 18-18,000), 3,361 cm^3 (±536) for mid reef sponges (range 32 - 9,040) and 2,526 cm^3 (±746) for outer reef sponges. (range 180 - 8, 262)

Male to female sex ratios equate to 1.9:1 (inner reef), 1.2:1 (mid reef) and 1.1:1 (outer reef). Inner reef sponges have male biased sex ratios with a significant departure from unity sex ratios (Pearson's χ^2 = 6.08 with Yates correction for 1 df, $p < 0.05$). In contrast, sex ratios for both mid and outer reef sponges have approximate equal sex ratios that do not significantly vary from unity.



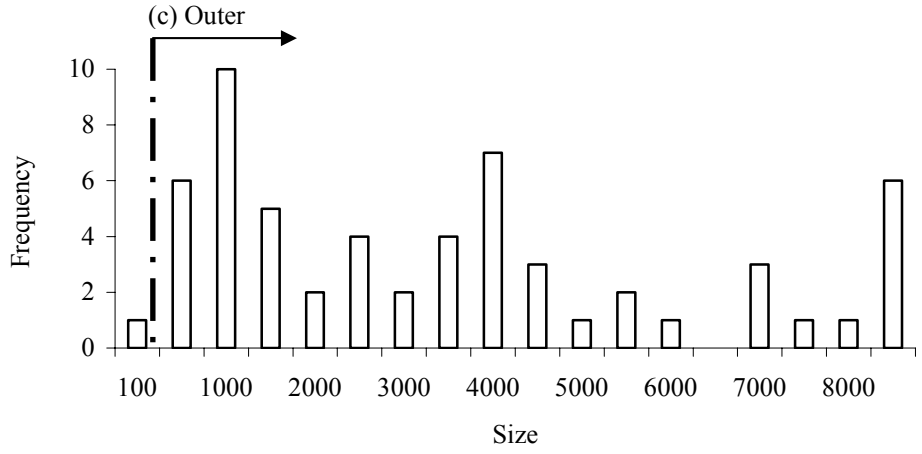


Fig. 3.6 (a-c). *R. odorabile* Size frequency histograms (cm^3) for all sampled sponges during Nov-Jan 2005/6 on the central sector of the GBR. Dashed line and direction of arrow indicates threshold reproductive size class (i.e. 500 > cm^3). (a) Inner is Pelorus Island, (b) Mid is Rib Reef and (c) Outer is Pith Reef.

3.4 Discussion

The key finding in this study was that there is a clear gradient of female reproductive effort across the shelf reefs with reproductive output increasing with increasing distance from the coastline. Female reproductive effort for *R. odorabile* varied significantly across the GBR on two levels. Firstly, sponges from the outer and mid reefs have an ROI of up to 15 times more than inner shelf reef sponges. Secondly, in conjunction (and co-related) to the reproductive output are increasing sizes of oocytes from inner to mid and outer reefs. Therefore, both increased oocyte size, and numbers contribute to a much higher reproductive output. Although egg quality was not measured, increased egg size confers a greater access to resources and/or energy to allocate to reproduction. Positive correlations between egg size and energy have been demonstrated for marine invertebrates. Larger eggs provide longer facultative feeding potential (McEdward & Morgan 2001, Miner et al. 2005), and presumably advantages for dispersal and settlement.

Environmental (resource availability, Lechowisz & Blais 1988) and

ecological impacts (competition, Tanner 1997, Uriz et al 1995) can impose differential directions of energy allocation into components of reproduction and growth. Microhabitat influences can regulate energetic allocations between defence and reproduction in sponges, with reproductive output decreasing when exposed to habitats with high levels of competition, suggesting energy is allocated to growth and reproduction unless faced with an event that requires a diversion of energy to maintain survival (Uriz et al 1995). The lower levels of reproductive output in *R. odorabile* females on inner reefs may therefore be a result of energy being allocated to other components of fitness (i.e. maintenance or survival at the expense of reproduction). Moreover, it is highly likely that the environmental microhabitat differences between the inner, mid, and outer reefs, in particular water quality and sedimentation, contribute to the reproductive output differences.

Turbid inner shelf reefs are commonly associated with coastal tropical environments, a result of particulate matter associated with terrigenous river run-off. Whilst inner shelf reefs of the GBR support a diverse coral reef community (Fabricius et al. 2005), the levels of sedimentation, nutrients and pollutants associated with terrigenous run-off have intensified with agricultural development (Haynes & Michalek-Wagner 2000, McCulloch et al. 2003). Excessive terrigenous run-off can have sub-lethal, and lethal consequences, for many coral reef taxa exposed to the shadow of terrigenous run-off plumes (Rogers 1990, Airoldi 2003). The effects of particulate sediment on filter feeding sessile invertebrates can be twofold. Firstly, suspended sediment can impact filter feeding organisms through the incidental intake of silt associated with water (Gerrodette & Flechsig 1979, Naranjo & Carballo 1996). Secondly, excess sediment leads to

the blockage of inhalant canals inhibiting feeding (Reiswig 1971, Gerrodette & Flechsig 1979). In addition, deposition of sediment can lead to burial of organisms (Wulff 1997) or increased metabolic drains to remove sediment (Telesnicki & Goldberg 1995). Therefore, for inner reef *R. odorabile* sponges it is proposed that conditions of turbidity require a higher degree of maintenance, and when faced with this energy drain other components such as reproduction are compromised. Turbidity associated with nutrients and suspended sediments from terrigenous-based run-off have been implicated in reduced fecundity, fertilisation, gametogenesis, embryonic development and larval survival for corals (review Fabricuis 2005). The effect of siltation associated with sewage outfall also reduces growth (weight) and reproductive activity in the sponge *Cymbastela concentrica,* through a combination of burial and clogging of inhalant canals (Roberts et al 2006). That *R. odorabile* occurs throughout the inner shelf reefs (Wilkinson & Cheshire 1989), but is still reproductive within these environments (see Chapter 2), suggests that the environmental conditions do not pose lethal impacts. However, the significantly lower investment of female reproduction at inner shelf reefs indicates these habitats are likely to be suboptimal when compared to mid and outer reefs.

In stark contrast to the trend of differential reproductive investment found in *R. odorabile* females was the finding that male reproductive output showed no significant variation between inner, mid and outer reef sponges. The conflicting patterns of reproductive effort between the sexes of *R.odorabile* may simply be a reflection of the different energy requirements to produce sperm or eggs (i.e. sperm are energetically cheaper to produce than eggs, Bateman 1948; Hall &

Hughes 1996.). Therefore, the question remains as to whether the environmental conditions at inner reefs are at a suboptimal threshold for reproduction in *R. odorabile* females only.

Sex ratios for *R. odorabile* also show differences across the shelf reefs, with a male dominated sex ratio for inner reefs sponge samples compared to approximate equal sex ratios for both mid and outer reef sponges. The finding of male dominated sex ratios of 1.9:1 at inner reefs in the present study is consistent with Chapter 2, although conducted with larger sample sizes and over two reproductive seasons. Nevertheless, these results may be indicative of the different environmental regimes with outer reefs providing optimum conditions. Equal sex ratios are purported to be the most evolutionary stable allocation (Fisher 1930) and comply with the theoretical expectations for optimal resource allocation in randomly mating populations (Williams 1975, Maynard Smith 1978).

Finally, consistent results of small sizes at sexual maturity were found at each location, in agreement with Chapter 2. Investing in growth to reach a refuge size and delaying sexual maturity is a common strategy reducing mortality risks associated with predation and competition (Babcock 1991).

In conclusion, there are marked differences in levels of reproductive output for *R. odorabile* across the shelf reefs of the central GBR. Whilst these results demonstrate reproductive output differences between sponges that are clearly occupying different microhabitats, it is difficult to conclusively determine a causal relationship between turbidity and reproductive output without appropriate experimental tests. Nevertheless, the turbid conditions of the inner reefs are highly likely to be a contributing factor to reduced reproductive effort for

inner shelf reefs. Indeed, given that reproductive output increases (in accordance with a corresponding increase in water quality) with increasing distance from the coast further reinforces this proposal. These findings highlight the necessity of examining reproduction across the spectrum of environmental conditions available to conspecifics to fully establish a complete understanding of reproductive variability, and ultimately larval supply.

Chapter 4: Larval Dispersal and Settlement

4.1 Introduction

Elucidating patterns of larval dispersal, settlement and recruitment of marine organisms is fundamental to our understanding of adult distributions. This is particularly relevant for marine sessile invertebrates with bi-partite life histories consisting of mobile larvae and sedentary adults. Moreover, larval dispersal for sedentary marine invertebrates has significant ecological and evolutionary implications providing both adaptive advantages and disadvantages (see review (Pechenik 1999, Strathmann et al. 2002).

Larval dispersal can be influenced by both oceanic processes (Sponaugle et al. 2002) and intrinsic biological traits, including larval competencies (Miller & Mundy 2003, Nozawa & Harrison 2005), swimming ability (Metaxas 2001), and vertical migration behaviours (Raimondi & Morse 2000, Queiroga et al. 2002). Importantly, the poor swimming abilities observed in many marine invertebrate larvae make directed horizontal dispersal challenging, particularly into opposing currents (Chia et al. 1984, Davis & Butler 1989). Therefore, the ability of larvae to migrate vertically underpins dispersal potential because it positions larvae in bodies of water subject to different flow regimes (Young 1995).

Beyond the processes contributing to wide ranging dispersal are finer scale habitat influences mediating settlement, recruitment and ultimately adult distributions. Successful recruitment, in part, relies on larvae identifying favourable habitats to settle. Therefore, for many invertebrate larvae, settlement is often in response to environmental cues signalling favourable habitats.

Light, salinity, temperature, pressure and gravity all contribute to the settlement process (Young 1995, Underwood & Keough 2000, Maldonado 2006). In addition, cues associated with physical surfaces and chemicals affect larval settlement. Surface micro-topography can provide settlement adhesion points (Verran & Boyd 2001) and micro refuges (Maldonado & Uriz 1998, Peterson et al. 2005). Chemical cues have been widely investigated for marine invertebrate larvae and include cues associated with biofilms of micro-organisms (Pawlik 1992, Hadfield & Paul 2001, Negri et al. 2001, Huang & Hadfield 2003), conspecifics (Raimondi 1991, Head et al. 2004), and host organisms (Swanson et al. 2004).

Larval settlement in many sessile marine invertebrates is well documented, however holistic approaches quantifying the entire pre-settlement phase from larval release to recruitment are rare (Harrison & Wallace 1990, Raimondi & Morse 2000). There are fewer studies demonstrating these processes in other conspicuous benthic taxa such as sponges (but see Maldonado & Young 1996; Maldonado & Uriz 1998; Maldonado 2006). This is surprising given their remarkable biodiversity estimated at 15,000 species (Hooper & Levi 1994) and their global distributions in benthic ecosystems.

Genetic data suggests limited dispersal abilities for sponge larvae (Duran et al. 2004a, Whalan et al. 2005) with relatively few species exhibiting wide scale dispersal (Lazoski et al. 2001). The duration of pre-settlement stages for sponge larvae are generally short ranging from one hour to several days (Ayling 1980, Maldonado & Young 1999). Furthermore, sponge larvae have poor swimming abilities and crawling is often observed (Bergquist & Sinclair 1968, Battershill &

Bergquist 1990, Woollacott 1993, Maldonado & Young 1996). Therefore, restricted dispersal of many species may be a result of short larval competencies coupled with poor motility.

Comprehensive settlement behaviours for sponges are few and there is a clear need to develop knowledge in this field (as highlighted by Maldonado 2006). Studies undertaken to date indicate depth regulation (Uriz et al 1998), phototaxis (Leys & Degnan 2001, Maldonado et al. 2003), and surface micro-refuge characters (Maldonado & Uriz 1998), to be important for successful sponge larval settlement. In addition some species respond to cues associated with micro-organism biofilms before successfully settling (Keough & Raimondi 1995).

Rhopaloeides odorabile (Thompson et al. 1987) is a tropical dictyoceratid sponge commonly occurring within the central GBR, Australia (Wlkinson and Cheshire 1989). This species is viviparous and sexually reproductive from September to February and larvae are dribble spawned during January and February (Chapters 2 and 3). Using manipulative laboratory experiments this study examined the behavioural characteristics of *R.odorabile* larvae from release to settlement and metamorphosis. Specifically, the vertical migration, phototactic responses and swimming abilities of larvae are determined. In addition settlement responses to cues associated with physical surface topographies, the degree of light exposure, and surfaces associated with biofilms and dead coral rubble are quantified.

4.2 Materials and Methods

4.21 Study site and sample collection

Larvae from the sponge *Rhopaloeides odorabile* were collected from the

fringing reefs off Pelorus Island (146° 29.58'E, 18°33.48'S) on the central GBR. Experimental manipulations were conducted at Orpheus Island Research Station (OIRS) over two spawning seasons in January 2005 & 2006.

Larvae were collected using larval traps on a modified design from (Lindquist et al. 1997) (Fig 4.1) which were placed over sexually mature sponges and checked once in the mid morning and then 4-6 hours later in the mid afternoon. *Rhopaloeides odorabile* is an afternoon dribble spawner (Chapter 3). Therefore, afternoon collections of larvae were used in all experiments to provide larvae that could be reliably aged. Following collections, larvae were transported to OIRS to examine pre-settlement larval mobility and settlement behaviour. Occasional difficulties in reaching field sites due to bad weather required the collection of larvae from several sponges maintained in flow through aquaria at OIRS. For the purpose of this study settlement and metamorphosis are assessed independently. A successful settlement event is defined as a larva attached to the substrate by its anterior pole. If agitation of the dish did not dislodge the larvae it was recorded as settled. Metamorphosed larvae had undergone clear metamorphic changes characterised by a flattened body plan. Each is defined separately.

Fig. 4.1. Larval trap that is placed over adult sponges used to collect larvae.

4.22 Larval behaviours

The general patterns of larval behaviour were initially qualitatively assessed over thirty hours by recording the majority response behaviours (i.e. > 50%) of twenty newly released larvae (i.e. less than two hours old) in a 100 mm Petri dish filled with 25 µm filtered seawater (FSW). Qualitative measures of behaviours were recorded for phototactic responses, mobility (categorised as swimming or corkscrewing), timing of substrate exploration and (or) temporary attachment, settlement, and metamorphosis. Observations were recorded for each behavioural characteristic every hour.

4.23 Vertical orientation in the water column over time

Qualitative observations (see methods & results - Larval behaviours) demonstrate *R.odorabile* larvae are phototactic during their pre-settlement phase. Therefore, manipulative experiments were designed to test whether larvae use vertical migration and if they do whether light mediates larval vertical migration. Experiments were based on methods modified from Uriz et al (1998). Fifty larvae

were placed in an experimental chamber consisting of a 1000 ml graduated glass cylinder filled with FSW. To remove bias associated with the placement of larvae to surface layers and initial static conditions, the tube was gently agitated at the commencement of the experiment. The cylinder was separated into three equal zones (top, middle and bottom). The positions of active larvae within each zone were recorded every 6 hours until larvae settled or died. To assess whether light influenced the position of larvae within the experimental chamber (i.e. vertical migration) one treatment exposed to a light cue (n = 6), and one excluding light (n = 6) was used. For both treatments, water temperature was maintained at ambient sea water temperatures by placing the cylinders in 1000 litre water baths supplied with flow through seawater. Observations at night (for the light cue treatment) or for the "without light cue" treatment required the use of a filtered red light torch to view and count larvae. Exposure to light was minimal and assumed to not affect larval behaviour for that time period record.

Treatment with a light cue

The first treatment exposed larvae to natural light cues by placing the experimental chambers (and larvae) in an outdoor field laboratory subject to natural photoperiods. To explicitly determine the effect of light on vertical migration larval behaviours this treatment was compared to a separate treatment with no light stimulus and as described in the following section.

Treatment without a light cue

A second treatment assessed whether light was a contributing cue to vertical migration behaviours. Here the premise was that if light was important in regulating positions within the water column, then removal of light should result

in a random distribution of larvae throughout the water column. To test this, experimental chambers were covered with black plastic so no light cue was available to larvae. However, larvae used in this treatment were held in larval traps for up to 2 hours before they could be collected, and therefore initial exposure to light was an unavoidable artefact in this treatment.

4.24 Swimming ability

Initial observations showed that larvae did not exhibit sustained swimming for more than twenty-four hours. Therefore, quantitative measurements were conducted for twenty-four hours. To quantify swimming speeds larvae were placed in a small glass aquarium (50 cm x 5 cm x 5 cm) with a 1x1 square cm grid superimposed to measure the swimming rate (secs cm^{-1}) for each larva over distances of up to 20 cms. Swimming speeds of individual larvae were recorded at 6, 12, 18 and 24 hours (n = 20 larvae for each time period). To avoid repeated measurements monitored larvae were removed following measurements.

4.25 Larval buoyancy

Given buoyancy may contribute to vertical migration passive buoyancy was tested over time. Larvae (n = 20) were euthanased at 0, 6, 12 and 24 hours post release by placing them into a solution of 10% phosphate buffered formalin followed by placement into a 50 ml graduated cylinder filled with seawater and recording whether they sank or floated.

4.26 Settlement and recruitment choices

Gregarious settlement

Settlement assays using potential cues were undertaken to assess influences on larval settlement. To reduce the risk of misinterpreting results

associated with gregarious settlement responses, an experiment was firstly undertaken to determine the influence of aggregative settlement. Larvae were placed into 5 ml Petri dishes containing 25 μm FSW at densities of 1, 2, 5, 10, 25 and 50 larvae (n = 4) and settlement rates were determined every 6 hours for 30 hours. Larval settlement was not significantly influenced by densities of larvae (repeated measures ANOVA, between subjects, $F_{5, 18} = 1.39$, $p > 0.05$). Therefore, settlement assays were conducted with 10 larvae per replicate sample with any significant effects detected being treatment rather than gregarious effects.

Settlement cues

To determine the behavioural response of larvae to different settlement substrates two series of experiments were undertaken. The first tested the settlement responses to shade and light exposed surface, surface micro-topography and biofilms. The second investigated the settlement response of larvae in the presence of potential chemical cues.

Settlement in response to light, surface micro-topography and biofilms.

PVC tiles (10 cm x 10 cm) were used as settlement substrata. To assess larval responses to micro-refuge exploitation three treatments were investigated; (1) 2-3 mm wide x 2 mm deep grooves were cut into the surface of the tile at equal intervals across the tile, and on both surfaces, (2) tiles were roughened with coarse (grade 80) sandpaper again on both tile surfaces to provide a random roughened surface. Grade 80 sandpaper produces a random pattern with consistent amplitude (Scardino and de Nys 2004) and (3) smooth tiles were used. Each surface topography category was then exposed to either a biofilmed or non-biofilmed surface and a light or shade exposure. Biofilms were developed by

placing tiles in a flow through aquaria for ten days at OIRS. To test for settlement responses to light (open habitats) or shaded surfaces (cryptic habitats) each tile was supported by a bolt at each corner so it could be suspended mid water. This provided light to the upper surface whilst the underside surface was shaded. The experiment therefore tested for three surface topographies in the presence/absence of biofilm, and in the degree of light exposure.

Each treatment was replicated 6 times and randomly assigned to one of 6 shallow plastic trays (40 cm x 30 cm x 4 cm) containing FSW to which 250 larvae were introduced. To eliminate the risk of interrupting larvae settling by making periodic observations I recorded settled larvae once at 48 hours. This time was based on results of previous experiments suggesting most larvae had either settled or died by 48 hours. After 48 hours settlement tiles were removed and observed with a stereo dissecting microscope, recording numbers of larvae settled. Larvae settling on the sides of tiles were also recorded.

Responses to chemical cues

The previous settlement experiment identified biofilms as a settlement inducer. This treatment was therefore tested further in addition to other potential inducers that are found in adult habitats. Initially larvae were exposed to two different treatments containing potential cues associated with (1) coral rubble, a common substratum found within the immediate vicinity of adult *R.odorabile* populations (pers ob), and (2) biofilmed surfaces of plastic 5 ml Petri dishes.

For the coral rubble treatments (hereafter termed rubble), rubble was collected from the immediate vicinity of adults and maintained in flow through seawater until required. Small portions (≅1 cm^2) of rubble were then placed into

Petri dishes. The presence of biofilms on both living and inanimate surfaces in the marine environment (including rubble used in this study) prompted me to test a third cue, being the effect of settlement to inanimate surfaces. Of specific interest was the larval settlement responses to coral rubble if the organic biofilm was not present. Rubble was autoclaved to kill any organic biofilm and small pieces of sterile rubble (≅ 1 cm) were then placed in Petri dishes with FSW. In conjunction with this, comparative treatments were run with both control and non-sterile rubble (≅ 1 cm).

Biofilm treatments were prepared by placing Petri dishes into a flow through aquarium for 10 days to allow a biofilm to develop. Prior to experimentation biofilmed Petri dishes were rinsed and shaken underwater to remove loose debris that had accumulated in the dish.

For each treatment 10 larvae were placed into 5 ml Petri dishes containing FSW and the treatment effect. Controls were simultaneously run by replicating the experiment without the treatment effect. Numbers of active, settled, metamorphosed or dead (and unaccounted) larvae were recorded each 6 hours until all larvae had settled or died.

4.27 Data analysis

Vertical orientation in the water column over time

To determine if larval vertical migration behaviour differed between treatments offered light and treatments excluding light, the data was analysed using a three factor repeated measures ANOVA. The 6 hourly counts (i.e. time) comprised the within factor treatment and the position in the experimental chamber (i.e. vertical orientation) and exposure to light being the between factor

treatment.

Swimming ability

To assess differences in swimming speed for each time period, a one factor ANOVA was performed. To discern differences between swimming speeds from each time period a Tukey's (HSD) post-hoc test was used.

Settlement in response to light, surface micro-topography and biofilms

To establish differences in larval settlement responses to biofilmed surfaces, degree of light exposure, and surface topography a three factor ANOVA was used with biofilm and degree of light and surface topography all fixed factors. Data was transformed (sqrt) to satisfy assumptions of normality and homoscedacity before ANOVA was executed.

Settlement in response to chemical cues

Time was a repeated measure in the chemical cue settlement experiments. This data was analysed using a repeated measures two factor ANOVA with time being the within factor treatment and cue as the between factor treatment. To assess differences of successful transitions from settlement to metamorphosis for each treatment (i.e. biofilm and rubble) a one-factor ANOVA was used to compare rates of settlement and metamorphosis for each cue at one time (48 hours).

The difficulties of collecting adequate numbers of larvae, due to the dribble spawning nature of *R. odorabile*, often did not permit replicate experiments to be run simultaneously on the same day of collection. However, each replicate was run under similar conditions, particularly the time of day the experiment commenced. To account for the potential of results showing "different

day effects" day was used as a blocking factor for all chemical cue experiments. As no significant day effect was detected the analysis was completed by consolidating experiments run over different days.

4.3 Results

Rhopaloeides odorabile dribble spawns larvae during the afternoon with each individual releasing 50 - 800 larvae each day over several days. *Rhopaloeides odorabile* produce typical parenchymella larvae approximately 250-300 μm long, ovoid in shape and white in colour with a dark anterior ring supporting a tuft of cilia, which is used to direct and control movement.

4.31 Larval behaviours

As an initial determination of behaviour larvae respond positively to directed natural light upon release. This behaviour gradually declines after twelve hours with a distinct movement away from a directed light source. However, larvae are active swimmers for up to 24 hours where continual swimming is occasionally interrupted for exploration of the substrate or surface-water interface. After 24 hours, periods of exploration become more frequent and longer. A large proportion of larvae exhibit corkscrewing swimming (i.e. rotational movement whilst positioned vertically) prior to settlement. Settlement occurs by orientating and attaching to the substrate via their anterior pole. Rapid beating of the posterior cilia maintains this position providing temporary attachment. Larvae either metamorphose or detach and undertake further exploration. Metamorphosis occurs by larvae using pulsating movements of the entire body eventually invaginating. Metamorphosis results in the flattened disk shaped form with the pigmented cilia ring occupying the central sector of the disk. Larval

metamorphosis occurred on the sides and bottom of the experimental chamber and in some instances at the surface water interface.

4.32 Vertical orientation in the water column

Treatment with a light cue

The majority of larvae collected during the day and introduced into experiments with exposure to natural photoperiods occupy the surface of the experimental chamber for the first 18 hours. At 6 hours 72% (±3.5) (mean ± 1SE) of larvae were congregated at the surface compared to 6% (±1.7) and 2% (±1.4) at mid water and the bottom respectively (Fig 4.2). Numbers of larvae at the surface decreased gradually from 12-18 hours and at 24 hours there was a majority shift with 42% (±4.4) of larvae migrating to the bottom compared to 20.29% (±1.1) at the surface, 4.57% (±0.7) mid water, and the remaining 33.1 (±7.5)% dead or settled. After 24 hours larvae congregated at the bottom and at 30 hours 42.6% (±6.2) of larvae occurred at the bottom compared to 4.3% (±0.46) at the surface. By 54 hours all remaining larvae were aggregated at the bottom. During the experiment less than 5% (±1.7) of larvae were observed mid water.

Treatment without a light cue

Prior to the experiment commencing larvae were exposed to daylight whilst held in larval traps for up to 2 hours before they could be collected and placed into the experimental treatment. Therefore, these larvae were initially exposed to a light cue. When larvae were placed into the treatment that subsequently removed light, 98% (±1.6) of larvae occupied the surface at 6 hours (Fig 4.3). However, after 12 hours larvae were dispersed throughout the water column (i.e. surface, mid water, bottom) and at 18 hours 41.3% (±10.4), 16%

(±5.4), and 28% (±7.4) of larvae occupied surface, mid and bottom layers respectively. Similarly, at 30 hours, 20.33% (±4.4), and 23% (±5.2) of larvae, occupied surface and bottom layers respectively. Larval migration behaviours are different when there is a light cue. A significant interaction between the position of larvae in the water column and a light cue (between factor) distinguishes the contrasting patterns of larval behaviours in light versus no light cue confirming light is an important determinant of larval migration behaviour (Fig 4.3; Table 4.1).

Table 4.1. Summary results of repeated measures ANOVA examining larval vertical migration behaviours for day spawned larvae. Time represents the 6 hourly record of larval position in the water column. Light indicates presence/absence of a light cue. Significance values based on the Greenhouse-Geisser corrections

Effects	**source**	**df**	**MS**	**F**	**p**
Within subjects	Time	3.53	750.17	12.85	<0.001
	Time*position	7.1	2382.2	40.81	<0.001
	Time*light	3.53	190.1	3.26	0.02
	Time*position*light	7.1	399.5	6.84	<0.001
	Residual	116.4	58.38		
Between subjects	Position	2	5057.3	71.97	<0.001
	Light	1	194.1	2.76	0.11
	Position*light	2	479.4	6.82	0.003
	Residual	33	70.27		

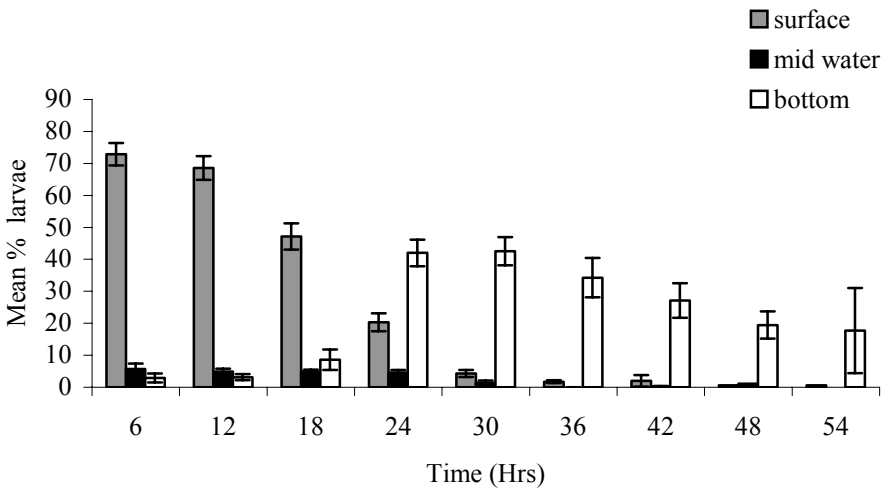


Fig. 4.2. Mean proportions (± 1SE, n = 6) of *R.odorabile* larvae and their orientation within a 1 litre experimental chamber over a period of 54 hours. Experimental chambers offer larvae full exposure to natural photoperiod regimes. Only active larvae were recorded therefore the decrease in larval numbers over time reflect larval settlement or death.

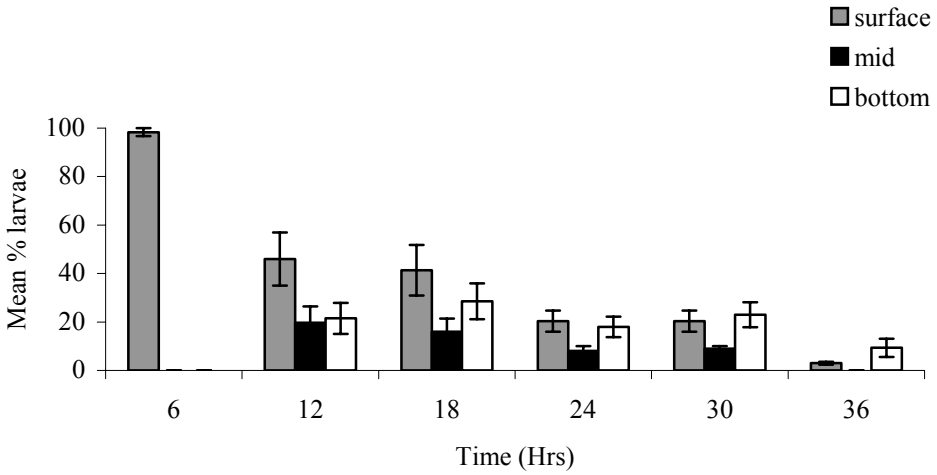


Fig. 4.3. Mean proportions (± 1SE, n = 6) of *R.odorabile* larvae and their orientation within a 1 litre experimental chamber over a period of 36 hours. Experimental chambers were covered in black plastic and offered no stimulus to light during the experiment. Larvae were collected post spawning and therefore exposed to light for up to 2 hours prior to the commencement of the experiment.

4.33 Larval buoyancy

Twenty larvae at 0, 6, 12, 24 and 36 hours post release were all negatively

buoyant and no inferential statistics were required.

4.34 Larval swimming ability

A maximum swimming speed of 0.4 cm sec^{-1} (±0.01) (mean ± SE) was recorded at the commencement of the experiment reducing to 0.3 cm sec^{-1} (±0.01) at 24 hours. Maximum mean swimming times showed a significant decrease over the experimental period (Fig 4.4. ANOVA: $F_{4, 95} = 28.78$, $p < 0.001$). Post-hoc tests (Tukeys HSD) identify the decrease in swimming speed between 6 and 12 hours after which there were no further changes in speed (Fig 4.4).

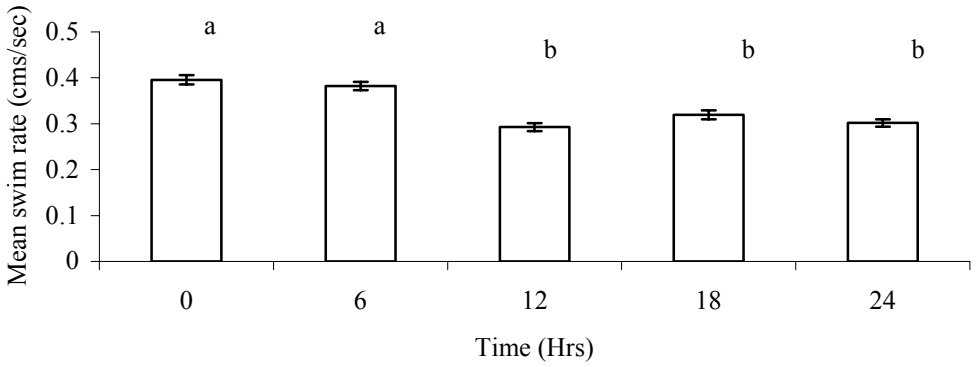


Fig. 4.4. Mean swimming rates (± 1 SE, n = 5) of *R.odorabile* larvae within an experimental aquarium at 6 hourly intervals for a period of 24 hours. Letters denote where significant differences (Tukey's post hoc) were found for swimming rates at each time period.

4.35 Larval responses to settlement treatments

Surface complexity, light and biofilm settlement responses

There was no significant interaction amongst treatments influencing settlement. However, larvae preferentially settled to treatments with a biofilm and also to light exposed treatments (Table 4.2, Fig 4.5). Larvae showed no preference for any of the physical surface treatments. Less than 0.01% of larvae settled on the edges of tiles and were not included in the analysis.

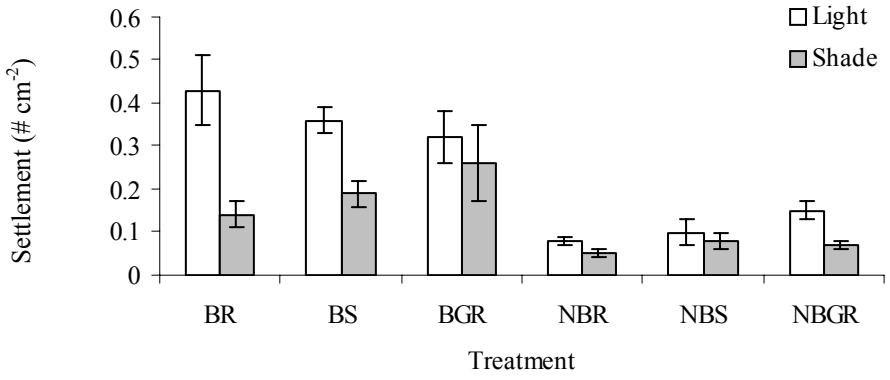


Fig. 4.5. Mean larval settlement per cm^2 (± 1 SE, n = 6) in response to a combination of substrate choices offering biofilmed (B), non biofilmed (NB), smooth (S), rough (R), or grooved surface (GR), in addition to choices of light or shade for each treatment.

Table 4.2. Summary three factor ANOVA results comparing settlement rates when larvae are offered choices of substrates including biofilmed or non biofilmed, light or shade or different surface textures (i.e. rough, smooth or grooved).

Source	df	MS	F	P
Biofilm (B)	1	0.27	22.25	< 0.001
Light (L)	1	920.92	77.58	<0.001
Surface (S)	2	0.008	0.71	0.49
BxL	1	0.19	1.56	0.22
BxS	2	0.009	0.74	0.48
LxS	2	0.006	0.49	0.62
BxSxL	2	0.19	1.57	0.22
Residual	57	0.12		

Biofilmed and coral rubble settlement responses

Larval settlement response was similar for both biofilm and rubble settlement cues with larval settlement being significantly higher for both biofilm and rubble in comparison to their respective controls (Figs 4.6 & 4.7; Table 4.3) Mean larval settlement was 33.9% (±5.2) and 23.3% (±6.3) for biofilm and rubble treatments respectively, at 6 hours compared to 82.78% (±4.6) and 80% (±5) by 48 hours. The mean rates of settlement contrasts sharply with respective controls with 8.24% (±3.2) and 10% (±3.2) at 6 hours for biofilm and rubble respectively,

and 39.4% (±6.6) (biofilm) and 40% (±5.1) (rubble) at 48 hours.

Whilst settlement rates are similar for biofilm and rubble (which also contains a biofilm) the presence of rubble appears to be required for metamorphosis. Total settlement rates (i.e. combined numbers of settled and metamorphosed larvae) were consistent for biofilm and rubble, however successful metamorphosis was significantly lower in biofilmed treatments. For example, larval metamorphosis reached 8.9% (±2.1) for biofilmed treatments, which is significantly lower than rubble 49.4% (±7.1) treatments (Figs 4.8 a & b, Table 4.4). Mortality was quantified at 7.2% (±3.7), and 16.9% (±3) for biofilm, and rubble treatments respectively.

Autoclaved rubble presented a sterile, inanimate surface with no biofilm cue. Larval settlement was significantly lower when compared to larval settlement with non-sterile (i.e. biofilmed) rubble (Fig 4.9; repeated measures ANOVA, time x cue, $F_{3.6, 17.8} = 6.4$ $p < 0.001$).

Table 4.3. Summary repeated measures ANOVA results comparing larval settlement in the presence of each of the cues associated with biofilms and rubble. Significance values based on the Greenhouse-Geisser corrections

Effects	source	df	MS	F	p
Biofilm					
Within subjects	Time	1.9	223.1	32.6	<0.001
	Time*Cue	1.9	29.8	4.3	0.02
	Residual	38.9	6.9		
Between subjects	Cue	1	937.6	44.4	<0.001
	Residual	21	21.42		
Rubble					
Within subjects	Time	2.9	92.14	27.16	<0.001
	Time*Cue	2.9	9.9	2.93	0.04
	Residual	75.5	3.4		
Between subjects	Cue	1	356.17	14.48	<0.001
	Residual	26	24.59		

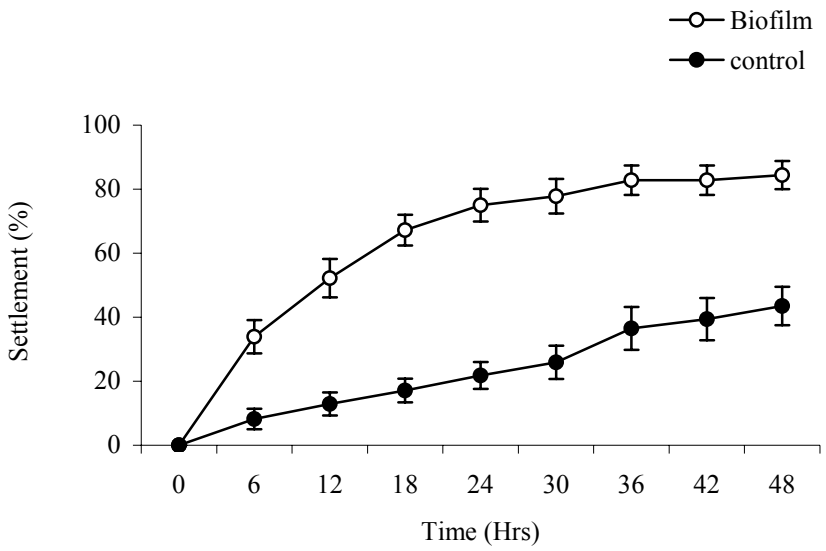


Fig. 4.6. Cumulative mean proportions (±1SE, n = 12 of settled *R.odorabile* larvae when exposed to a biofilmed substrate (Petri dish) compared to settlement in control treatments representing Petri dishes with FSW and no biofilmed substrate.

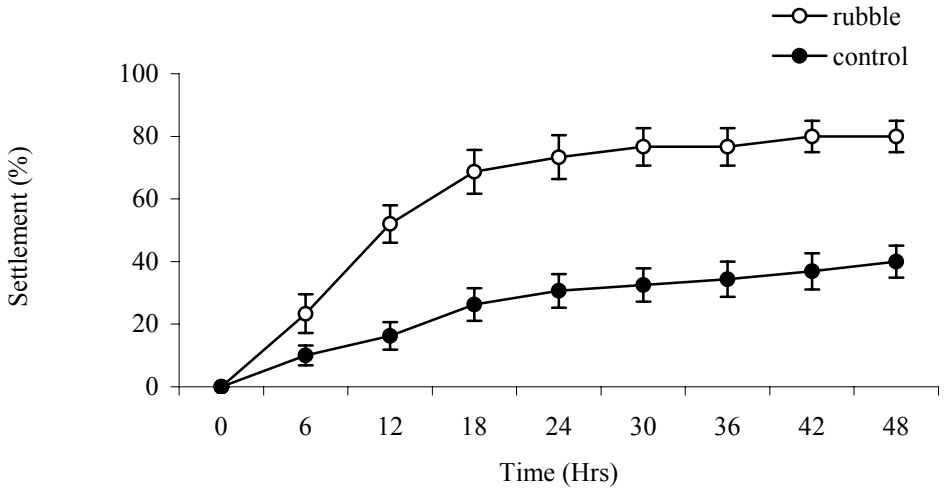


Fig. 4.7. Cumulative mean proportions (±1SE, n = 16) of settled *R.odorabile* larvae when exposed to experimental conditions with rubble compared to settlement in control treatments with FSW and no rubble.

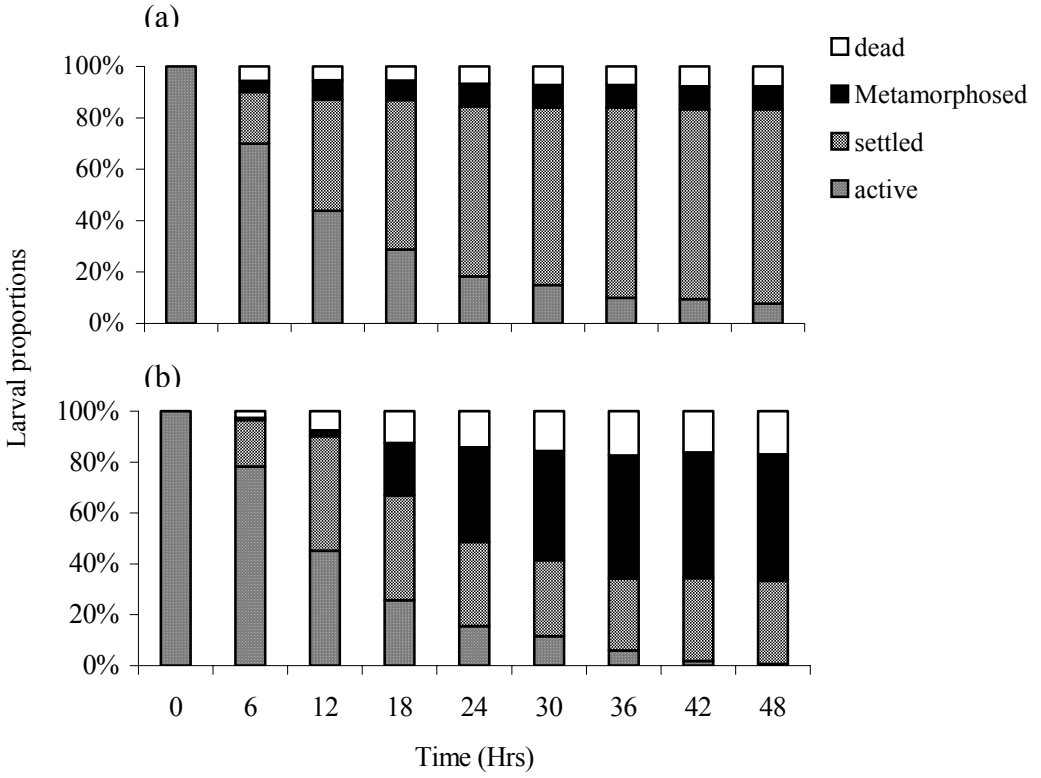


Fig. 4.8 (a-b). Proportions of larvae represented as active, settled, metamorphosed or dead when exposed to treatments of: (a) biofilmed surfaces over time, and (b) rubble over time.

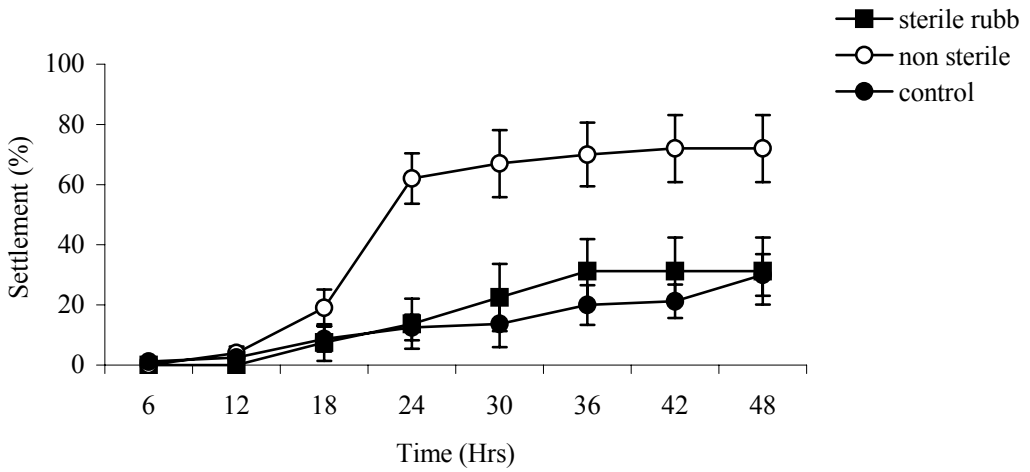


Fig. 4.9. Cumulative mean proportions (±1SE, n = 5) of settled *R.odorabile* larvae when exposed to experimental conditions of non-sterile rubble compared to settlement in sterile rubble and control treatments with FSW and no rubble.

4.4 Discussion

This is one of the few studies that outline a hierarchical range of related larval behaviours that contribute to the dispersal and settlement patterns of sponge

larvae. Based on qualitative and quantitative measures and with the aid of a conceptual model, the sequence of events for pre-settlement larval behaviours in *R. odorabile* larvae is described (Table 4.4). Following an afternoon release larvae are cued by light to direct their active swim to the surface, maintaining this position during the night and returning to the benthos at dawn 12 - 18 hours post release. A change to negative phototaxis provides a mechanism to return to the benthos facilitated by their negative buoyancy. Larvae swim to explore the benthos 18-30 hours post-release intermittently stopping to test the substrate for settlement. After 30 hours directed horizontal swimming decreases and is replaced by a corkscrewing behaviour. Larvae are able to temporarily attach to the substrate and have the capability to dislodge and continue exploring for favourable habitats. Settlement occurs from 12 hours post-release, and is associated with light exposed surfaces and biofilmed substratum (signalling open space). Metamorphosis occurs between 24-48 hours. Consistent with other benthic marine invertebrates (Zimmer & Butman 2000) *R. odorabile* larvae use hydrodynamics to passively disperse over wide distances during a planktonic phase. This is followed by a demersal phase where larvae actively disperse over finer microhabitat scales whilst exploring the benthos for settlement sites.

Table 4.4. Conceptual model based on both qualitative and quantitative larval behaviours depicting the sequence of events that *R. odorabile* larvae use from larval release to settlement. Circles represent presence and relative strength of larval behaviour for that time period (2-48 hours)

midday			6			midnight 12			dawn 18			midday 24			30			36			42			48		
Light (+ve)	#	#	#	#	#	#	#	#	#																	
Light (-ve)				#	#	#	#	#	#	#	#	#	#	#												
Buoyancy (-ve)	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#								
Swim	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#									
Corkscrew swim							#	#	#	#	#	#	#	#	#											
Explore substrate												#	#	#	#											
Temp attachment									#	#	#	#	#	#	#											
Settlement					#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
Metamorphosis							#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#

Wide scale dispersal: vertical migration & phototaxis

Vertical migration is an important dispersal strategy for many invertebrate larvae (Young 1995) and this is also the case for *R. odorabile*. The swimming abilities of sponge larvae (Maldonado & Young 1996) are unlikely to facilitate independent horizontal dispersal. However, vertical migration positions larvae in bodies of water that provides greater probabilities of passive transport (Oliver & Willis 1990; Kingsford & Choat 1996). Vertical migration has been observed in many marine invertebrate larvae, including sponges (Uriz *et al.* 1998), and is invoked as a process that facilitates dispersal (Oliver & Willis 1990). Although the results of this study are limited to laboratory manipulations the movement of larvae to surface waters in the field would promote passive dispersal via wind driven surface currents.

Whilst it is clear *R. odorabile* larvae use vertical migration, a comprehensive understanding of the mechanisms driving this behaviour is less clear. A variety of biological mechanisms can regulate vertical migration behaviours in sponges including phototaxis (+ve and –ve), geotaxis, and changes to buoyancy (see review Maldonado 2006). Despite no real neural capacity, tufted parenchymella sponge larvae utilise pigment cells in their propelling cilia to respond to light (Leys & Degnan 2001, Maldonado et al. 2003). *Rhopaloeides odorabile* larvae subject to natural photoperiod, move to the surface following release and maintain this position for up to 18 hours suggesting positive phototactic behaviour. After 18-24 hours larvae display negative phototactic behaviours and move to the bottom. When light is excluded larvae exhibit different larval movement patterns. Specifically, without light larvae do not show

clear congregation patterns and instead disperse throughout the water column, confirming light contributes to the direction of vertical migration for *R. odorabile* larvae. The finding that 98 % of larvae congregated at the surface at 6 hours for larvae that were subsequently placed into a treatment without a light cue supports the notion that initial exposure to light provides a sufficient imprint to direct upward movement despite the light cue being subsequently removed. Furthermore, the dispersion of larvae throughout the experimental chamber after 6 hours suggests any initial light imprint is eventually lost. Finally, the significant interactive effects of light and time distinguish larval behaviours in the presence/absence of light demonstrating light plays an important role in directing larval vertical migration for *R. odorabile*.

Larval vertical migration can also be mediated by gravity or buoyancy (review Maldonado 2006). Whilst gravity has been implicated in vertical orientation movements in some sponge species (Warburton 1966) there is no evidence of receptors that monitor gravity in sponges (Leys & Degnan 2001). However, pre-settlement spicule development may change the centre of gravity in sponge larvae and aid in directed movement. For example, *Sigmadocia caerulea* larvae undergo physiological changes to its larval skeleton over time altering the differential weight of spicules at the posterior end of the larvae, reducing buoyancy and aiding descent (Maldonado *et al.* 1997). *Rhopaloeides odorabile* is a dictyoceratid sponge and therefore does not contain spicules so this explanation is not valid. *Rhopaloeides odorabile* is however negatively buoyant for the duration of its larval life, therefore a predisposition to sink coupled with downward swimming would facilitate descent where it presumably continues to

disperse over finer spatial scales during a demersal phase.

Fine scale dispersal active exploration for settlement sites

After 24 hours *R.odorabile* larvae cease to display continuous swimming, replacing it with intermittent behaviour of short swimming bursts and periods where they probe the substratum. These results were measured in static water and represent maximum swimming capabilities which peak at the equivalent of 4 mm sec^{-1} immediately post release and decrease to 2.7 mm sec^{-1} at 24 hours. They compare well with swimming speeds of other sponge larval species. *Haliclona tubifera* swims at 3.6 mm sec^{-1} (Woollacott 1993), *Halichondria melanodocia* 2.1 mm sec^{-1} (Woollacott 1990), *Sigmadocia caerulea* 3.6 mm sec^{-1} and *Halichondria magniconulosa* 2.7 mm sec-^{1} (Maldonado & Young 1996). More importantly the swimming speeds of *R.odorabile* larvae are greater than the range observed for marine invertebrates classified as weak swimmers (i.e. 0.1-0.4 mm sec^{-1}) and compare favourably with stronger swimming invertebrates (i.e. 3-7 mm sec^{-1}), including those that use vertical migration (Metaxas 2001). For *R. odorabile* larvae the gradual decrease in swimming ability to 2.7 mm sec^{-1} at twenty-four hours is still greater than other weaker swimming invertebrates. When *R. odorabile* commences descent active swimming coupled with negative buoyancy would facilitate both the vertical descent to the benthos, and active substrate exploration thereafter.

When presented with a range of settlement surfaces *R. odorabile* larvae preferentially settled to plates exposed to light with bio-films regardless of the physical surface textures. The settlement to light exposed areas is consistent with observations of adult distributions in the field. *Rhopaloeides odorabile* is most

commonly found attached to the walls of massive coral structures or directly to benthic surfaces that are exposed to relatively higher light levels. The apparent indifference of larvae to settle on surfaces affording some level of micro-refuge was unexpected as micro-refuges minimises the risk of being incidentally grazed by indiscriminate grazers (Maldonado & Uriz 1998).

Biofilms are clearly important in initiating a settlement response for *R. odorabile* larvae. The presence of a biofilm decreases the time to settle and increases the number of larvae settling. Rubble showed similar results, however larval settlement to rubble is almost certainly a response to the biofilm associated with the rubble. Surface complexity can contribute to settlement in marine invertebrates (Kohler et al. 1999). However, higher settlement to treatments containing non-sterile rubble, compared to those containing sterile rubble, demonstrates settlement to the surface of the rubble to be cued by a biofilm.

Although settlement rates were highest in biofilmed treatments, the numbers of settled larvae successfully undergoing metamorphosis were lower than on rubble. This demonstrates that successful recruitment for *R.odorabile* relies on larvae firstly identifying favourable surfaces (i.e. biofilms) to promote settlement, and for those surfaces to have specific complexity (i.e. physical structure) to promote metamorphosis. That *R.odorabile* has lower larval settlement rates to sterile rubble surfaces further strengthens the interpretation that cues associated with biofilms, and the surface complexity associated with rubble, are equally important for successful recruitment. Larval metamorphosis in corals is influenced by a combination of cues associated with settlement surfaces (Negri et al 2001), but this strategy has not been previously reported in sponges.

An alternative or additional explanation for metamorphic success between biofilm (treatments) and rubble may rely on differences in biofilm ages or microbial community compositions. Rubble for the experiments was collected from the immediate vicinity of adult populations of *R.odorabile* and may contain cues important for promoting metamorphosis that were not available on the 10 day old biofilm used in this study. This study was not designed to collect empirical data on settlement responses to biofilms of different ages or compositions. However, both specific communities of organisms and ages of biofilms have been implicated in determining settlement patterns (Keough & Raimondi 1995, Wieczorek & Todd 1997, Huggett et al. 2006) and may explain the higher rates of metamorphosis for *R.odorabile* larvae in rubble treatments.

Positive responses to biofilms are well documented for numerous marine invertebrates (Pawlik 1992) and have been observed for a handful of sponge larval species both in field and laboratory manipulations (Keough & Raimondi 1995) . Surface biofilms of micro-organisms (eg bacteria, diatoms) signal favourable habitats, most notably unoccupied space (Keough & Raimondi 1995). In addition, bacterial biofilms are reported to supply exopolymers that aid in adhesion to the substrate (Maki et al. 1989). For *R. odorabile* the importance of settlement cues (biofilms) may be critical because their vertical migration behaviours suggests larvae are likely to disperse beyond favourable parental habitats (Pechenik 1999).

Levels of larval settlement at rates of up to 40 % in controls was unexpected, although indiscriminate settlement of sponge larvae is common (Bergquist & Sinclair 1968, Keough & Raimondi 1995), as they presumably will attempt to settle on any surface eventually. This is consistent with the control

treatments in this study where larvae settled onto plastic surfaces with no apparent cue. Whilst there is a clear preference for settlement in response to the cues examined in this study, the levels of indiscriminate settlement observed in the corresponding controls poses questions related to how essential the cue is in inducing settlement. Indiscriminate settlement in the absence of cues may result in settlement to sub-optimal habitats and consequently lower chances of survival. Nevertheless, it extends an opportunity for settlement to 'desperate' lecithotrophic larvae before valuable energy reserves are exhausted precluding successful metamorphosis (Raimondi & Morse 2000, Marshall & Keough 2003).

Conclusion

The weak swimming ability and behavioural response associated with marine invertebrate larvae has provided arguments that for these groups of organisms hydrodynamic regimes rather than behaviours influence dispersal and settlement. This study clearly demonstrates that larval behaviour is a contributing factor to successful dispersal and settlement. Indeed hydrodynamics when coupled to larval dispersal behaviour is critical to dispersal and settlement. The sequential behaviour of vertical migration, the use of phototaxis, swimming and subsequent preferential selection of habitat cues provides important information on the processes determining adult distributions of *R.odorabile*.

Chapter 5: Inferring Larval Dispersal from Population Genetics

5.1 Introduction

The central focus of this thesis has been to examine underlying processes that contribute to population genetic structure. Chapters 2 & 3 provided data that established reproductive processes influencing levels of larval supply and behaviours that contribute to dispersal and settlement. However, to assess the influence of larval dispersal on population distribution we need to confidently identify the spatial and temporal extent of larval dispersal.

The small size of many marine invertebrate larvae complicates our objective of elucidating true levels of larval dispersal and how that determines adult population distributions. Directly tracking or tagging larvae has been successful for some marine invertebrate taxa (Levin 1990). However, our understanding of larval dispersal is predominantly linked to outcomes from indirect measures. For marine invertebrate larvae this includes assessing dispersal potential in accordance to hydrodynamics, and pre-settlement larval behaviours including mobility, vagility, and pre-competency periods (Underwood & Keough 2001). Here, traditional paradigms suggest dispersal abilities are a reflection of larval developmental modes and planktonic periods (Jackson 1986). Emerging genetic data, however, suggests traditional paradigms associating planktonic periods with dispersal potentials are not necessarily an accurate predictor of dispersal potential (Ayre & Hughes 2000). Marine invertebrate larvae showing poor relationships between larval development modes and dispersal potentials have recently been demonstrated, with several examples of larvae often dispersing

over far less spatial distances than their dispersal potentials suggest (Ayre & Hughes 2000).

Whilst our understanding of larval dispersal is best approached with a composite of data, including larval behaviours and hydrodynamics, arguably the most convincing data allowing us to indirectly infer levels of larval dispersal comes from population genetic data. Various molecular methods have been employed over several decades (eg allozyme electrophoresis and mtDNA) to more recent techniques (eg microsatellites), providing highly informative data on population genetic structure, demographic histories, migration and gene flow, phylogeography, parentage and mating systems (review Parker et al 1998; Frankham et al. 2002).

Population genetic data for sponges has predominantly indicated genetic subdivision across varying spatial scales but with very few examples of panmixia. To date analysis of sponge population genetics indicates patterns of genetic subdivision over distances ranging from a few kilometres (Whalan et al. 2005), to tens and hundreds of kilometres (Benzie et al. 1994, Sole Cava & Boury-Esnault, 1999; Duran et al. 2002, Duran et al. 2004a, Duran et al. 2004b). Examples of sponge species showing either panmictic structure or mild genetic differentiation over large spatial scales are limited. For example, for the sponge *Chondrosia reniformis,* comparisons of populations reveal genetic homogeneity over a distance of 8000 kms (Lazoski et al. 2001). More recently, mtDNA data analysis from the sponge *Crambe crambe* showed little genetic differentiation existed between populations separated over several thousand kilometres (Duran et al. 2004b). However, further analysis of population genetic structure using

microsatellites for *Crambe crambe*, over similar geographical boundaries, contrasted sharply with the mtDNA results indicating evidence of genetic differentiation over smaller spatial scales (Duran et al. 2004a).

Our understanding of larval dispersal, as inferred from population genetic structure in sponges, whilst compelling is to a large degree still fragmented. The current data suggests that sponges appear to be a group with restricted dispersal capabilities and there is a clear need to address a more valid cross section of sponge species that encompass different life history (reproductive) traits and larval behaviours before generalisations can be drawn. Furthermore, the testing of different classes of molecular markers are also necessary if we are to further develop our understanding, particularly for comparisons of genetic variation over very fine (within site) spatial scales. The aims of this study were to elucidate the patterns of genetic variation for the ubiquitous Great Barrier Reef sponge *Rhopaloeides odorabile.* Specifically, I use allozyme electrophoretic and mtDNA (cytochrome oxidase I) data to detect the spatial scale of genetic variation within and among reefs of the inner and mid shelf reefs of the GBR, and determine the patterns of connectivity between examined reefs separated between 1 – 140 kms.

5.2 Materials and Methods

5.21 Study sites and sample collection

Samples from 719 sponges were collected by SCUBA, between August 2003 and September 2004. Collections were undertaken from a total of four sites within the Palm Island group situated on the inner shelf region of the central GBR, and from 11 sites on the mid and outer shelf reefs of the central GBR (Fig 5.1 & Table 5.1).

Rhopaloeides odorabile is more abundant and easier to locate on the mid and outer shelf reefs and on average 56 sponges were collected from each of these reef sites in comparison to 43 sponges from inner shelf reef sites. The habitats that sponges were collected from were similar, although inner shelf reef sites of the Palm Islands occur on either the reef flat or slope regions of fringing reefs in water 5-12 meters in depth. Sponges on the mid and outer shelf reef sites were collected from back and front reef slope regions in water ranging from 5-18 meters in depth.

To enable a hierarchical assessment of genetic variation a range of geographic scales were sampled. This included broad scale comparisons between outer, mid and inner shelf reefs separated by distances of tens of kilometres (up to 140 kms Fig 5.2). Medium and fine scale differences were assessed by examining variation amongst sites within each region, and for reefs within the reef complexes at Davies, Broadhurst and Lynch reefs, which provided comparisons over distances ranging from kilometres to tens of kilometres.

Sponges were opportunistically located using SCUBA and a sharp knife was used to excise samples approximately 27 cm^3 in size from each sponge. To reduce risks of contamination associated with symbiotic organisms sections were further dissected to provide samples with no pinacoderm, a region in the sponge commonly associated with symbiotic algae. Replicate samples were then placed in both liquid nitrogen and 70% ethanol within two hours of collection until required for genetic analysis. On return to the laboratory liquid nitrogen preserved samples were transferred to –80 ^{o}C freezer storage pending allozyme electrophoresis. Ethanol preserved samples were stored at room temperature pending DNA

analysis. Geographic distances between each pair of collection sites were determined using the software Ozi Explorer version 3.95.4e (D&L Software Pty Ltd) and are based on the shortest straight line distance between sites as determined by their respective GPS coordinates.

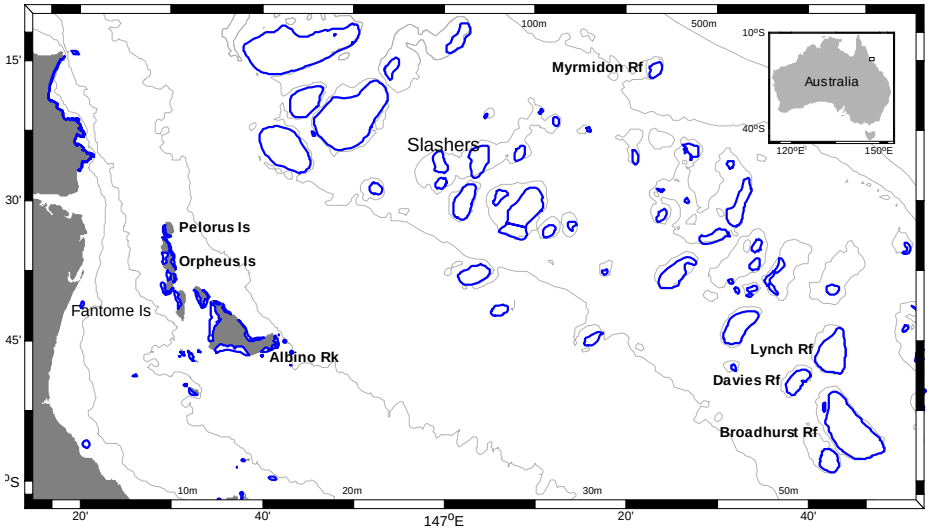


Fig. 5.1. Map of the central GBR with sampled sites for genetic analysis. Collections from Broadhurst, Lynch and Davies Reef included three collections within each of these reef complexes.

Table 5.1. Sampling sites, collection sizes (n) and dates collected for *R.odorabile*.

SITE	**Latitude**	**Longitude**	*Date*	n
Mid-/Outer Reefs				
Lynch 1 (L1)	18°44.244'S	147°42.938'E	Sept 2003	55
Lynch 2 (L2)	18°45.958'S	147°41.232'E	Jun2004	59
Lynch 3 (L3)	18°45.670'S	147° 41.226'E	Jun2004	54
Davies 1 (D1)	18°49.010'S	147° 37.968'E	Sept 2003	60
Davies 2 (D2)	*	*	Jun 2004	50
Davies 3 (D3)	*	*	Jun 2004	60
Broadhurst 1 (B1)	18°52.261'S	147° 42.350'E	Sept 2003	61
Broadhurst 2 (B2)	18°52.908'S	147° 42.203'E	Jun2004	54
Broadhurst 3 (B3)	18°53.010'S	147° 42.071'E	Jun2004	51
Slashers (SL)	18°29.485'S	147°08.313'E	Aug 2003	49
Myrmidon (M)	18°16.827'S	147°29.934'E	Aug 2003	36
Inner Reefs				
Albino Rock (A)	18°45.996'S	146°42.871'E	Sept 2003	57
Fantome Isl (F)	18°40.424'S	146° 31.639E'	Sept 2004	27
S. Pelorus Isl (SP)	18°31.955'S	146° 29.625E'	Mar 2004	50
N. Pelorus Isl (NP)	18°33.624'S	146° 29.722E'	Sept 2004	53

Abbreviations for sites listed adjacent to site name and are used throughout the remainder of this chapter. * Exact Co-ordinates unable to be collected for these sites due to a GPS malfunction but are estimated to be in the vicinity of 2-5 kilometres from Davies 1.

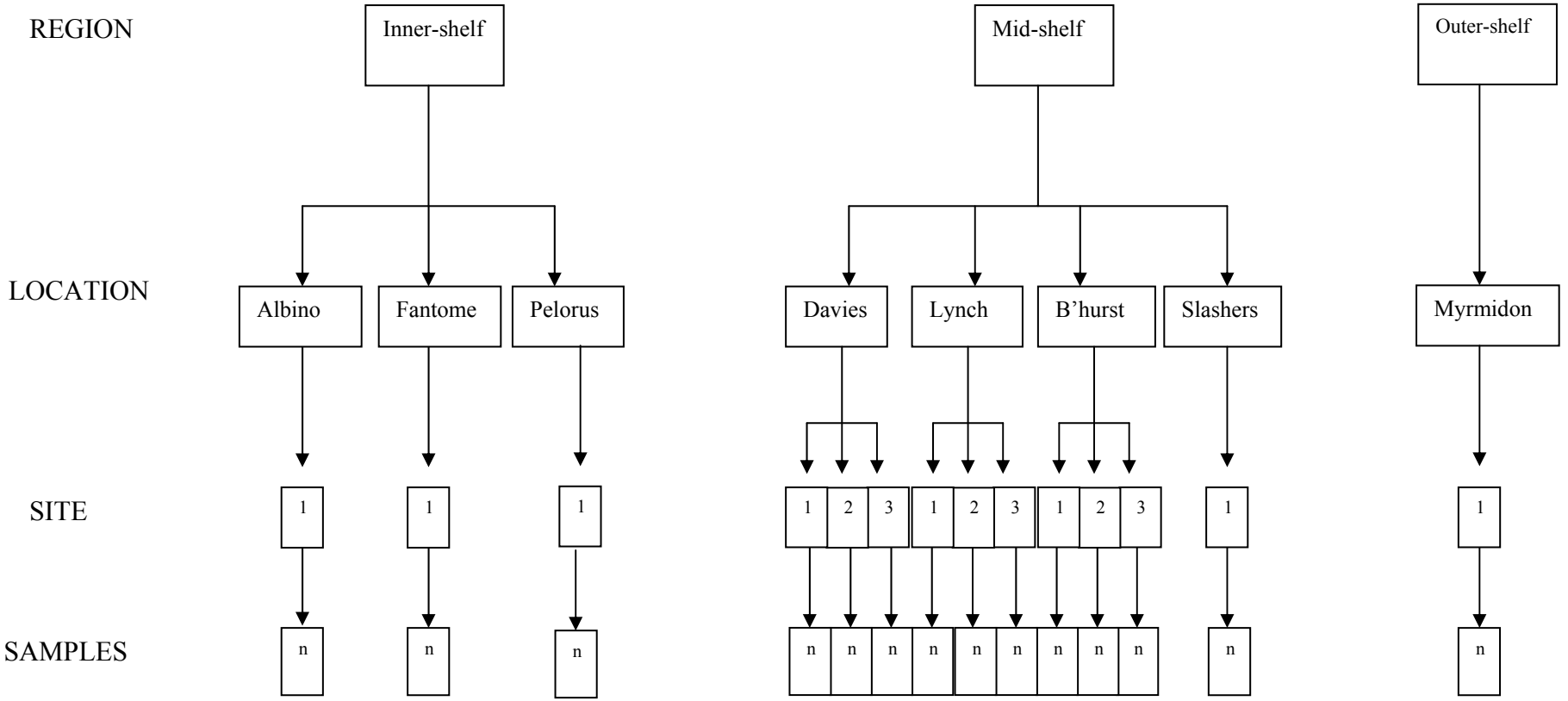


Fig. 5.2. Hierarchical sampling design depicting geographic scales examined to assess genetic variation in the GBR sponge *R.odorabile*. Samples could therefore be pooled at each hierarchy of the design enabling comparisons of genetic variation for samples within sites, sites within locations, among locations within regions and among regions.

5.22 Molecular methods

Allozyme electrophoresis

Allozyme electrophoresis was used to compare the genetic structure of *R. odorabile* populations within and among 15 sites. To determine which enzyme encoding loci were polymorphic and therefore would be informative for a population genetic study, a preliminary survey of 34 enzymes using two different electrophoretic buffers was undertaken (Table 5.2). Thirty two sponges representing a random selection of individuals from all sample sites were examined for each enzyme tested.

Allozymes were assayed on 12% w/v horizontal starch gels and a sub selection of these was also tested on cellulose acetate gels (Cellogel). Methods for starch and cellulose acetate were followed as described in Richardson et al. (1986). Enzymes were extracted by homogenising small pieces of sponge (8-10 mm^3) with a stainless steel rod in two volumes of homogenising buffer [(0.2 M sucrose, 0.02 M Tris–HCL (pH 8). 0.1 % mercaptoethanol, and 0.02% (w/v) bromophenol blue)] until no whole tissue remained. Filter paper (Whatman) inserts (5 mm x 6 mm) were used to absorb the extract solution. Tissue inserts were then loaded onto gels for electrophoresis using either Tris-citrate pH 8 or Tris-EDTA borate (TEB) pH 8.4 buffers.

Four out of the 32 enzymes tested could be reliably scored. However, the monomorphic expression at one locus (isocitrate dehydrogenase) precluded informative data collection. Difficulty in resolving enzymes for other sponges has been previously reported (Whalan et al 2005). Following electrophoresis, gels were stained for the polymorphic enzymes phosphoglucose isomerase (PGI; EC 5.3.1.9), malate dehydrogenase (MDH; EC 1.1.1.37) and an unidentified dehydrogenase (now abbreviated as NDH) using an enzyme recipe for xanthine dehydrogenase (EC

1.1.1204). Phosphoglucose isomerase was highly variable showing 6 alleles. Malate dehydrogenase was less variable exhibiting 2 alleles. The remaining locus was not identified. This type of activity has been reported for other sponges (Klautau et al. 1999; Lazoski et al 2001) and these authors did not include this activity in their analysis due to uncertainty over its origin. In this study, however, the positions where alleles separated on the starch gels were distinctly different to the other two loci (i.e. PGI & MDH). Whilst the protein responsible for this activity (locus) could not be identified this type of expression is often a result of unknown hydrogenase activity (Richardson et al. 1986). The variable nature of this unknown dehydrogenase, represented with 2 alleles, has warranted inclusion for analysis in this study. Alleles were labelled alphabetically according to their relative rates of electrophoresis mobility.

DNA analysis

Nucleic acids (DNA) were extracted from sponges from 15 locations across the central GBR (Fig 5.1 & Table 5.1) for amplification of partial sequences of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit 1 gene (CO1). Ethanol preserved tissue yielded degraded DNA, therefore, liquid nitrogen preserved tissue was used for all extractions.

DNA was extracted by placing approximately 100 mg of tissue into a 1.5 ml eppendorf tube which was then ground with a pestle in 750 µl of grinding buffer [(100mM Tris; 100 mM EDTA; 1% SDS; 100 mM NaCl; Milli Q H_2O)] and incubated at 65^{o}C for 2 hours. Following this Potassium Acetate was added (187.5 ul of 5M to attain a final concentration of 1M) and the extract was then stored on ice for 30 minutes before the extract was centrifuged at 15,000 rpm for 15 minutes and the supernatant collected. Nucleic acids were precipitated by adding 0.8 volumes of 100% isopropanol

to the supernatant and standing at room temperature for 5 minutes, followed by centrifuging at 15,000 rpm for 15 minutes. Pelleted DNA was washed twice with 70 % ethanol and dried in a heated vacuum centrifuge on medium heat for 3 minutes. Dried DNA was re-suspended in 0.5 ml of molecular grade water and quantified using a spectrophotometer (Nanodrop). Standardised solutions were prepared at concentrations of 5 ng/ul of DNA and stored at –20 ^{o}C pending use in polymerase chain reactions (PCR).

The universal primers HCO2198 and LCO1490 were initially tested according to the cycling conditions suggested by Folmer (1994). However, PCR products using these primers yielded poor sequences. To obtain more informative sequences PCR products based on the Folmer (1994) primers, were subsequently cloned into a pGEM-T (Promega) vector following the manufacturers instructions. PCR reactions using standard vector primers (T7 and SP6) were carried out for 24 sponge colonies and the products cloned and sequenced by a commercial operator (Macrogen Inc. Korea). The resulting sequences were then aligned using Sequencher (version 4.5, GeneCodes Corporation) and based on conserved sequence alignments, species specific primers were designed using the software Oligo (Version 4) Primers are identified with the following sequences RO-CO1F 5'GGAACGGTGGGTTTATCTTTAAGTTTG3'and RO-COIR 5'GGAACAATA TGGGGTCTCCGCCTC 3'

Amplifications were carried out on a Pellier Thermocycler (DNA engine Tetrad 2, MJ Research) in 25 µl total reaction volumes consisting of 10x Quigen Buffer with 1.5 mM $MgCl_2$, 0.2 µM of forward and reverse primer, 5ng μl^{-1} template DNA, 0.2 µM dNTPs and 0.5 units of Taq polymerase (Quigen). Amplifications were performed at the following temperature cycling regime; an initial denaturation of 94^{o}C for 2 mins; 35

cycles at 94°C for 30 secs, 50°C for 30 secs, 72°C for 45 secs, and a final extension of 72°C for 5 mins. This protocol yielded strong amplification products allowing direct sequencing of Sephadex G-50 purified PCR products. Sequencing of PCR products on both forward and reverse sequences were carried out by Macrogen Inc. Sequences were aligned and edited uisng the software Sequencher (version 4.5, GeneCodes Corporation), providing a final sequence length of 547 bp for analysis.

5.23 Data analysis

Descriptive statistics

Tests for departures from Hardy-Weinberg equilibrium (HWE) were obtained for each allozyme locus using the exact test (Raymond & Rousset 1995) implemented in Arlequin version 3.1 (Schneider et al 1997). Heterozygote excess or deficits were determined using the equation D = (Ho-He)/He (Selander 1970), where Ho is observed hetrozygotes and He is expected heterozygotes under HWE. as calculated in Arlequin. Measures of haplotype diversity were also obtained from Arlequin version 3.1

Detecting population structure - allozymes and mtDNA

A hierachical analysis of molecular variation (AMOVA) was undertaken to detect the spatial scale of genetic differentiation both within and among populations. Firstly, reefs from the inner shelf were assigned to one geographical group and reefs from the mid/outer shelf region to another geographical group. A second AMOVA analysis ignored geographical groupings. The matrix of pairwise F_{ST} values were then plotted in Multi Dimensional Space (MDS) to determine groupings of sites and to provide a graphical picture of the spatial patterns associated with genetic structure. Using the groupings determined in MDS ordination a further AMOVA was undertaken to analyse the partioning of molecular variance within and among groups. To aid in the

interpretation of differences among sites in MDS the methods of (Johnson et al. 2001) were followed. This procedure provides a scale of significance within MDS space that is a useful approximation for detecting differences between sites.

5.3 Results

5.31 Allozyme diversity

Alleles for all 3 loci were consistently found in all populations, with only minor differences in allele frequencies present among sites. No alleles were found with a frequency greater than 0.85 (Table 5.3). Significant deviations from Hardy Weinberg Equilibrium (HWE) were present with heterozygote deficits occurring at 9 of the 15 sites. Most heterozygote deficits (6 out of 9) were recorded at the most diverse locus, *PGI* (Table 5.4*)*.

Table 5.2. Summary of enzymes screened to detect genetic variation in *R.odorabile* populations.

Enzyme	EC #	Activity	Scorable
Aspartate aminotransferase	Aat; EC 2.6.1.1	faint	no
Acid phosphatase	Acph; EC3.1.3.2	blurred	no
Alcohol dehydrogenase	Adh; EC 1.1.1.1.	blurred	no
Adenylate kinase	Ak; EC2..4.3	nil	no
Arginine phosphokinase	Apk; EC 2.7.3.3	nil	no
Catalase	Cat;EC1.11.1.6	nil	no
Diaphorase	Dia; EC 1.8.1.4	nil	no
Esterase (α and β)	Est; EC 3.1.1	no	no
α -Glycerophosphate dehydrogenase	aGpd; EC 1.1.18	nil	no
Glucose-6-phosphate dehydrogenase	G6pd; EC 1.1.1.49	nil	no
Glutamate dehydrogenase	Gdh; EC 1.4.1.2.	blurred	no
Hexokinase	Hk; EC2.7.1.1	blurred	no
Isocitrate dehydrogenase	Idh; EC 1.1.1.42	monomorphic	yes
Lactose dehydrogenase	Ldh;EC 1.1.1.27	inactive	no
Leucine aminopeptidase	Lap; EC 3.4.-.-.	nil	no
Malate dehydrogenase	Mdh; EC1.1.1.37	polymorphic	yes
Malic enzyme	Me;EC 1.1.1.40	blurred	no
Mannose- 6-phosphate isomerase	Mpi; EC 5.3.1.8	nil	no
Nucleoside phosphorylase	Np	faint	no
6 phosphogluconate dehydrogenase	Pgd; EC 1.1.1.44	blurred	no
Phosphoglucose isomerase	Pgi; EC 5.3.1.9	polymorphic	yes
Phosphoglucomutase	Pgm; EC 5.4.2.2	faint	no
Peptidase Leucyl tyrosine Leucyl proline Leucyl valyl Leucyl leucine	Pep;EC3.4.-.-.	no	no
Sorbitol dehydrogenase	Sdh; EC 1.1.1.14	nil	no
Superoxide dismutase	Sod; EC 1.15.1.1	blurred/nil	no
Triose-phosphate isomerase	Tpi; EC 5.3.1.1	blurred	no

Table 5.3. Allelic frequencies at 3 allozyme loci in all 15 populations of *R.odorabile* from the inner and mid shelf reefs of the central Great Barrier Reef. n = numbers of *R.odorabile* individuals sampled at each site. Site abbreviations as detailed in Table 5.1. PGI, Phosphoglucose isomerase; MDH, malate dehydrogenase, NDH, unidentified enzyme activity.

	Inner shelf reefs				Mid-Outer shelf reefs										
	NP	SP	F	A	D1	D2	D3	L1	L2	L3	B1	B2	B3	SL	M
n	50	49	25	43	48	44	54	52	52	39	52	51	36	39	25
PGI															
A	0.07	0.00	0.06	0.06	0.07	0.18	0.09	0.07	0.09	0.22	0.10	0.03	0.06	0.07	0.02
B	0.34	0.35	0.42	0.26	0.25	0.17	0.25	0.26	0.36	0.26	0.21	0.19	0.28	0.26	0.36
C	0.07	0.09	0.12	0.10	0.03	0.16	0.08	0.13	0.03	0.01	0.02	0.05	0.04	0.10	0.16
D	0.03	0.03	0.04	0.09	0.02	0.05	0.20	0.09	0.03	0.03	0.08	0.05	0.01	0.06	0.04
E	0.17	0.15	0.08	0.19	0.20	0.12	0.16	0.14	0.26	0.24	0.17	0.16	0.11	0.20	0.24
F	0.32	0.38	0.20	0.31	0.43	0.32	0.40	0.30	0.23	0.22	0.41	0.53	0.50	0.31	0.18
Ho	*0.60*	*0.73*	*0.84*	*0.77*	*0.67*	*0.79*	*0.74*	*0.71*	*0.71*	*0.74*	*0.71*	*0.71*	*0.69*	*0.74*	*0.68*
He	*0.75*	*0.72*	*0.74*	*0.80*	*0.73*	*0.70*	*0.61*	*0.79*	*0.74*	*0.79*	*0.75*	*0.67*	*0.67*	*0.77*	*0.79*
MDH															
A	0.53	0.44	0.27	0.57	0.53	0.21	0.43	0.49	0.18	0.36	0.50	0.30	0.36	0.39	0.47
B	0.47	0.56	0.73	0.43	0.47	0.79	0.57	0.51	0.82	0.64	0.50	0.70	0.64	0.61	0.33
Ho	*0.32*	*0.38*	*0.35*	*0.25*	*0.45*	*0.46*	*0.41*	*0.32*	*0.33*	*0.52*	*0.44*	*0.39*	*0.50*	*0.18*	*0.50*
He	*0.40*	*0.44*	*0.29*	*0.29*	*0.43*	*0.54*	*0.39*	*0.34*	*0.35*	*0.46*	*0.42*	*0.45*	*0.45*	*0.34*	*0.47*
NDH															
A	0.26	0.19	0.17	0.16	0.31	0.30	0.32	0.20	0.20	0.40	0.30	0.30	0.36	0.20	0.33
B	0.74	0.81	0.83	0.84	0.69	0.70	0.68	0.80	0.80	0.60	0.70	0.70	0.64	0.80	0.67
Ho	*0.52*	*0.53*	*0.18*	*0.43*	*0.16*	*0.36*	*0.50*	*0.44*	*0.36*	*0.64*	*0.42*	*0.54*	*0.45*	*0.37*	*0.39*
He	*0.51*	*0.47*	*0.44*	*0.54*	*0.53*	*0.36*	*0.54*	*0.50*	*0.30*	*0.46*	*0.52*	*0.43*	*0.45*	*0.55*	*0.51*
Mean															
Ho	0.48	0.55	0.46	0.48	0.43	0.54	0.55	0.49	0.47	0.63	0.52	0.55	0.55	0.43	0.52
He	0.55	0.54	0.49	0.54	0.56	0.53	0.51	0.54	0.46	0.57	0.56	0.52	0.52	0.55	0.59

Table 5.4. Heterozygote deficits for 3 allozyme loci and as calculated by (Ho-He/Ho) where Ho and He are observed and expected heterozygotes respectively. Negative values indicate deficits. Significance at p<0.05* and p<0.01**

Site	***PGI***	***MDH***	***NDH***
Lynch 1	-0.101**	-0.060	-0.100
Lynch 2	-0.040	-0.003	0.200
Lynch 3	-0.006	0.130	0.180*
Davies 1	-0.007	0.005	-0.720**
Davies 2	-0.125**	0.230	-0.006
Davies 3	-0.187*	-0.007	0.080
Broadhust 1	-0.100	0.050	-0.210
Broadhurst 2	0.090	-0.119	0.182
Broadhurst 3	0.030	0.111	0.020
Myrmidon	-0.105**	0.030	-0.235
Slashers	-0.052	-0.454*	-0.309
Fantome	0.135	0.207	-0.614*
Albino	-0.038**	-0.508	-0.208
S Pelorus	0.028	-0.159	0.130
N.Pelorus	-0.200*	-0.200	0.040

5.32. Genetic diverstity mtDNA

Nucleotide sequences, 547 bp in length were amplified from the CO1 gene. Three positional changes along the sequence produced a total of 3 haplotypes equating to less than 1 % sequence divergence (Tables 5.5). Haplotype 1 was the most dominant haplotype (range 55 - 100%) and was common to all samples, whilst haplotype 2 was found at the majority of sites at varying frequencies (range 4 - 40%). Haplotype 3 was found at lower frequencies (range 4 - 28%) and at only 6 out of the 15 sites (Table 5.6).

Table 5.5. Partial COI sequence alignment showing positions of variability (bold) in the 3 haplotypes for *R.odorabile* from the central GBR.

	Sequence and positional change		
	239	360	495
Haplotype 1	GTACAAA**A**GTCCAG	AGACCC**G**ATATAC	CGGGGAA**A**GACAAC
Haplotype 2	**A**	**T**	**A**
Haplotype 3	**T**	**T**	**C**

Table 5.6. Haplotypic diversity for all 15 populations of sampled *R.odorabile* populations on the GBR.

Sampling site	Sample size	Haplotype frequencies		
		H1	H2	H3
Inner shelf Reefs				
Albino	20	0.60	0.40	-
Fantome	23	0.96	0.04	-
S.Pelorus	26	0.96	0.04	-
N.Pelorus	12	1.00	-	-
Mid Shelf Reefs				
Slashers	27	0.74	0.22	0.04
Davies 1	27	0.68	0.04	0.28
Davies 2	28	0.82	0.10	0.08
Davies 3	22	1.00	-	-
Lynch 1	27	0.89	0.11	-
Lynch 2	23	1.00	-	-
Lynch 3	26	0.77	0.19	0.04
Broadhurst 1	20	0.55	0.40	0.05
Broadhurst 2	26	0.92	0.04	0.04
Broadhurst 3	20	0.80	-	0.20
Myrmidon	23	0.80	0.20	-

5.33 Population genetic structure

AMOVA comparisons among all 15 populations without predetermined geographic groupings were undertaken separately for both genetic markers. There were significant genetic differences among all 15 sites, however patterns of fine scale geographic subdivision were different for allozyme and mtDNA data sets. Therefore, results on population structure for allozymes are presented separately to the results for mtDNA data.

Allozymes

Small, but significant F_{ST} values from 3 allozyme loci (Table 5.7, F_{ST} = 0.014, $p < 0.05$) suggest genetic differentiation among all populations. AMOVA partitions the variance among populations at 1.41%. Most of the variation is explained within populations (86.1%). Comparisons of pairwise F_{ST} values for reef sites (i.e. bommies) within the reef complexes of Davies, Lynch and

Broadhurst indicate that there was no genetic differentiation at several of these sites. Despite pooling these sites little change to the underlying patterns of genetic variation or to the clarity of geographical groupings for any of the spatial scales examined was observed (Table 5.7).

The matrix of pairwise F_{ST} comparisons reveal that large scale differences between most sites are responsible for levels of heterogeneity (Table 5.8). Both significance levels of raw data, and Bonferroni corrections for multiple tests are presented, due to the conservative and often restrictive nature of appying multiple testing adjustments like Bonferroni (Moran 2003). For a total of 78 pairwise comparisons 52 showed significant genetic differentiation (raw data). Following Bonferroni corrections the number of significant pairwise comparisons reduced to 24. Noteably, four sites (L2, L3, B23,and D23) are genetically differentiated in the majority of their pairwise site comparisons. The MDS ordination shows the pattern clearly (Fig 5.3). Whilst there are no clear groupings of sites, the position of L2, L3, B23, D23, are situated on the periphery of the ordination and explain a large component of the heterogeneity for pairwise population comparisons. Although no clear geographical separation of sites were evident in the ordination, a chaotic genetic pattern exists where differentiation is a result of both localised and regional subdivision. The genetic analysis based on 3 allozyme loci therefore, does not support groupings that distinguish sites of the inner shelf from those on the mid shelf.

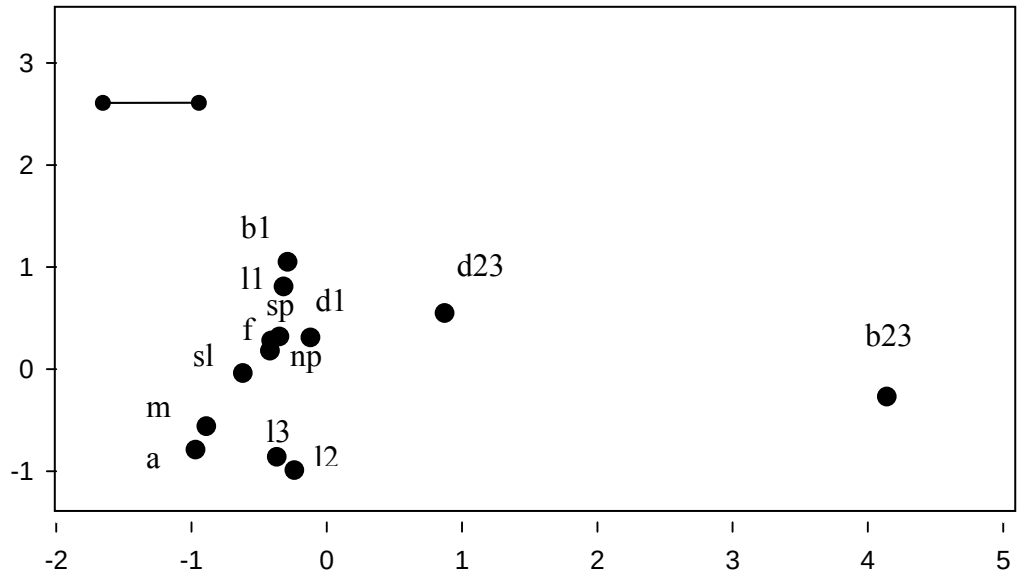


Fig. 5.3. Multidimensional scaling of pairwise F_{ST} (allozymes) among 13 populations of *Rhopaloeides odorabile* from the inner and mid shelf reefs of the central Great Barrier Reef. The scale bar represents a measure within MDS space where F_{ST} is significantly different to zero at p = 0.05.

Table 5.7. Summary results of AMOVA for allozymes and mtDNA data showing levels of genetic structuring and variation (%) attributable to each spatial scale analysed. Groups were determined from exploratory AMOVA and MDS of pairwise F_{ST} to detect obvious groupings within the MDS ordination.. P<0.01*

Spatial comparison	**Allozymes**		**mtDNA (CO1)**	
	F_{ST}	% variation	F_{ST}	% variation
Between groups	0.01*	1.3	0.15*	16.2
Reefs within groups	0.12*	12.6	-0.01*	-0.5
Within reefs	0.13*	86.1	0.12*	84.3

Table 5.8. Matrix of pairwise F_{ST} for 3 allozyme loci for *R.odorabile* among sites on the mid and inner shelf reefs from the central section of the Great Barrier Reef. Significant values of F_{ST} are indicated in italics (raw values) and after Bonferroni corrections (italicised and bold). P<0.05*, P<0.01**. Site abbreviations are as detailed in Fig 5.1.

	L1	L2	L3	D1	D23	B1	B23	M	SL	F	A	SP
L1												
L2	***0.016***											
L3	***0.014***	***0.016***										
D1	*0.005**	***0.017***	***0.016***									
D23	0.004	***0.021***	***0.017***	*0.007***								
B1	0.003	*0.010***	*0.021***	0.001	*0.009***							
B23	*0.016***	***0.039***	***0.043***	***0.025***	*0.008***	***0.025***						
M	*0.013**	*0.011***	*0.007**	*0.011***	***0.190***	*0.090***	***0.052***					
SL	*0.003*	*0.012***	***0.012***	*0.009***	***0.130***	0.003	***0.032***	*0.007**				
F	0.003	0.008	*0.015***	0.005	0.006	0.006	*0.023*	0.004	0.007			
A	*0.007**	*0.010***	***0.015***	*0.009**	***0.023***	0.002	***0.046***	*0.010***	0.003	*0.001**		
SP	0.004	***0.015***	***0.014***	0.003	*0.005**	0.004	***0.026***	*0.006**	*0.008**	0.002	*0.012***	
NP	0.002	*0.010***	*0.008***	*0.006**	***0.013***	0.005*	***0.024***	*0.012***	0.005	0.006	*0.007**	*0.004*

mtDNA (CO1)

Given the absence of geographical groupings of mid and inner shelf reef sites in the allozyme analysis an initial examination was undertaken to assess genetic variation without classifying sites into pre-determined geographical groups (i.e. mid shelf reefs V inner shelf reefs). Here, AMOVA showed significant genetic differentiation among all 15 sites (F_{ST} = 0.1 $p < 0.001$), however, in contrast to the allozyme results a larger component (10.5%) of variation is attributable among populations.

The pairwise matrix of F_{ST} values revealed no significant differences between several sites within their respective reef complexes of Davies, Lynch and Broadhurst (Davies 2 & 3; Lynch 1 & 2; Broadhurst 1 & 3) so these sites were pooled before undertaking further analysis. Using pooled data, (now comprising 12 sites) to further clarify geographical groupings, a further MDS ordination revealed 3 clear groups, which were used in AMOVA to detect group structure (Fig 5.4). AMOVA revealed significant subdivision among groups (Table 5.7, F_{ST} = 0.15, $p < 0.001$). The pairwise matrix of F_{ST} values showed 28 out of the 66 pairwise comparisons to be significant (raw data), although following Bonferroni corrections only 2 pairwise comparisons remained significant (Table 5.9). Levels of heterogeneity appear to be driven by 3 notable sites, Davies 1, Myrmidon and Albino, each of which record significant pairwise F_{ST} values for more than half of their pairwise site comparisons. These patterns are also shown clearly in the MDS ordination with Albino and Myrmidon clustering as one group on the periphery of the MDS and with clear separation from all other sites (Fig 5.4). Whilst Davies 1 is associated with other mid shelf reefs sites (i.e. B13, L3 & SL), and identified as

a group in the ordination, its separation from other sites within this group also highlights its determining power in explaining levels of differentiation. Overall, grouping of sites in the ordination appear chaotic, with inner shelf reef sites grouping with mid shelf reef sites suggesting a poor relationship with geographic genetic variation. For example, closely aligned mid shelf reef sites are differentiated whilst other mid shelf reef sites show homogeneity with inner shelf reef sites although separated by tens of kilometres. Broadhurst 2 is within the same reef complex as Broadhurst 1 & 3 and although separated by 1.1 km shows significant genetic differentiation (Tables 5.9 and 5.10). However, Broadhurst 1 & 3 shows genetic connection to other inner and mid shelf reefs despite being separated by tens of kilometres.

Table 5.9. Matrix of pairwise F_{ST} values following analysis of mtDNA (CO1) for 12 populations of *R.odorabile* on the inner and mid shelf reefs of the central GBR. Significant F_{ST} values are in italicised (raw), and bold and italicised following Bonferroni corrections. P<0.01. Site abbreviations as detailed in Fig 5.1.

	L12	L3	D1	D23	B13	B2	M	SL	F	A	SP
L12											
L3	*0.109*										
D1	*0.169*	*0.061*									
D23	-0.003	0.037	*0.136*								
B13	*0.142*	-0.014	0.017	*0.087*							
B2	-0.022	0.048	*0.130*	-0.027	*0.089*						
M	*0.338*	0.034	*0.146*	*0.238*	0.022	***0.242***					
SL	*0.141*	-0.037	0.065	0.064	0.018	0.074					
F	-0.004	0.073	*0.184*	-0.016	*0.119*	-0.031	*0.271*				
A	***0.357***	0.038	*0.148*	*0.253*	0.023	*0.256*	0.049	0.014	*0.289*		
SP	-0.007	0.088	*0.199*	-0.009	*0.132*	-0.026	*0.292*	*0.115*	-0.042	*0.313*	
NP	0.011	0.099	0.179	-0.002	0.132	-0.019	*0.285*	0.124	-0.138	*0.302*	-0.034

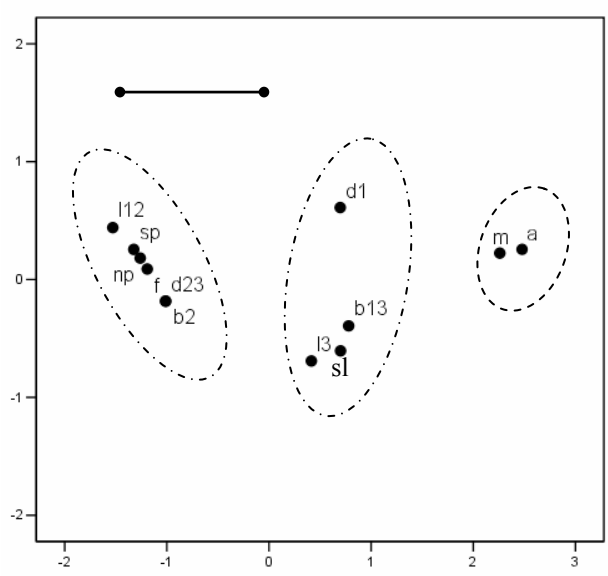


Fig. 5.4. Multidimensional scaling of pairwise F_{ST} (mtDNA) among 12 populations of *R. odorabile* from the inner and mid shelf reefs of the central sector of the Great Barrier Reef. The scale bar represents a measure within MDS space where F_{ST} is significantly different to zero at p = 0.05. Site abbreviations as described in Table 5.1.

Table 5.10. Matrix of geographical pairwise distance between each of the sampled sites for *R.odorabile* on the GBR. Due to a malfunctioning GPS coordinates for D2 and D3 could not be determined. However, for comparison purposes pairwise distances for both D2 and D3 (highlighted in table) are based on D1, which is estimated to be 2-5 kms from both the reefs, D2 and D3. Distance comparisons using D2 or D3 are therefore ±2-5km.

	L1	L2	L3	D1	D2	D3	B1	B2	B3	M	SL	F	A	SP	NP
L1		2.4	3.4	8.4			15.2	16.2	16.7	61.5	66.7	125.4	105.3	129.4	130.9
L2			1.9	9.5			11.5	12.6	12.9	64.1	67.4	124.3	104.3	129.4	130.3
L3				8.7			12.3	13.3	13.7	62.3	65.5	122.9	102.4	127.5	128.4
D1							9.8	10.5	10.3	65.1	63.5	117.8	96.8	123.1	124.2
D2							9.8	10.5	10.3	65.1	63.5	117.8	96.8	123.1	124.2
D3							9.8	10.5	10.3	65.1	63.5	117.8	96.8	123.1	124.2
B1								1.1	1.5	73.9	73.3	104.6	132.1	132.1	133.6
B2									0.9	75.1	74.1	126.8	105.4	132.3	133.8
B3										76.1	75.7	125.9	104.4	133.4	134.9
M											32.8	100.4	88.7	98.4	98.5
SL												67.4	53.7	67.8	68.1
F													22.4	12.9	15.9
A														32.7	35.2
SP															3.0

5.4 Discussion

Results from allozyme and mtDNA data support genetic differentiation at both the broadest (i.e. 10's kms) and smallest spatial scales (1km) examined in this study. In addition, levels of genetic connectedness are also observed across the same spatial scale comparisons providing an overall pattern of chaotic genetic structure. Whilst extensive larval dispersal would explain genetic homogeneity over broad spatial scales, fine scale population subdivision would also suggest larval dispersal is restricted between some of the sites examined.

Given the patterns of chaotic genetic patchiness it is difficult to invoke single models of larval dispersal to explain genetic structure. Open system models of planktonic dispersal, resulting in panmictic population structures, are commonly reported for sessile marine invertebrates (Williams & Benzie 1997), including sponges (eg Laszoski et al 2001). In contrast closed system models of larval dispersal resulting in population subdivision over small spatial scales are also observed for sessile marine invertebrates including sponges (eg Duran et al 2004; Whalan et al 2005). Larval dispersal depicting chaotic patchiness which show large scale patterns of homogeneity in addition to small scale differentiation are also observed in marine invertebrate populations (Johnson & Black 1984; Watts et al. 1990; Moberg & Burton 2000).

Interpreting population structures for marine taxa relies on an understanding of a complex matrix of parameters that influence larval dispersal. Complicated life histories, observed in many marine sessile invertebrates, often result in dynamic population structures influenced by several parameters

including life history traits (larval development mode), larval biology, larval behaviours and hydrodynamics (Grosberg & Cunningham 2001).

Dispersal ability has been related to planktonic competency periods with lecithotrophic larvae spending less time in the plankton and therefore having less dispersal ability than broadcast spawned gametes (Hellberg 1996, Ayre & Hughes 2000). Whilst there are clear examples confirming breeding mode contributes to dispersal potential (eg Williams & Benzie 1997), there are equal numbers of examples that do not support this model (eg Ayre and Hughes 2000). *Rhopaloeides odorabile* is a brooding sponge species (Chapter 2) and shows genetic homogeneity between sites separated by tens of kms. This contrasts with the single other study of sponge population genetics undertaken on the Great Barrier Reef (Benzie et al. 1994). The dictyoceratid brooding sponges *Phyllospongia lamellosa*, *P. alcicornis*, *Carterospongia flaellifera* and *Collospongia auris* all recorded genetic differentiation, as determined by allozymes, for populations separated by tens to hundreds of kilometres (Benzie et al. 1994). Benzie et al. (1994) also found genetic divergence for each of the four sponge populations increased with increasing geographical separations, which clearly contrasts with the genetic patchiness observed in *R.odorabile* populations. When other viviparous taxa are compared the relationship between larval development mode and dispersal potential (as indicated by genetic structure) remain unconvincing (Ayre & Hughes 2000, Severance & Karl 2006). The apparent homogeneity between populations of *R. odorabile* separated by tens of kilometres suggests the brooded larvae of this sponge are capable of dispersing beyond natal reefs. Nevertheless, fine scale heterogeneity over distances as small

as 1 km (eg. sites within Broadhurst Reef Fig 5.4, Tables 5.8 and 5.9) also suggests levels of endogenous recruitment. The apparent lack of relationship between geographical separation and genetic structure suggests that these patterns may be best interpreted in terms of larval dispersal in accordance to reef specific hydrodynamics.

The behavioural strategies of vertical migration and pre-competency periods of up to 54 hours for *R. odorabile* larvae (Chapter 3) provide a mechanism for passive dispersal via current systems throughout the sampled sites in this study. However, recognising that the GBR comprises a patchy mosaic of reef systems may be of critical importance in explaining patterns of genetic variation and therefore larval dispersal in *R. odorabile*. Localised recruitment patterns of *R. odorabile* larvae at several of the mid shelf reef sites (eg Davies & Broadhurst) may be a result of hydrodynamics contributing to water retention around specific reefs during the larval dispersal phase.

Retention of water to reefs of the GBR can occur over periods that in many cases exceed the competency periods of dispersing larvae (Black et al. 1991). Furthermore, water retention to reefs may also prevent water exchange from external currents moving around reefs (Black et al. 1991). Hydrodynamics therefore presumably restrict larvae to natal reefs and limit the exchange of water carrying larvae from more distant reefs. However, the ephemeral nature of currents can contribute to differential hydrodynamic regimes providing variable patterns of retention or indeed advection depending on reef size, shape, aspect (leeward or windward side) and time further highlighting reef specific hydrodynamics (Black et al. 1991). *Rhopaloeides odorabile* females release larvae

over several weeks and therefore presumably under different environmental conditions (i.e. tidal & current regimes). It is highly likely that the degree of larval dispersal may be subject to reef specific hydrodynamics and current patterns at the time of larval release which, may explain the genetic patchiness observed in this species. Therefore, despite localised recruitment at some sites the occurrence of random or occasional dispersal over extensive distances may explain genetic homogeneity between distant sites. Furthermore, the mosaic patchwork of reefs on the GBR may provide intermediate points (stepping stones) of colonisation thereby moderating genetic divergence between distant populations. Dispersal between distant sites separated by over 10's of kms via a series of adjacent reefs is a plausible proposal given the relatively short larval pre settlement periods of up to 54 hours in *R. odorabile*.

Although both allozymes and mtDNA (CO1) data for *R. odorabile* support an overall similar genetic pattern of localised subdivision and connectivity between distant sites the levels of variation are lower (although still significant) for allozyme data. Mitochondrial DNA data however reveal more obvious patterns of differentiation. Similar disparities of genetic structure are reported for other marine invertebrates using multiple markers (Palumbi 1994, and references within) and these discordant patterns have been explained in terms of reflecting historical patterns of gene flow rather than contemporary events.

Historical events associated with sea level fluctuations can expose populations to different current regimes (Williams & Benzie 1997) leading to isolation of populations and variable patterns of gene flow (Palumbi 1994). It is well established that present day GBR sea levels are 100 metres higher than those

during the last glaciation event 18, 000 years ago. Re-colonisation of the present mid and inner shelf reef communities occurred from 12, 000 years ago, when sea levels commenced their rise, to their present level 8, 000 years ago. Present genetic patterns therefore, may be far from equilibrium in respect of migration and genetic drift, and rather than reflecting levels of current gene flow (or larval dispersal strategies) represent historical footprints (Palumbi 1994). Despite our conclusions that larval dispersal behaviours support genetic structure for *R. odorabile*, the interpretation of past genetic signatures cannot be overlooked. Indeed the low rates of variability for many sponges and cnidarians have been interpreted in terms of their low rates of evolution in association with long generation times and low metabolic rates (Worheide 2006)

Conclusion

Taken together both markers demonstrate patterns that indicate homogeneity and localised genetic subdivision for *R. odorabile*. This chaotic genetic patchiness suggests endogenous recruitment is occurring at fine spatial scales with levels of occasional long distance dispersal (or dispersal potentially using a series of closely linked reefs as stepping stones) at frequent enough levels to prevent divergence between the most distant sites. The genetic patterns for *R. odorabile* are consistent with larval behaviours of vertical migration and passive dispersal in accordance with reef specific hydrodynamics present within the mosaic patchwork of reefs that determine the GBR. However, the interpretation of contemporary levels of gene flow, as reflected in genetic structure, can also be complicated by historical imprints if populations are yet to reach equilibrium between migration and genetic drift. Population histories can have lasting effects

and can shadow interpretations of contemporary gene flow with genetic patterns reflecting periods of past levels of gene flow rather than present day dispersal.

Chapter 6: General Discussion

Whilst there is an ever-growing record detailing the biology and ecology for sponges there is a paucity of fundamental information on processes that determine population dynamics. Developing an understanding of parameters that regulate sponge populations are becoming increasingly important with recognised threats of over exploitation and human mediated climate/habitat fluctuations (Castritsi-Catharios et al. 2005). In the context of addressing some of the critical components of population dynamics, this thesis provides key data on the reproduction, larval dispersal, larval settlement, and population genetic structure for the common Great Barrier Reef sponge, *Rhopaloeides odorabile*.

Early proposals of population connectivity in the marine environment depended heavily on the notion of demographically open populations that rely on recruitment from exogenous supplies (Caley et al. 1996). Although there are numerous examples of taxa that adhere to this model, for some taxa, patterns of connectivity are more complicated because they display both widespread dispersal and endogenous recruitment. Indeed, these genetic patterns are observed for *R.odorabile*.

Rhopaloeides odorabile displays chaotic genetic patterns with genetic differentiation detected at the broadest (10's kms), and smallest spatial scales (1km) examined in his study. In addition, genetic connectedness also occurs over the same spatial scale comparisons. The apparent contrasting patterns of genetic structure suggest localised recruitment, but with enough wider scale dispersal to prevent divergence between the most distant sites examined in this study.

Hydrodynamics can play an important role in the passive dispersal of weak swimming marine invertebrate larvae (Underwood & Keough 2001) and is likely to be an important component explaining the population genetic structure for *R.odorabile*. Importantly, the complicated mosaic of reefs that structure the GBR can result in differential hydrodynamic regimes (Black et al. 1991) and this highlights the important role reef specific currents may play in passive larval dispersal. The complicated patterns of genetic structure emerging for many taxa, including this study, also illuminate the importance of interpreting genetic patterns with due reference to other factors, including the influence of the intrinsic biology of adults when explaining patterns of connectivity (i.e. reproductive output, mode of larval development, and larval behaviours).

The apparent lack of barriers to dispersal is thought to promote open systems of genetic exchange in marine environments (Palumbi 1994). This view is often used to argue that local reproductive output is unimportant for population maintenance because recruitment is largely determined via exogenous larval sources (review Caley et al 1996). For many taxa, this proposal highlights the importance of exogenous recruitment. However, contrary to his view there is increasing evidence demonstrating patterns of self recruitment (review Levin 2005), indicating that for some taxa localised reproductive effort is an important process for population maintenance. Estimations of reproductive output in sponges are described for several species, and whilst they are critical in developing our understanding of biology, their informative value in interpreting population dynamics are limited unless the spatial scales of recruitment can be estimated. This area is largely unexplored for sponges, but at least one study has

demonstrated that for some sponges higher levels of reproductive output are associated with wide scale dispersal and adult distributions consistent with demographically open systems, suggesting population maintenance relies on recruitment from neighbouring sites (Uriz et al 1998). For other sponges lower reproductive output and more restricted larval dispersal are consistent with aggregated adult distributions suggesting population maintenance is more important at localised scales (Uriz et al 1998). For *R. odorabile,* reproductive output varies in accordance with environmental habitat conditions, with low levels of reproductive output for sponges inhabiting the turbid waters of inshore reefs (Chapters 2 & 3) and with offshore sponges showing levels of reproductive output that are up to 15 times higher (Chapter 3). Reef environments exposed to excessive turbidity from terrigenous run-off adversely affect reproductive characteristics for coral reef organisms (Fabricius 2005), and this is a likely explanation for the reduced reproductive output for inner reef populations of *R. odorabile*. However, the importance of reduced levels of fecundity for inner reef populations, in terms of population maintenance, may be a moot point because genetic connectivity occurs over both fine and broad geographical scales (Chapter 5). Therefore, recruitment both from neighbouring and offshore sites contribute to population maintenance and to a degree may negate any effects of lower levels of reproductive output for sponges occurring on inner reefs.

Although reproductive effort in *R. odorabile* is unlikely to be important on a localised scale, other reproductive characters may be important influences for the population dynamics of *R. odorabile.* For example, mode of larval development may play a role in determining larval dispersal potential.

Rhopaloeides odorabile is a brooding sponge and produces well developed larvae with competency periods typically ranging between 48 and 54 hours (Chapters 2 & 4). Competency periods of marine invertebrate larvae (i.e. planktonic durations) are often reflected in the spatial scale of dispersal (Shanks et al. 2003, Siegel et al. 2003), and therefore the patterns of genetic variation. Accordingly, the short competency periods of *R. odorabile* (Chapter 4) should result in limited dispersal and therefore high levels of population subdivision. However, patterns of genetic homogeneity between sites separated by tens of kilometres suggests larvae have the capability of dispersing beyond natal or closely separated neighbouring reefs. The mode of larval development and larval competency periods may not always be a sensible predictor of larval dispersal potential, as the genetic results for *R. odorabile* suggest. Importantly, current genetic data highlights both examples of site retention for broadcast spawners with comparatively long larval competency periods, and widespread dispersal for brooding taxa producing larvae with short competency period (Ayre and Hughes 2000). Recognising the influence of the mode of larval development and hydrodynamics on the passive larval dispersal for *R. odorabile* may only be part of the explanation for interpreting genetic patterns. Other mechanisms acting in concert with hydrodynamics, such as pre-settlement larval behaviours, may be a critical component to their dispersal capabilities.

Consistent with other marine invertebrates the cumulative evidence from this thesis demonstrates a two tier dispersal strategy for *R. odorabile* where hydrodynamics influence passive planktonic dispersal over wider scales followed by a demersal phase of substratum exploration occurring over microhabitat scales

(Butman 1987, Pawlik 1992). The use of vertical migration by *R. odorabile* larvae coupled with hydrodynamics would support the patterns of genetic connectivity (over 10's of kilometres) for this sponge. It is also proposed that following a return to the benthos, fine scale microhabitat dispersal occurs during a demersal exploration phase where larvae actively select suitable settlement sites.

The final stages of larval dispersal for many marine invertebrates include a period of demersal exploratory behaviour, where active swimming often occurs over meters or cms (Zimmer & Butman 2000). Larvae of *R. odorabile* explore the benthos for suitable settlement sites for approximately 24-48 hours. An active, demersal exploratory phase may be important given their apparent discriminatory strategy for selection of suitable settlement sites. *Rhopaloeides odorabile* show clear settlement responses to cues associated with biofilms. Settlement to biofilms has been demonstrated in many marine invertebrates (review Pawlik 1992) and promotes settlement to favourable habitats, most notably open space (Keough & Raimondi 1995). Whilst biofilms induce larval settlement in *R. odorabile* one of the most intriguing findings in this thesis was the importance of coral rubble in promoting a successful transition from initial settlement to metamorphosis, highlighting the hierarchical selectivity of several cues to determine settlement to favourable habitats.

Conclusions and future directions

The work embodied within this thesis represents a cross disciplinary study detailing reproductive biology, larval ecology and genetics, which adds to the knowledge of sponge biology and ecology from release to settlement and how these processes interplay to determine levels of population connectivity. This

information is a significant contribution to our understanding of how sponge populations are connected and maintained by critical processes during the larval phase. However, the findings within this thesis have also opened up many new and unexpected areas for research.

Reproductive biology

The finding of differential investment of reproductive output in relation to levels of water quality was expected. It would be informative to expand the sampling to include reefs exposed to higher levels of turbidity associated with terrigenous input (i.e. closer to the coastline). If *R. odorabile* can survive on reefs with higher levels of turbidity than those examined in this thesis, it poses the question as to whether exposure to higher levels of turbidity inhibits reproduction further than what was determined for sponges occurring on the inner reef of Pelorus Island. Indeed, does reduced reproductive output matter if population maintenance is determined by larval recruitment largely from an exogenous larval supply?

Larval ecology

One of the most intriguing results presented in this thesis was the hierarchical settlement strategy in response to several cues, particularly the higher levels of metamorphosis when coral rubble is present. What does the coral rubble contain that promotes higher levels of metamorphosis? Is it mediated by chemistry or the complexities of organic biofilm ages and/or community structure associated with the rubble? These types of questions are particularly informative for elucidating key components of settlement, and recruitment processes, not only for sponges, but also for other dominant benthic reef organisms.

Population genetics

Sampling to detect patterns of genetic variation was conducted over limited spatial scales. Questions of historical footprints on population genetic data may well be resolved by expanding sampling over broader spatial scales. Our knowledge of distributional boundaries for *R. odorabile* are hampered by a lack of extensive surveys, but it does occur at Lizard Island, several hundred kilometres to the north of the region examined in this thesis. Comparisons over broader spatial scales would be a valuable addition to interpretations of genetic patterns for *R. odorabile*. Moreover, development of different molecular markers would also be informative, particularly if they show higher levels of variation (eg ITS region or microsatellites). Finally, given the chaotic patterns of genetic structure it would be valuable to investigate the role of hydrodynamics on passive particle dispersal at the same sites investigated genetically.

In conclusion, developing our understanding of key processes that occur along entire life cycle continuums for benthic organism with a bi-partite life cycle is a critical component to our overall understanding of how populations are maintained and regulated. This thesis has addressed key components of the planktonic phase of *R. odorabile*, and is a first step in completing an important picture of how populations are maintained and ultimately provides pivotal information on how to best conserve and manage important benthic invertebrates.

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