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Natural products and their supply from the tropical sponge *Luffariella variabilis*

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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STATEMENT OF THE CONTRIBUTION OF OTHERS

Research funding for this study was provided through the Australian Institute of Marine Science, the James Cook University Finfish and Emerging Aquaculture Research Advancement Programme, CRC Reef, a Queensland Government 'Growing the Smart State PhD Funding' award, and my supervisor Professor Rocky de Nys. An Australian Postgraduate Award and a James Cook University write up grant provided stipend support.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Professor Rocky de Nys. Rocky's intellectual guidance, encouragement, support and enthusiasm, all mixed with a healthy dollop of pragmatism have been pivotal and are very much appreciated. He has been an excellent supervisor and ultimately I leave his lab with not only a PhD submitted, but with an understanding of what it takes to function as an early career scientist. I sincerely hope we will be able to collaborate in the future.

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Finally, I would not have undertaken a PhD without the support of my wonderful parents Haley Epstein and Geri Ettinger and sister Sascha Ettinger-Epstein. Thankyou so much for all your enthusiasm and love.

ABSTRACT

This thesis examines the critical link between the fundamental biology and chemical ecology of the Great Barrier Reef sponge *Luffariella variabilis* (Poléjaeff 1884) for the aquaculture based supply of bioactive metabolites. *Luffariella variabilis* produces manoalide, a high value bioactive sesterterpene used as a molecular probe. The sponge is cryptic and distributed widely through the Indo-Pacific -- this study was done on on the central Great Barrier Reef at Orpheus Island in the Palm Islands group, Queensland, Australia where *L. variabilis* is common.

The first objective of the study was to examine the natural products chemistry of *L. variabilis*. Three new acetylated compounds, 25-acetoxyluffariellin A, 25-acetoxyluffariellin B and 25-acetoxyseco-manoalide were obtained from *L. variabilis* and the structures of the three new compounds elucidated on the basis of their spectroscopic data. The known major metabolites, manoalide monoacetate, manoalide, luffariellin A and seco-manoalide were also identified.

The known major metabolites were then monitored temporally and spatially to determine the potential yield from wild harvest or aquaculture. Production of the major metabolites was hardwired with little variation in space and time at the population level in the Palm Islands. Manoalide monoacetate (35 - 70 mg g^{-1} dry weight of sponge) was always the most abundant compound followed by manoalide (15 - 20 mg g^{-1} dry weight of sponge). Luffariellin A and seco-manoalide were always 10 –70 times less abundant and varied between 0 – 3 mg g^{-1} dry weight of sponge. Collections of *L. variabilis* made at Davies Reef and Magnetic Island yielded the same rank order and yields of compounds indicating a generality of pattern over at least 100 km. The ‘hardwiring’ of metabolite production at the population level by *L. variabilis* was also reflected in the lack of any inductive effect on metabolite production. In

addition, individually monitored sponges produced fixed ratios of the major metabolites over time. However, these ratios varied between individuals with some individuals consistently producing high levels of manoalide and manoalide monoacetate. The potential for selection of high yielding stocks is discussed.

In order to explore the sustainable production of natural products via wild harvest or aquaculture, the reproductive output of *L.variabilis* was quantified and correlated with sea temperature over two reproductive seasons (2004 and 2005). *Luffariella variabilis* is gonochoristic and viviparous. Gametogenesis commenced for females at a water temperature of 21 ºC, the lowest water temperature of the year, and spermatogenesis occurred above 22.5 ºC (with sperm asynchronously developed and released from August or September to October). Females asynchronously developed oocytes from July to September, embryos from September to December, and larvae from November to December. Female reproduction terminated in December (after larval release) prior to the highest mean annual water temperature of 30 ºC in January. There was a significant (30 %) decrease in female reproductive output in 2005 compared to 2004 as measured by the reproductive index (0.91 ± 0.14 female reproductive propagules mm^{-2} of choanoderm in 2005 compared with 1.27 ± 0.11 mm^{-2} in 2004). This corresponded with delayed oogensis and spermatogenesis, and a shortened larval development cycle because of a delayed minimum temperature (21 ºC) in August of 2005 compared with July 2004. Correspondingly, the maximum percentage of the choanoderm occupied by female reproductive propagules (eggs, embryos and larvae) was also reduced by 33 % in 2005 (5.09 % in October 2004 compared with 3.44 % in October 2005). However, the mean sizes of individual female propagules remained the same from year to year. Males in contrast, showed no overall difference in either reproductive index or percentage occupation of the choanoderm between 2004 and 2005. The significantly lower reproductive output (~30 %) for *L. variabilis* associated with delayed minimum water temperatures has significant implications for population reproductive success

where oogenesis, spermatogenesis and larval release are cued by minimum and maximum water temperatures, given the predicted increases in water temperatures associated with climate change.

Determining the settlement responses of *L.variabilis* larvae is crucial in determining on-growth potential for aquaculture. The response of *L. variabilis* larvae to a hierarchy of settlement cues was examined from mid-November to late December 2005. Light cued the daytime release (0700 – 1600 hrs) of up to 830 larvae day^{-1} $sponge^{-1}$ over 5 – 6 weeks. Newly released larvae initially swam upwards. However, at 20 - 40 min post release, larvae exhibited a clear negative phototaxis and light strongly influenced their settlement. Irradiance levels of 55 µmol m^{-2} s^{-1} and 14 µmol m^{-2} s^{-1} slowed the settlement rate of larvae and inhibited overall settlement after 18 hours by ~ 60 % and 35 % respectively compared with controls. The rate of settlement and overall settlement were still significantly reduced at irradiances of >3 µmol m^{-2} s^{-1}. This corroborated with the adult distribution of *L. variabilis* in dark areas. *Luffariella variabilis* larvae are gregarious settlers with increasing rates of settlement and overall settlement with increasing densities of larvae. Gregarious settlement of *L. variabilis* larvae is associated with a conspecific larval settlement cue(s). Individual and groups of ten larvae placed in 'conditioned' water (water in which 200 larvae had previously settled) initially settled faster than controls. Furthermore, this effect was highest on single larvae with a four fold increase in overall settlement. While the rate of settlement was faster for groups of ten larvae, overall settlement totals were similar to those of controls. In contrast, cues often associated with invertebrate larval settlement such as biofilms, crustose corraline algae and adult conspecifics had no effect on settlement at any time.

In summary, the production of the major *L. variabilis* metabolites was fixed in time and space. Manoalide monoaceteate and manoalide were produced in high amounts making the sponge an ideal target for either wild harvest or aquaculture. *Luffariella variabilis* is gonochoristic, released sperm in August,

September and October and asynchronously brooded embryos over six months culminating with larval release in November and December. Larvae settled rapidly in the dark and at faster overall rates, and higher overall totals with increasing density. This was because settling larvae release a settlement cue (athough there was no effect of other common invertebrate settlement cues). The rapid settlement of larvae in dark areas corroborates with the adult distribution of the sponge and strongly suggests that biomass of *L. variabilis* for the production of manoalide could be augmented by ongrowth and culture of larvae.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter Two

Chapter Three

Chapter Four

Chapter Five

LIST OF TABLES

Chapter Two

Chapter Three

Chapter Four

CHAPTER ONE – GENERAL INTRODUCTION

Sponge Secondary Metabolites

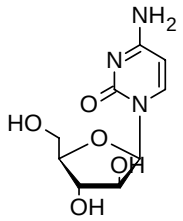
Sponges are a rich source of structurally diverse, biologically active, natural products, and they account for more than 50 % of all the metabolites reported from marine invertebrates (Faulkner, 2002). These metabolites have a range of biosynthetic origins being derived from isoprenoid, acetogenin, shikimate, amino acid, nucleic acid and carbohydrate pathways (reviewed in Ireland, 1993, Harper et al., 2001)

The diversity of marine natural products found within sponges is attributed to their sedentary filter feeding existence where they face a suite of ecological pressures including competition for space (Pawlik, 1993), predation (Pawlik et al., 1995), fouling (Engel and Pawlik, 2000, Dobretsov et al., 2005, Lee et al., 2006, Yang et al., 2006) and disease (Corriero et al., 2004, Castritsi-Catharios et al., 2005). Proposed ecological roles of sponge metabolites include anti-feedants, anti-foulants, antibiotics and photoprotective agents (reviewed in Garson, 2001, Paul and Puglisi, 2004, Paul et al., 2006). However, the putative ecological roles of most sponge secondary metabolites are not empirically demonstrated (Paul et al., 2006).

The diverse biosynthetic capability of sponges has driven intense interest in sponge metabolites for use in medicine and industry (Blunt et al., 2003, Newman et al., 2003, Piel, 2004, Paterson and Anderson, 2005, Paul et al., 2006). Sponge natural products are putative therapeutic agents in the treatment of human diseases and are currently the most important marine source of biologically active natural products for the development of therapies (Newman and Cragg, 2004a).

By far the most prominent interest in sponge natural products is as anti-cancer agents (Paterson and Anderson, 2005, Marris, 2006). This is because of their commercial potential to large pharmaceutical companies (reviewed in Newmann

and Cragg, 2004a). However, arabinosyl cytosine or Ara C **(1)** (1-ß-D-arabinofuranosyl cytosine) sold by Pharmacia & Upjohn (under the brand name Cytosar-UR) is the only marine-derived anticancer agent and is in clinical use as a potent anti-leukaemic drug (reviewed in Newman and Cragg, 2004b, Thakur and Muller, 2004). It is the result of seminal studies more than 50 years ago resulting in the discovery of spongothymidine and spongouridine from the sponge *Tethya crypta* (Bergmann and Feeney, 1951, Bergmann and Burke, 1955) from which spongouridine was modified to develop Ara C.



(1) Ara C

Sponge Natural Products And Derivatives In Pre-Clinical Evaluation And Clinical Trials For Cancer

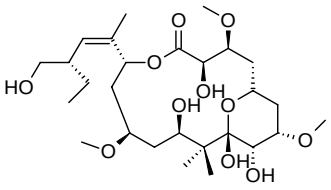
While the development of anti-cancer drugs from sponges and other marine sources has had limited success to date, there are a significant number of sponge derived compounds in preclinical evaluation, Phase I and Phase II clinical trials. There are also promising lead compounds which have yet to progress to pre-clinical evaluation. Pre-clinical evaluation assesses the pharmacological activity of the compound. If appropriate it then passes into Phase I clinical trials, where potential new drugs are assessed to evaluate safety, determine safe dosage ranges, and identify side effects. In the subsequent Phase II clinical trials safety and efficacy are tested within the intended patient population by administering the drug to up to 300 people. This process culminates in Phase III studies which administer the drug to up to 3000 people to confirm its effectiveness and monitor side effects.

Preclinical evaluation

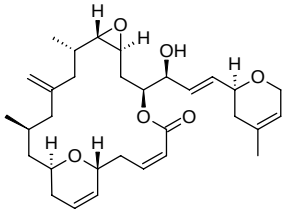
Preclinical testing is usually carried out by a pharmaceutical company and this phase demonstrates that the compound exhibits pharmacological activity in animal models consistent with its intended use in humans. Furthermore, toxicological studies carried out in at least two species (e.g. rat and dog, rat and monkey) must not show any acute toxicities. The preclinical evaluation phase contains the largest numbers of sponge derived compounds as these have yet to be rigorously tested in humans.

Of the five published sponge derived compounds in preclinical evaluation, two interfere with cell division as microtubule stabilising agents. These are laulimalide **(2)** from *Cacospongia mycofijiensis* and other sponges (Mooberry et al., 1999, Mooberry et al., 2004) and peloruside A **(3)** from *Mycale hentschelli* (Gaitanos et al., 2004, West et al., 2000). The third, dictyodendrin **(4)** from *Dictyodendrilla verongiformis* (Warabi et al., 2003) interferes with DNA replication and cell division by inhibiting telmomerase. The remaning two compounds are cytotoxins, the first of which, salicylihalamide A **(5)** from *Haliclona* sp. and other sponges, demonstrates activity against breast, colon, renal and melanomas (Erickson et al., 1997, Erickson et al., 2001, Beutler and McKee, 2003). The second, variolin B **(6)** from *Kirkpatrickia variolosa* (Perry et al., 1994), is anti-leukaemic.

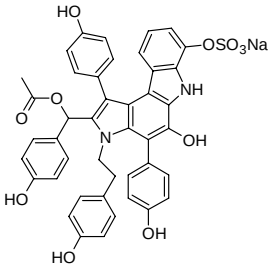
Although these compounds show strong potential as anti-cancer agents their supply for further clinical evaluation is mostly unresolved and this will need to be developed via synthesis (Newman and Cragg, 2004a,b). This is because it is difficult to collect *K. variolosa* (for the variolins) from the Antarctic and there is a lack of *Haliclona* sp. (for salicylihalamide A and other salicylihalamides) from Western Australia. This has prevented any *in vivo* work with salicylihalamide A (Newman and Cragg, 2004a,b). There is also no supply mechanism for the dictyodendrins from *D. verongiformis*. However, in one case the aquaculture of *M. hentschelli* has the potential to supply enough biomass for the extraction of peloruside A (Page et al., 2005b).



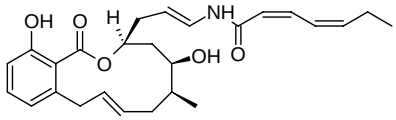
(2) Laulimalide



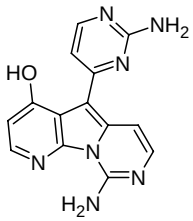
(3) Peloruside A



(4) Dictyodendrin A



(5) Salicylihalamide A



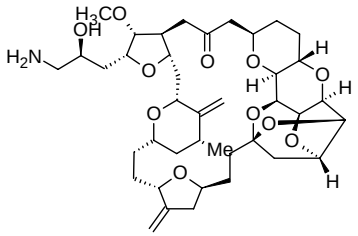
(6) Variolin B

Phase I trials

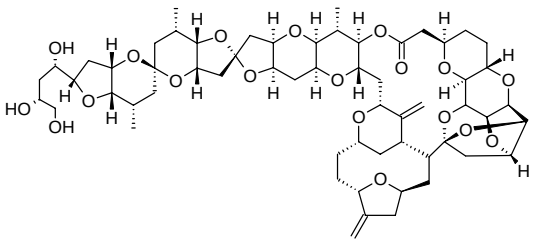
Only three sponge derived compounds are published in Phase I trials and this is a reflection of these agents completing the *in vitro* and *in vivo* (non human) preclinical testing phase. Two of these compounds are tubulin interactive agents interfering with cell division. The first of these, E7389 **(7)** is a halichondrin B **(7a)** derivative from *Lissodendoryx* sp. and other sponges (Pettit, 1994, Choi et al., 2003, Munro et al., 1999, Simmons et al., 2005) and it affects tubulin depolymerisation (Bai et al., 1991). The second is discodermolide **(8)** from *Discodermia dissolute* (Mickel et al., 2004, Mickel, 2005) which binds to microtubules more potently than the plant derived anti-cancer compound Taxol. The active site at which discodermolide binds in the microtubules may be the same as with Taxol (He et al., 2001, Paterson and Florence, 2003). Discodermolide is also immunosuppressive and acts as an incidental cytotoxin (He et al., 2001). The

third compound, KRN 7000 **(9)**, an agelasphin **(9a)** derivative from *Agelas mauritianus* (Natori et al., 1993, Natori et al., 1994, Kobayashi et al., 1995) demonstrates anti-tumor (melanoma) and potential immunostimulatory activities and is targeted as a cancer immuno-therapy agent (Freemantle, 2004, Mickel et al., 2004).

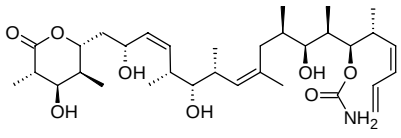
The supply of the Phase I trial compounds is better resolved compared with the preclinical compounds with discodermolide already produced from a large scale synthesis by Novartis, while aquaculture of *Lissodendoryx* has shown promise for a partial supply of halichondrin B. However, extensive synthetic work with E7389 which is efficacious at very low doses may resolve the supply of this compound (Hart et al., 2000). Similarly, synthetic research with agelasphin to produce KRN 7000 may also resolve the supply of this compound (Newman and Cragg, 2004a,b).



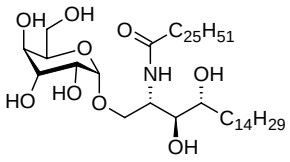
(7): E7389



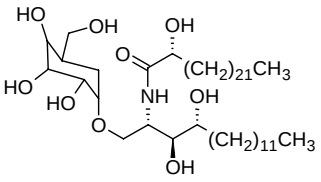
(7a) Halichondrin B



(8) Discodermolide



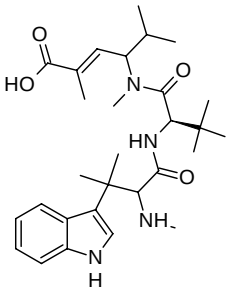
(9) KRN 7000



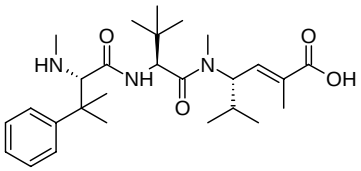
(9a) Agelasphin

Phase II trials

If Phase I trials do not demonstrate any toxicities that would preclude subsequent testing in patients compounds move into Phase II trials in which safety and efficacy are tested within the intended patient population. There is only one sponge derived compound, HTI 286, a hemiasterlin derivative from *Cymbastela* sp. and other sponges (Talpir et al., 1994) under Phase II reflecting the more rigorous nature and larger sample size of of Phase II trials. This compound interacts with tubulin to produce microtubule depolymerisation and can only be supplied by synthesis.



(10) Hemiasterlin

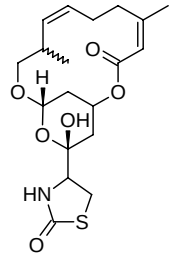


(10a) HTI 286

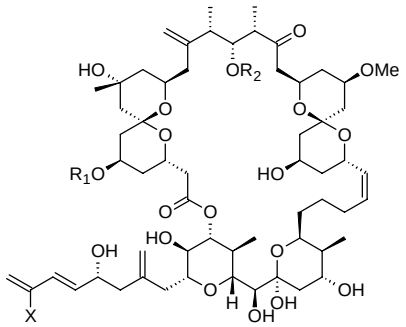
Sponge Natural Products As Anti-Cancer Lead Compounds

While sponge compounds and their derivatives feature heavily in pre-clinical, Phase I and Phase II trials, they are also a consistent source of lead compounds. Two of the best examples of potent lead compounds are Latrunculin B from *Latrunculia magnifica* (White and Kawasaki, 1992, El Sayed et al., 2006), and the spongistatins from *Spongia* spp. (Bai et al., 1993). Latrunculin B is a potent actin-active agent which interferes with the ability of cells to maintain their shape and inhibits cell division. Similarly, the spongistatins from Indian ocean *Spongia* spp. (Bai et al., 1993, Pettit et al., 1993) disrupt tubulin polymerisation interfering with mitotic divisions and induce cell death at very low concentrations (Luduena et al., 1995, Uckun et al., 2001).

A supply of latrunculin B is possible in principle via aquaculture although no scaled up ventures are currently reported (Hadas et al., 2005). In contrast, the spongistatins are found in minute quantities from 10^{-7} to 10^{-8} % of wet weight and there is no current supply mechanism. However, other *Spongia* spp. are extensively cultivated in the Meditteranean as bath sponges and transfer of aquaculture technology may be possible (Osinga et al., 1999b).



(11) Latrunculin B



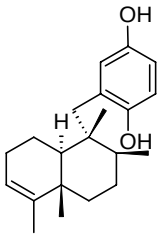
(12) Spongistatin 1 (X=Cl R_1=R_2=Ac)

Sponge Natural Products And Derivatives Under Evaluation For Other Applications

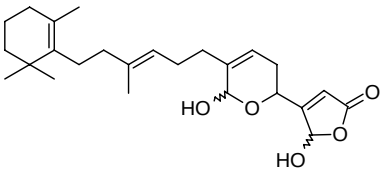
The economic realities of the drug discovery process most prominently drive interest in sponge compounds as anti-cancer agents. However, structurally diverse sponge compounds act in a wide variety of other roles, for example as anti-inflammatory agents. Two of the best anti-inflammatory examples are avarol **(13)** from *Dysidea avara* (Muller et al., 2004a) and manoalide from *Luffariella variabilis* **(14)** (Soriente et al., 1999). There are also synthetic anti-allergy/asthma compounds based on the metabolite contignasterol **(15)** from *Petrosia continginata* in Phase II and III clinical trials (Coulson and O'Donnell, 2000, Shen and Burgoyne, 2002).

In addition, the anti-herpes (HSV1) triphosphate derivative of Ara A **(16)** (1-ß-D-arabinofuranosyl adenine) derived from spongothymidin isolated from *Cryptothethya crypta* is a commonly used anti-HSV1 agent in Japan (Newman and Cragg, 2004b). There are other compounds under evaluation such as the ilimaquinone **(17)** which is anti-HIV active (reviewed in Tziveleka et al., 2003).

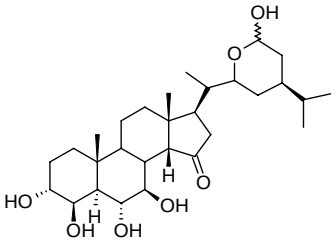
Other potential uses for sponge compounds include anti-tuberculosis (De Oliveira et al., 2006) and other anti-infective agents (Donia and Hamann, 2003, Gochfeld et al., 2003, Hamann, 2003). Furthermore, sponge natural products can also be used as bioprobes. For example, manoalide (Soriente et al., 1999) irreversibly binds to the enzyme phospholipase A_2 involved in the inflammation process in mammals but which also has a broad range of other functions. Manoalide is the industry standard compound in determining PLA_2 inhibition (Gomez-Paloma et al., 2005). Furthermore, sponge metabolites are also of interest as scaffolds for in multi-component reaction synthesis (Tietze et al., 2003, Newman and Cragg, 2004b). These promise to reduce synthesis times for compounds compared with standard linear synthesis approaches.



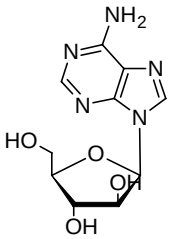
(13) Avarol



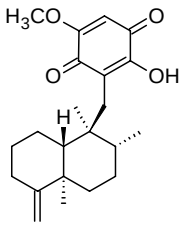
(14) Manoalide



(15) Contignasterol



(16) Ara A



(17) Ilimaquinone

The Supply Issue

After the natural product bio-discovery process of a potential therapeutic agent, demonstration of its *in vitro* and/or clinical efficacy usually precipitates a crisis of supply (Cragg et al., 1993) as grams to kilograms of natural products are often required for clinical evaluations (based on the biological activity of the compound). This is seen for many of the potential compounds in clinical trials described above (Baker et al., 1995, Hart et al., 2000, Duckworth and Battershill, 2003a, Piel, 2004) and studies have commonly ground to a halt through a lack of available material for research (Baker et al., 1995, Proksch et al., 2002). The concentrations of active compounds from marine invertebrates vary and sometimes relatively large amounts are produced. Quantities range from 3 % of the wet weight (Muller et al., 1986) down to less than 10^{-8} % (Pettit, 1996)

In addition to sometimes minute concentrations of desirable compounds, organisms that produce desirable bioactives are also often not found in abundance. Large-scale harvesting of marine organisms, such as sponges, is neither practical nor ecologically acceptable, and multi-step chemical syntheses are generally not economically viable (reviewed in Sennett, 2001, Proksch et al., 2003). This often makes supply difficult if not impossible. However, isolated cases of large scale syntheses are in place with the sponge drug target discodermolide being synthesised in commercial quantities (Freemantle, 2004, Mickel et al., 2004).

Desirable sponge metabolites are also often only found in trace amounts. For example, the concentration of latrunculin B an anti-microbial and anti-cancer lead compound ranges between 0.35 – 1.2 % of the dry weight of *Negombata magnifica* (Schmidtz, 1983, White and Kawasaki, 1992, Hadas et al., 2005). Moreover, the chemotherapeutic leads spongistatin 1 and spongistatin 9 are found in only 2.2 x 10^{-7} % and 3.4 x 10^{-7} % of sponge dry weight respectively (Norcross and Paterson, 1995).

Addressing The Supply Issue

Given the critical issue of supplying compounds for clinical trials, sponge cell culture (Pomponi, 1994, Pomponi, 1997b, Rinkevich et al., 1998, Rinkevich, 1999), microbial production of metabolites (Jensen and Fenical, 1996, Flowers et al., 1998, Garson et al., 2000, reviewed in Piel, 2004), the cloning of biosynthetic genes and their subsequent transfer to microbes (reviewed in Salomon et al., 2004, Hildebrand et al., 2004), and aquaculture (Duckworth and Battershill, 2003b, Mendola, 2003) have all been proposed to contribute to the production of bioactive compounds from sponges.

Of these options, there is only limited evidence that sponge cell cultures can be maintained in culture (Rinkevich, 1999, reviewed in Pomponi, 2006) due to microbial contamination, a lack of understanding of media in which to grow cells (Pomponi, 1994, Pomponi, 1997b, de Rosa et al., 2003), and contamination by other eukaryotes (Klautau et al., 1994). Furthermore, there are even fewer demonstrations of the synthesis of bioactive compounds from sponge cell cultures (Pomponi, 1994, Pomponi, 1997a, Muller et al., 1999, Kelve et al., 2003, Muller et al., 2004a). Similar issues exist for the isolation and culture of imputed symbiotic, commensal, or parasitic organisms to obtain bioactive metabolites from the originally invertebrate associated microbial assemblages (Piel, 2004, Piel et al., 2004, Piel, 2006). It has been estimated that only very small numbers (0.1 to 1 %) of marine microbes can be isolated and fermented using techniques modified to approximate marine conditions (Lee et al., 2006). Gene transfer is also problematic due to determining which genes are involved in the synthesis of the target metabolite before the stages are reached of developing clone libraries and the appropriate vectors (reviewed in Hildebrand et al., 2004, Muller et al., 2004a, Piel, 2006).

Herein lies the potential of aquaculture to deliver cost-effective production methods for the production of target metabolites. Aquaculture thus far, has had the best track record in the production of large amounts of biomass of target organisms

for the extraction of their metabolites (Mendola, 2003). Thus, the focus of this thesis will be on the potential of aquaculture and the critical link between fundamental biology, chemical ecology, and the supply of bioactive metabolites by aquaculture.

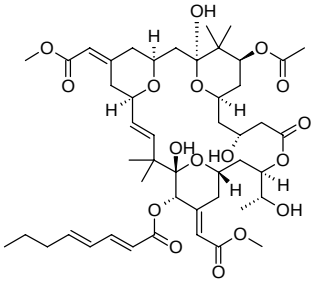
Aquaculture

The proven capability of aquaculture to provide biomass of tunicates (Mendola, 2003), bryozoans (Mendola, 2003) and sponges (Munro et al., 1999, Duckworth and Battershill, 2003b, Hadas et al., 2005, Page et al., 2005b) which produce bioactives relies on understanding the closure of lifecycles, selective breeding potential, reproductive biology, food requirements, and the environmental requisites of these target organisms.

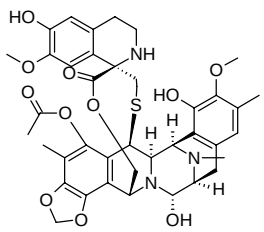
The two best cases for land and sea based systems in aquaculture are for culturing the bryozoan *Bugula neritina* for bryostatin 1 **(18)** and the colonial ascidian *Ecteinascidia turbinata* for ET 743 **(19)** (Carballo et al., 2000, Mendola, 2003) both of which have potential as anti-cancer therapies. More than 70 tons year^{-1} of *E.turbinata* have been harvested from the Mediterranean and Atlantic using simple apparatus and by fragmenting colonies to maximize growth rates (Carballo et al., 2000). Fragmentation appeared to stimulate growth rates through the production of small daughter colonies that individually have higher relative growth rates than large colonies. Therefore, absolute growth rates of a series of smaller colonies are larger than those of a single large colony of similar biomass (Stoner 1989). Neither Carballo et al. (2000) or Mendola (2003), however, reported the amount of ET 743 produced per kilogram of cultured ascidian and how this related to yields of compounds from wild harvest collections.

The aquaculture of *B. neritina* for the production of the anti-cancer compound bryostatin 1 has highlighted the importance of understanding the biology and reproductive ecology of target aquaculture species. There are two documented chemotypes of *B. neritina* (Pettit, 2002) with one chemotype living in shallow water and not producing bryostatin 1, while the other deep-water chemotype does

produce bryostatin 1 (Davidson and Haygood, 1999, McGovern and Hellberg, 2003, Mendola, 2003). Furthermore, the two chemotypes reproduce at different rates with the deep chemotype releasing only 1 – 3 % of the number of larvae of the shallower chemotype (Mendola, 2003). Moreover, the larvae of *B. neritina* have also been shown to be the lifestage of the bryozoan which contain the highest concentrations of bryostatins 10, 20 and a further uncharacterised bryostatin (Lopanik et al., 2004). These compounds are not present in the adults, protect larvae against predation by fish, and are produced by an endosymbiont *Endobugula sertula* (Lopanik et al., 2004). Therefore, the symbiont may be induced to produce additional metabolites, or its biosynthetic genes cloned to optimise bryostatin production, for biomedical applications (Haygood et al., 1999). Moreover, the bryostatin in *B. neritina* larvae could be exploited to increase bryostatin yields by harvesting larvae instead of the adult colonies. There is also a patent for the extraction and purification of bryostatins from the larvae of *Bugula neritina* and the novel bryostatin mentioned above (Lindquist and Lopanik, 2005).



(18) Bryostatin 1



(19) Ecteinascidin 743

Sponge aquaculture

In contrast to the culture of *B. neritina* and *E. turbinata* for extraction of bioactive compounds, sponge aquaculture has focused almost exclusively on the production of bath sponges in the Mediterranean Sea and the Caribbean (Osinga et al., 1999b). Large scale cultivation methods for the production of bath sponges have been well described (reviewed in Osinga et al., 1999a) and focus on the optimal methods of cutting bath sponges, attaching them to lines and site selection. The most common way of providing stock for culture is to cut a mature sponge into a number of pieces, or explants, each of which was then on-grown to generate additional biomass.

A serious complication in using explants for on-growth is that the lifecycle of the target sponge is not closed, and that wild harvest material must continually be collected. This is becoming increasingly difficult in traditional sponge grounds such as the Florida Keys and the Mediterranean as stocks are either overexploited, reduced in numbers by disease, or both (Corriero et al., 2004, Castritsi-Catharios et al., 2005). The closure of a target sponge's lifecycle frees the grower from collecting wild harvest material and may also provide the basis for the selection of desirable traits if they are heritable (Duckworth and Battershill, 2003a). However, the knowledge capital in closed lifecycle aquaculture is clearly lacking in sponges with little information on larval release, settlement, recruitment and on-culture of larvae (reviewed in Maldonado, 2006).

Moreover, the closure of lifecycles will be critical in the cultivation of sponges for the production of secondary metabolites where wild harvest cannot provide enough biomass for the extraction of bioactives (Hart et al., 2000, Simmons et al., 2005). For example, halichondrin B, a compound isolated from the sponges *Halichondria okadai* and *Lissodendoryx* sp. passed the first pre-clinical test phase as an anti cancer agent, but was suspended pending resolution of the supply issue (Munro et al., 1999, reviewed in Hart et al., 2000). It has been estimated that an annual production of several tonnes of *Lissodendoryx* sp. will be required for the clinical evaluations to proceed (Munro et al., 1999, Simmons et al., 2005) and the total available stock of wild *Lissodendory*x sp. is far smaller than this (Hart et al., 2000, Simmons et al., 2005).

Aquaculture can provide a partial solution to supplying *Lissodendoryx* sp. via explants which deliver significant gains in biomass in culture. Farms can also be scaled up into a quasi-commercial production similar to that of mussels and production of five tonnes of sponge per 100 meters of longline is possible at rates of 30 – 60 % of the halichondrin B content of wild *Lissodendoryx* sp. (reviewed in Munro et al., 1999). However, to upscale, an understanding of the lifecycle and

larval on-growth potential of *Lissodendoryx* sp. is critical as there is not enough wild supply to produce explants for culture.

Whilst the aquaculture of *Lissodendoryx* sp. has only delivered a small supply of halichondrin B, it has partially bridged the supply gap in order to allow extensive work in the synthesis of halichondrin B **(7a)** structural analogues including E7389 **(7)** (Hart et al., 2000). In this molecule halichondrin B's left hand side is substantially truncated, and in the right half of the molecule a destabilizing ester is replaced with a ketone (reviewed in Simmons et al., 2005). This synthesis of the structural analogue (a yield of < 1% and a 35 step synthesis) is currently supplying milligram quantities for clinical trials and E7389 still remains in contention in clinical trials due to its potency at very low concentrations (reviewed in Simmons et al., 2005).

Another major gap in the knowledge of sponge explant culture is a lack of understanding of the drivers in the variability in their chemical production. For example, explants of the New Zealand marine sponge *Mycale hentscheli* have been cultured to assess the production of the cytotoxic metabolites mycalamide A, pateamine and peloruside A (Page et al., 2005b). High growth rates and survival in culture were demonstrated yet there was highly differential production of metabolites. Peloruside A production (mean of 0 – 250 μg g^{-1} dry weight of sponge) was variable among wild sponges with only 50 % of the individuals sampled containing detectable concentrations. Furthermore, when explants of peloruside A containing sponges were transplanted to other sites they no longer produced the compound after approximately six months, suggesting site specific environmental influences on metabolite production. This was in contrast to both mycalamide A and pateamine which were present in all individuals. This highlights that putative environmental drivers of the variation in natural products need to be understood before any attempt at large scale supply can be undertaken. Similar considerations were required in the venture for the cytotoxic and anti-microbial latrunculins from the Red Sea sponge *Negombata magnifica* (Hadas et al., 2005). While explant growth, survival and yields of latrunculin B were

encouraging, explants were sourced from only nine broodstock and these individuals may not have reflected the true production capability of *N. magnifica*.

Critical Pre-Requistites For Sponge Aquaculture

To develop aquaculture for the production of sponge metabolites, an understanding of the variability in the production of bioactive metabolites and closing of life cycles is required. Understanding mechanisms driving variability in the production of metabolites by sponges provides quantitative grounds for the selection of high yielding stocks and optimisation of seeding, cloning, somatic growth, and production protocols for the aquaculture of desirable sponges (Pomponi, 1999, Duckworth and Battershill, 2003a).

Understanding variability in production

Understanding intraspecific variation in the qualitative and quantitative production of secondary metabolites in sponges is required as variation can occur within single members of a population (Pisut and Pawlik, 2002, Puyana et al., 2003), among members of different populations (Swearingen and Pawlik, 1998) or among populations of a given species (Page et al., 2005). Moreover, differences in the production of metabolites have been attributed to factors as wide ranging as predation (Chanas and Pawlik, 1997), habitat (Engel and Pawlik, 2000) and symbiont profile (Lee et al., 2003). Furthermore, up-regulation of sponge metabolite production occurs in response to bacterial toxins and wounding (Richelle-Maurer et al., 2003), (Muller et al., 2004b) and conversion of metabolites is also known after tissue damage (Thoms et al., 2006). It must also be taken into account that many natural products isolated from sponges and other marine macro-invertebrates are also putatively produced by symbionts (Pomponi, 1999) and cyanobacteria, and heterotropic bacteria have been demonstrated to be the producers of natural products originally attributed to the invertebrate host (Unson et al., 1994, Bewley et al., 1996).

Understanding sponge reproduction and recruitment

A critical prerequisite in determining the culture potential of sponges is their reproductive biology. Sponges employ an impressive variety of both asexual and sexual reproductive approaches (Battershill and Bergquist, 1990) despite lacking specialised reproductive tissues and organs (Knox et al., 1994). Viviparity (Ereskovsky et al., 2005, Whalan et al., 2005), oviparity (Fell, 1983, Mariani et al., 2001), gonochorism (Witte and Barthel, 1994) and forms of hermaphroditism (Fromont, 1999) are reported. Female to male (and vice versa) switches between successive reproductive seasons are also noted (Gilbert and Simpson, 1976). Subsequent to understanding the reproductive approach of a target sponge, sustainable production strategies call for the closing its lifecycle (Mendola, 2003). A key component of closing lifecyles is quantifying the factors that affect larval behaviour, settlement and metamorphosis. This then allows the heritability of compound production to be assessed from cultured larvae. Moreover, if a desired chemical makeup is heritable, it may then be cultured to optimise the production of desirable metabolites and result in the development of high yielding stocks (Mendola, 2003). Tradeoffs between growth and metabolite production can also be optimised after the development of high yielding broodstock.

Thesis Aims

In this thesis the sponge *Luffariella variabilis* (Porifera: Demospongiae) is used to address key questions relating to the supply of marine natural products. *Luffariella variabilis* is a cryptic, coral reef associated sponge distributed widely through the Indo-Pacific (Bergquist, 1980, Bergquist, 1995). Understanding the natural products chemistry (Chapter 2 & 3), reproductive biology (Chapter 4) and early life stages (Chapter 5) are key steps in the production of pharmacologically active compounds. *Luffariella variabili*s produces the potent anti-inflammatory compound manoalide (Soriente et al., 1999), a molecular tool used in the study of the inhibition of phospholipases involved in inflammation processes (Potts et al., 1992) which is extracted from wild harvest material (Biomol International LP) and is the target metabolite of this study.

In Chapter Two, the known and novel compounds of *Luffariella variabilis* are described. Previously reported compounds and the structures of three new acetylated compounds, 25-acetoxyluffariellin A, 25-acetoxyluffariellin B and 25-acetoxyseco-manoalide are elucidated on the basis of their spectroscopic data.

In Chapter Three, patterns of quantitative variation of the four major metabolites of *L. variabilis*, manoalide monoacetate, manoalide, luffariellin A and seco-manoalide were monitored seasonally over one year in the sponge at three locations (separated by kilometres to 10s of kilometres) in the Palm Islands, Queensland, Australia. Collections were also made at Magnetic Island and Davies Reef. This was used to quantify variation in *L. variabilis* metabolite production in populations separated by ~ 100 km and determine the potential yield of metabolites from wild harvest or aquaculture.

A temporal study also measured variation in the production of the major compounds at monthly intervals at Orpheus Island, Queensland over one year. As part of the design, some sponges were repeatedly sampled to determine whether amounts of the major compounds were maintained within individuals over time. Furthermore, any induction of metabolite production was quantified including the transformation of acetylated metabolites to their alcohols through hydrolysis.

In Chapter Four, the sustainable production strategies for manoalide by describing the reproduction of *L. variabilis* over two reproductive seasons quantifying the relationship between mode of reproduction, gametogenesis, larval release and temperature is explored.

In Chapter Five, the lifecycle of *L. variabilis* is closed. The release of *L. variabilis* larvae, their behaviour and settlement responses are then quantified in response to a hierarchy of cues including light and conspecifics.

The findings in each chapter are presented in a broader context and discussed in Chapter Six. This chapter argues that a multidisciplinary approach to sponge

aquaculture for the sustainable production of natural products requires a focus on chemical and reproductive ecology of target organisms. Additionally, directions for future research are proposed.

CHAPTER TWO – THE NOVEL NATURAL PRODUCTS CHEMISTRY OF *Luffariella variabilis* ▲

Introduction

An understanding of the qualitative and quantitative production of metabolites by a target organism is the first key question in the supply of any marine natural product. Therefore, the first step in determing variation in the production of metabolites by *L. variabilis* is an understanding of its natural products chemistry, specifically from the Palm Island Group of the Great Barrier Reef. This will provide the platform for quantitative studies of the spatial and temporal variation of natural products from *L. variabilis*.

Sponges of the genus *Luffariella* are widespread throughout the Indo-Pacific (Bergquist, 1980) and have afforded a wealth of bioactive sesterterpenes (de Silva and Scheuer, 1980, de Silva and Scheuer, 1981, Koenig et al., 1992, Namikoshi et al., 2004). Manoalide (**14**) was the first of a series of related compounds reported from the Palauan sponge *Luffariella variabilis* by de Silva and Scheuer (1980) who subsequently isolated seco-manoalide (**20)**, and (*E*)- and (*Z*)-neomanoalide (**21**, **22**) (de Silva and Scheuer, 1981). Kernan et al. (1987) reported the presence of two new sesterterpenes, luffariellin A (**23**) and luffariellin B (**24**), in addition to manoalide (**14**) and seco-manoalide (**20**). They also quantified variation of chemistry in 410 Palauan sponges all assigned as *Luffariella variabilis* with the ratio of these four metabolites being found to vary significantly between different sponge samples. In the current study, the isolation of three new acetylated compounds (**25-27**) and the previously reported manoalide (**14**) (de Silva and Scheuer, 1980), seco-manoalide (**20**) (de Silva and Scheuer, 1981), luffariellin A (**23**) (Kernan et al., 1987), and manoalide monoacetate (**28**) (Cambie et al., 1988) are reported.

▲ This chapter is adapted from Ettinger-Epstein, P., Battershill, C. N., de Nys, R., Motti, C., Wright, A. and Tapiolas, D. (in press) *Journal of Natural Products*.

Furthermore, it has been found that sponge storage protocols, have a significant effect on the isolated chemistry. This chapter details the isolation and structure elucidation of three new compounds and the effect of allowing samples to thaw on the presence of these secondary metabolites.

Materials And Methods

General Experimental Procedures

Optical rotations were recorded on a Jasco 715 CD Polarimeter. UV spectra were measured on a Shimadzu SPD-M10AVP PDA detector. Infrared spectra were taken on a Nicolet Nexus FTIR. ^{1}H and ^{13}C NMR spectra were recorded in neutralised $CDCl_3$ using a Bruker Avance 600 MHz NMR spectrometer with cryoprobe. The $CDCl_3$ used was slightly acidic based on a litmus paper test and was neutralized prior to use by passing it through basic alumina. Spectra were referenced to residual ^{1}H (δ 7.27) and ^{13}C (δ 77.0) resonances in the deuterated solvents. Both 1D and 2D NMR spectra were recorded using standard Bruker pulse sequences. High-resolution mass spectra were measured with a Bruker BioApex 47e FT-ICR mass spectrometer fitted with an Analytica of Branford electrospray source. Ions were detected in positive mode within a mass range of *m/z* 200-1000. Direct infusion of the sample (0.2 mg ml^{-1}) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 80 $\mu l\ h^{-1}$. HPLC was performed with a Shimadzu LC10-AT pump coupled to either a SPD-M10AVP PDA detector (analytical analyses) or a Shimadzu SPD-10A UV/VIS detector (preparative isolations). HPLC columns were purchased from Phenomenex. Compressed gases came from BOC Gases (Townsville, Australia) and were at least 99.99% pure. Purified water was obtained from a MilliQ water purification system (Millipore, Billerica, MA), all other solvents used were HPLC grade (Mallinckrodt, Hazelwood, MO, USA).

Sponge Material

Luffariella variabilis (Order: Dictyoceratida, Family: Thorectidae) was collected by hand using SCUBA at depths between 5 - 10 m at Orpheus Island (18º 35' 37" S 146º 29' 07" E) in the Palm Islands group, Great Barrier Reef, Queensland, Australia. *Luffariella variabilis* is common at the site and occurs in densities greater than 1 m^{-2}. A voucher specimen is lodged at the Australian Institute of Marine Science, Queensland, Australia (# 27405). Freshly collected sponges were frozen as soon as returned to the surface by immersion in liquid N_2 and kept frozen at -176°C. The sponges had a dark brown/black exterior and were extensively covered in fouling organisms, interiors were orange-brown.

Extraction and Isolation

The frozen sponge sample for preparative isolation was freeze-dried and extracted with CH_2Cl_2 (3 x 200 ml) at RT. The combined dried extracts (358 mg) were dissolved in MeOH and chromatographed using preparative RP HPLC [Phenomenex, Luna C18 (2), 5µm, 250 x 21 mm; gradient of CH_3CN-H_2O 70:30 to 100:0 over 60 min as eluent, flow rate 10 ml min^{-1}; UV detection at 254 nm]. A late eluting fraction was found to be rich in manoalide monoacetate (**28**) and was not further purified. The fractions containing compounds **25-27** were further purified using semi-preparative HPLC [Phenomenex, Luna C18 (2), 5µm, 250 x 10 mm; gradient of CH_3CN-H_2O 73:27 to 81:21 over 15 min as eluent with flow rate 4 ml min^{-1} and UV detection at 254 nm] to afford 25-acetoxyluffariellin A (**25**), 25-acetoxyluffariellin B (**26**), 25-acetoxyseco-manoalide (**27**), manoalide (**14**), seco-manoalide (**20**) and luffariellin A (**23**). Compounds **14**, **20**, **23** and **28** were identified by comparison of their NMR data with literature data (de Silva and Scheuer, 1980, de Silva and Scheuer, 1981, Kernan et al., 1987, Cambie et al., 1988).

25-Acetoxyluffariellin A (25): (4.41 mg, 0.08 % dry weight); colourless oil; $[\alpha]^{21}_D$= -38.1 (*c* = 0.11, $CHCl_3$); UV (PDA, CH_3CN-H_2O 70:30 to 100:0) λ_{max} (relative absorption) 196 (1), 228 (0.67) nm; IR (film) ν_{max} 3490 (br), 1797, 1766,

1211, 1026, 999 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 2.1 and 2.2; HRESIMS *m/z* 481.2577 ($C_{27}H_{38}O_6Na$ $[M + Na]^+$ required 481.2561).

25-Acetoxyluffariellin B (26): (1.14 mg, 0.02% dry weight); colourless oil; $[\alpha]^{21}_D$ = -156.1 (*c* = 0.06, $CHCl_3$); UV (PDA, CH_3CN-H_2O 70:30 to 100:0) λ_{max} (relative absorption) 200 (1), 226 (0.84) nm; IR (film) ν_{max} 3518 (br), 2362, 2335, 1761, 1679, 1210 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 2.1 and 2.2; HRESIMS *m/z* 481.2547 ($C_{27}H_{38}O_6Na$ $[M + Na]^+$ required 481.2561).

25-Acetoxyseco-manoalide (27): (3.36 mg, 0.06% dry weight); colourless oil; $[\alpha]^{21}_D$ = -42.0 (*c* = 0.11, $CHCl_3$); UV (PDA, CH_3CN-H_2O 70:30 to 100:0) λ_{max} (relative absorption) 193 (1), 228 (0.70) nm; IR (film) ν_{max} 3480 (br), 1799, 1681, 1208 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 2.1 and 2.2; HRESIMS *m/z* 481.2556 ($C_{27}H_{38}O_6Na$ $[M + Na]^+$ required 481.2561).

Analyses of sponge extracts

Each sponge sample (n = 15) was divided into two portions immediately after collection under water. Both portions of each sponge were placed separately in liquid N_2 on return to the surface. In the laboratory, one portion of each sponge was freeze dried. The remaining portion of each sponge was allowed to thaw at RT for three h before being freeze dried. All freeze dried sponge samples were extracted with CH_2Cl_2 (3 x 10 ml). The combined dried extracts were dissolved in DMSO and analysed using analytical HPLC [Phenomenex, Luna C18 (2), 5µm, 250 x 4.6 mm; gradient of CH_3CN-H_2O, 73:27 + 0.1% TFA to 81:21 + 0.1% TFA, over 35 min as eluent, flow rate 1 ml min^{-1}; UV detection at 254 nm].

Results And Discussion

The sponge *Luffariella variabilis* collected off Orpheus Island, Australia, was frozen on return to the surface by immersion in liquid N_2 and kept frozen at -

176°C. The frozen sponge was freeze-dried and extracted with CH_2Cl_2 (3 x 200 ml) at RT. The combined dichloromethane extracts (358 mg) of *Luffariella variabilis* were purified using a series of preparative and semi-preparative HPLC chromatography. This process yielded three new sesterterpenes **25-27**, as well as the known compounds, manoalide (**14**) (de Silva and Scheuer, 1980), seco-manoalide (**20**) (de Silva and Scheuer, 1981), luffariellin A (**23**) (Kernan et al., 1987), and manoalide monoacetate (**28**) (Cambie et al., 1988). A number of modifications to previous structural assignments of the known metabolites were recorded during the elucidation of these compounds. The previous isolation of luffariellin A reported doubled proton and carbon signals in the NMR spectra in purified $CDCl_3$ for the protons and carbons around the α-hydroxybutenolide and δ-lactol rings. This was consistent with a mixture of two diastereoisomers in these regions. However, in slightly acidic $CDCl_3$, only a single set of broad signals were observed. This doubling of signals has also been reported for manoalide (Tsuda et al., 1992), and related compounds (Tsuda et al., 2002). The broadening of signals in the NMR spectra for the compounds reported herein were likely the result of ring opening and closure at C-24 and C-25 under acidic conditions.

Compound **25** showed a $[M + Na]^+$ ion in its HRESIMS, consistent with the molecular formula $C_{27}H_{38}O_6$, and therefore, nine degrees of unsaturation in the form of multiple bonds and rings. The ^1H and ^{13}C NMR data of **25** showed it to contain six double bonds and therefore be tricyclic, and thus, very similar to luffariellin A (**23**) (Kernan et al., 1987). When the ^1H NMR data of **25** and **23** were compared, the differences between the two data sets were the presence of an additional methyl singlet signal at δ_H 2.18 and the downfield shift of the H-25 resonance (δ_H 7.12 in **25** compared to δ_H 6.22 in luffariellin A). The ^{13}C NMR spectral data of **25** compared to that of luffariellin A (**23**) showed additional carbon signals at 168.8 (qC) and 20.1 (CH_3) ppm and the signals associated with C-3 and C-25 were shifted (δ_C 165.5, 92.4 in **25** compared to δ_C 169.0/168.3, 98.3 in luffariellin A). These differences were consistent with **25** being the 25-acetoxyl derivative of luffariellin A (**23**). The gHMBC NMR data of **25** confirmed this and confirmed the position of the acetoxyl function. A correlation from the methyl

signal at δ_H 2.18 to C-25 (δ_C 92.4) was observed as well as correlations from H-25 (δ_H 7.12) to C-2 (δ_C 118.6) and C-3 (δ_C 165.5). These correlations are in agreement with the NMR data for both manoalide monoacetate (**28**) and thorectolide monoacetate (**29**), both of which have an acetoxybutenolide terminus (Tsuda et al., 1992). The Δ^{10} geometry was determined to be *E* based on the ^{13}C NMR chemical shift of C-23 (δ_C 16.2) (Couperus et al., 1976). Compound **25** is therefore 25-acetoxyluffariellin A. Selected ^{13}C NMR assignments and relative configurations were confirmed from the gCOSY, gHSQC and 1D selective TOCSY spectra of **25**. The chemical shifts of C-13 and C-18 resonated at δ_C 34.2 and 28.8, respectively, in contrast to the values previously reported (Kernan et al., 1987) (C-13 and C-18 at δ_C 25.9 and 34.3, respectively). The stereochemistry at C-14 relative to C-15 was determined by a 1D selective gNOESY experiment. When the H_3-22 signal (δ_H 0.72) was irradiated, NOEs were observed to the H_3-21 signal (δ_H 1.68), the signal for the adjacent methine H-15 (δ_H 1.77), and to both of the signals associated with the exo-methylene H_2-20 (δ_H 4.84, 4.65), indicating these groupings to be on the same side of the ring, and gives **25** the same relative stereochemistry at C-14 and C-15 as that previously reported (Kernan et al., 1987). The relative stereochemistry at C-4 or C-25 was unable to be conclusively determined. The axial nature of H-4 was deduced from its coupling constants (10.9, 4.0 Hz). When the H-4 signal (δ_H 4.77) was irradiated, small NOEs were observed to the signals for H-2, H_2-5, H-6, H-24, H-25, H_3-25-OAc as well to the signal for the exchangeable proton 4-OH. Consequently the relative stereochemistry at both C-4 and C-25 remain undetermined.

Compound **26** analysed for $C_{27}H_{38}O_6$ by HRESIMS. Comparison of the NMR data of compound **26** (Tables 2.1 and 2.2) with those of luffariellin B (**24**) (Kernan et al., 1987) showed the two data sets to be very similar. Where differences were apparent, they were consistent with **26** being the C-25 acetoxyl derivative of luffariellin B (**24**). These differences included additional signals for an acetate group [δ_C 169.1 (qC), 20.6 (CH_3), δ_H 2.21 (s)], and the shift of the C-3, C-25 and H-25 signals (δ_C 166.1, 93.2 and δ_H 7.21 in **26** compared to δ_C 170.4/ 169.3,

98.3/97.9 and δ_H 5.40 in luffariellin B). These differences showed **26** to be 25-acetoxyluffariellin B. As for luffariellin A the chemical shifts of C-13 and C-18 for luffariellin B required revision (Kernan et al., 1987). The relative stereochemistry of C-14 and C-15 was determined by selective gNOESY experiments to be the same as that reported for luffariellin A (Kernan et al., 1987). The relative stereochemistry at C-4 and C-25 remain unassigned.

Compound **27** had the molecular formula $C_{27}H_{38}O_6$ as determined by HRESIMS, and thus was isomeric with compounds **25** and **26**. Comparison of the 1D NMR data of compound **27** (Tables 2.1 and 2.2) with that of seco-manoalide (**20**) (de Silva and Scheuer, 1981) showed the two data sets to be similar. In the data set for **27** additional resonances in both the ^1H and ^{13}C NMR spectra [δ_H 2.20 (s); δ_C 169.1 (qC), 20.7 (CH_3)], and a shift in the signals assigned as C-3, C-25, and H- 25 (δ_C 165.9, 93.2; δ_H 7.07 respectively in **27**, compared to δ_C 169.0, 99.0; δ_H 6.15 respectively in seco-manoalide) were consistent with the presence of an acetoxyl function at C-25 in **3** rather than the OH of seco-manoalide. As for **25** and **26**, the geometry of the C-10 olefin-was determined to be *E* on the basis of the chemical shift of C-23 (δ_C 15.7). These data and all of the other physical data recorded were consistent with **27** being 25-acetoxyseco-manoalide. The relative stereochemistry at C-4 and C-25 remain unassigned.

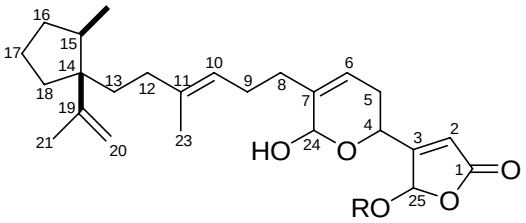
Optical rotations measured for manoalide **(14)** and manoalide monoacetate (**28**) in this study agree with published values (de Silva and Scheuer, 1980, Cambie et al., 1988) indicating that the absolute stereochemistry at C-4 in both can be assigned as R. The optical rotation of seco-manoalide isolated in this study ($[\alpha]_D$ -65.3) differed in magnitude and sign to that reported in the literature ($[\alpha]_D$ +16.2) (de Silva and Scheuer, 1981). However, because both compounds are mixtures of diastereoisomers at C-25, it is not possible to comment on the significance of this finding or the absolute stereochemistry of seco-manoalide. The optical rotation measured for luffariellin A ($[\alpha]_D$ -32.0) was of similar magnitude and opposite to that reported in the literature ($[\alpha]_D$ +40.1) (Kernan et al., 1987). The relative

stereochemistry at C-14 and C-15 in the luffariellin A isolated in the current investigation was shown by selective gNOESY experiments to be the same as reported (Kernan et al., 1987). Due to both compounds being isolated as mixtures of diastereoisomers, it is not possible to confirm unequivocally the current compound as the antipode of that previously reported (Kernan et al., 1987).

The presence of the new acetylated compounds (**25-27**) in the sponge extracts was significantly affected by the sponge tissue work-up protocols. A small number of sponge samples, which were known to contain the acetylated compounds (**25-27** and **28**), were sub-sampled and thawed before being lyophilized and extracted. Only the non-acetylated compounds (**14**, **20** and **23**) were isolated from the DCM extracts of these samples. Subsequently, sponge samples (n=15) were divided into two *in situ* and immediately returned to the surface where they were immersed in liquid N_2. In the laboratory, one piece of each collected sponge was allowed to thaw for three hours prior to being freeze-dried, with the remaining piece kept frozen until freeze dried. In the sponge samples that were kept frozen before being freeze dried and extracted, the acetylated compounds (**25-27** and **28**) together with compounds **14**, **20** and **23** were isolated. However, in the sponge samples that were allowed to thaw before being freeze dried and extracted, only the non-acetylated compounds **14**, **20**, and **23** were detected. Once isolated, the acetylated compounds **25-27** and **28** are stable, suggesting that their "instability" in the sponges is enzyme mediated. Presumably the enzymes that are active in the freshly collected sponge remain viable in the frozen material. As such, the acetylated compounds may be precursor storage metabolites that can be hydrolyzed enzymatically to the alcohols that have a predetermined function, for example, defense. Activated defenses where biologically inactive acetylated metabolites are enymatically hydrolysed to biologically active alcohols or aldehydes have been previously reported in the algae *Halimeda* spp. (Paul and Van Alstyne, 1992) and *Caulerpa taxifolia*, *C. prolifera* and *C. racemosa* (Jung and Pohnert, 2001). Recent reports have also postulated enzymatic cleavage of brominated isoxazoline alkaloids into more active monocylic nitrogenous compounds (aeroplysinin-1 and dienones) as an

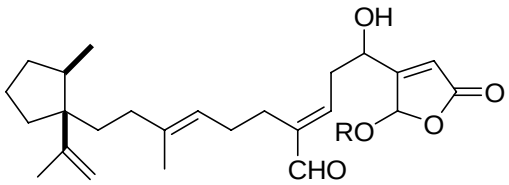
activated defense mechanism after mechanical wounding in the sponge *Aplysina* sp. (Thoms et al., 2006).

In conclusion, this qualitative determination of the natural products chemistry of *L. variabilis* from the central Great Barrier Reef provides the platform for quantitative studies for the spatial and temporal variation of the production of natural products from *L. variabilis*.



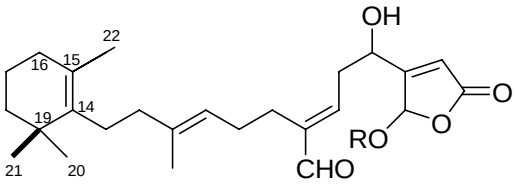
R = Ac **25**

R = H **23**



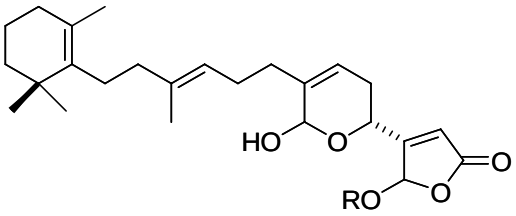
R = Ac **26**

R = H **24**



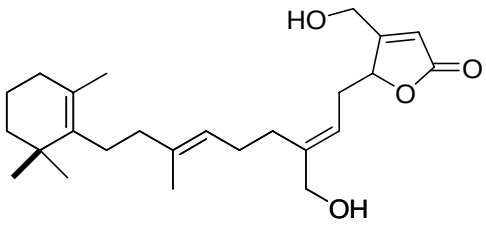
R = Ac **27**

R = H **20**

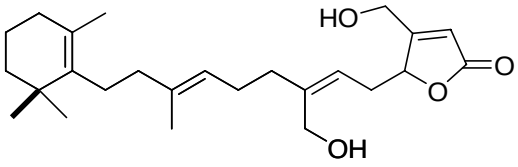


R = Ac **28**

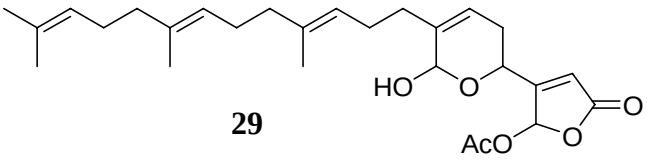
R = H **14**



21



22



29

Figure 2.1: Novel and known natural products reported from *L. variabilis.*

Table 2.1: ^{1}H NMR Spectroscopic Data (600 MHz, $CDCl_3$) for compounds **25-27**

	25		26		27	
Pos'n	δ_H (*J* in Hz)	gHMBC	δ_H (*J* in Hz)	gHMBC	δ_H (*J* in Hz)	gHMBC
1						
2	6.10 (s)	1, 3, 4, 25	6.15 (s)	1, 3, 4, 25	6.15 (s)	1, 3, 4, 25
3						
4	4.77 (dd, 10.9, 4.0)	2, 3, 25	4.68 (dd, 7.0, 4.5, 4.0)	2, 3, 5, 6	4.68 (dd, 7.1, 5.2, 4.0)	2, 3, 5, 6,
4-OH			2.42 (d, 4.5)		2.40 (d, 5.2)	
5	2.28 (ddd 17.1, 4.3, 4.0) 2.32 (dd 17.1, 10.9)	3, 4, 6, 7	2.81 (dt, 15.4, 7.1) 2.89 (ddd, 15.4, 7.1, 4.0)	4, 6	2.82 (dt, 15.4, 7.1) 2.91 (ddd, 15.4, 7.1, 4.0)	4, 6
6	5.71 (d, 4.3)	4, 5, 8, 24	6.53 (t, 7.1)	4, 5, 7, 8, 24	6.55 (t, 7.1)	4, 5, 7, 8, 24
7						
8	2.14 (m)		2.32 (t, 7.6)	7, 9, 10, 24	2.35 (t, 7.6)	7, 9, 10, 24
9	2.15 (m)		2.08 (m)	8, 10, 11	2.11 (m)	8, 10, 11
10	5.10 (dt, 1.0, 6.7)	8, 9, 12, 23	5.08 (br t, 7.2)	8, 9, 12, 13, 23	5.13 (br t, 7.2)	8, 9, 12, 23
11						
12	1.71 (m) 1.76 (m)		1.68 (m) 1.74 (m)		1.98 (m)	
13	1.36 (m) 1.40 (m)		1.32 (m) 1.38 (m)		2.01 (m)	
14						
15	1.77 (m)		1.76 (m)			
16	1.30 (m) 1.94 (m)		1.32 (m) 1.94 (m)		1.91 (t, 6.2)	
17	1.61 (m) 1.72 (m)		1.61 (m) 1.72 (m)		1.56 (m)	
18	1.48 (m) 1.75 (m)		1.46 (m) 1.72 (m)		1.42 (m)	
19						

Table 2.1: continued

	25		26		27	
20	4.65 (s)	14, 19, 21	4.64 (s)	14, 19, 21	0.99 (s)	18
	4.84 (s)	14, 19, 21	4.83 (s)	14,19, 21		
21	1.68 (s)		1.68 (s)		0.99 (s)	18
22	0.72 (d, 7.0)	16	0.71 (d, 7.0)	16	1.60 (s)	16
23	1.60 (s)	10, 12	1.56 (s)	10, 12	1.62 (s)	10, 12
24	5.31 (d, 4.5)	6, 8	9.44 (s)	6, 8	9.45 (s)	6, 8
24-OH	2.82 (d, 4.5)					
25	7.12 (s)	2, 3	7.07 (s)	2, 3	7.07 (s)	2
25-OAc	2.18 (s)		2.21 (s)		2.20 (s)	

Table 2.2: ^{13}C NMR Spectroscopic Data (125 MHz, $CDCl_3$) for compounds **25-27**

Position	**25**	**26**	**27**
	δ_C, mult.	δ_C, mult.	δ_C, mult.
1	169.2, qC	168.7, qC	168.6, C
2	118.6, CH	119.5, CH	119.8, CH
3	165.5, qC	166.1, qC	165.9, qC
4	61.5, CH	65.9, CH	65.8, CH
5	27.9, CH_2	34.2, CH_2	33.9, CH_2
6	120.6, CH	146.6, CH	146.2, CH
7	136.9, CH	146.2, qC	146.5, qC
8	32.3, CH_2	24.3, CH_2	24.4, CH_2
9	25.6, CH_2	26.4, CH_2	26.7, CH_2
10	122.6, CH	122.1, CH	121.9, CH
11	136.4, qC	137.7, qC	137.7, qC
12	34.7, CH_2	34.8, CH_2	40.1, CH_2
13	34.2, CH_2	34.2, CH_2	27.8, CH_2
14	55.1, qC	55.1, qC	136.9, qC
15	41.8, CH	41.9, CH	127.1, qC
16	30.6, CH_2	31.0, CH_2	32.7, CH
17	20.2, CH_2	20.6, CH_2	19.5, CH_2
18	28.8, CH_2	29.5, CH_2	39.8, CH_2
19	148.0, qC	148.1, qC	35.0, qC
20	111.6, CH_2	111.8, CH_2	28.6, CH_3
21	20.2, CH_3	20.7, CH_3	28.6, CH_3
22	17.7, CH_3	18.2, CH_3	19.8, CH_3
23	16.2, CH_3	16.3, CH_3	15.7, CH_3
24	91.4, CH	194.6, qC	194.2, qC
25	92.4, CH	93.2, CH	93.2, CH
25-OAc	168.8, qC	169.1, qC	169.1, qC
	20 .1, CH_3	20.6, CH_3	20.7, CH_3

CHAPTER THREE – SPATIAL AND TEMPORAL PRODUCTION OF THE MAJOR METABOLITES OF *Luffariella variabilis*

Introduction

Both marine and terrestrial taxa produce suites of secondary metabolites, with broad ecological (reviewed in Rosenthal and Berenbaum, 1992, Pawlik, 1993, Paul et al., 2006) and applied (Newman and Cragg, 2004a,b, Paterson and Anderson, 2005) roles. Sponges account for more than 50 % of marine metabolites (Faulkner, 2002) and provide a rich source of structurally diverse, biologically active natural products derived from a variety of metabolic pathways (reviewed in Ireland, 1993, Harper et al., 2001). Bioprospecting for natural products, many of them sponge derived, is an important source of drug leads (Capon, 2001, Tan et al., 2006, Hunt and Vincent, 2006) and the diverse biosynthetic capability of sponges has driven intense interest in their metabolites for use in medicine and industry (Duckworth and Battershill, 2003a, Newman et al., 2000, Newman et al., 2003, Paterson and Anderson, 2005).

However, there have been major issues in supplying many of the compounds with putative bio-medicinal properties for clinical evaluation, a critical next step in the drug development process (Baker et al., 1995, Hart et al., 2000, Piel, 2004). This is because many metabolites are produced in extremely small quantities and wild collection simply cannot meet demand (Norcross and Paterson, 1995, Duckworth and Battershill, 2003a, Page et al., 2005b). In order to address this supply issue, mechanisms to increase yield are important, and as a first step understanding the interspecific variation of sponge natural products and the mechanisms driving their production is critical. This will then permit the selection of high yielding stocks allowing for the optimisation of seeding, cloning, somatic growth, and production protocols for the aquaculture of desirable sponges (Pomponi, 1999, Duckworth and Battershill, 2003a).

Despite the intense interest in the supply of sponge metabolites for pre-clinical and clinical trials, descriptions of the variation in their production (which form the basis for any harvest effort) are rare (Paul et al., 2006). The most rigorous descriptions of variation in the production of metabolites from marine organisms is for the brown algal phlorotannins (Hay and Steinberg, 1992, Hay, 1996, Targett and Arnold, 1998), which provide important models of how these metabolites co-evolved under selective biotic pressures such as herbivores (Van Alstyne, 1988, Cronin and Hay, 1996b, Targett and Arnold, 1998) and pathogens (Dixon, 2001), and how environmental factors such as nutrients, temperature, light, desiccation and salinity affect their production (Yates and Peckol, 1993, Arnold et al., 1995, Cronin and Hay, 1996a, Pavia et al., 1997, Van Alstyne and Pelletreau, 2000, Jormalainen et al., 2001).

While there is a complex interplay between the biological and environmental factors influencing the production of phlorotannins by the brown algae, such information is scarcely understood in sponges (Pawlik, 1993, Paul et al., 2006). Spatial variation in the quality and quantity of sponge metabolites can occur within single members of a population (Pisut and Pawlik, 2002, Puyana et al., 2003); among members of different populations (Swearingen and Pawlik, 1998) or among populations of a given species (Page et al., 2005a). Moreover, the differences in the production of metabolites have been attributed to predation (Chanas et al., 1997, Swearingen and Pawlik, 1998, Dunlap and Pawlik, 1998), habitat (Engel and Pawlik, 2000), ontogeny (Swearingen and Pawlik, 1998), alleopathic interactions (Thacker et al., 1998), geographic location and depth (Chanas et al., 1997, Duckworth et al., 1997) and symbiont profile (Lee et al., 2003, reviewed in Piel, 2004, Ridley et al., 2005).

Variability in sponge metabolite production is also driven by their chemical defence mechanisms either induced (Steel et al., 2002, Muller et al., 2004b); (Richelle-Maurer et al., 2003) or activated (Thoms et al., 2006).When metabolite biosynthesis is induced, the effects develop slowly over hours to days, and factors driving changes in metabolite production mostly relate to the presence of harmful

microorganisms (i.e. the priorities of resource allocation in the affected organism may be shifted from growth to defence) (Muller et al., 2004b, Richelle-Maurer et al., 2003). In contrast, attack-induced transformation (e.g. after wounding) of stored inactive precursor compounds yielding more active transformation products with defensive activity is rapid (Thoms et al., 2006). Such reactions are known to be catalysed by enzymes in macroalgae (Paul and Van Alstyne, 1992, Jung and Pohnert, 2001, Van Alstyne et al., 2001, Van Alstyne and Houser, 2003) and phytoplankton (Wolfe and Steinke, 1996, Wolfe et al., 1997, reviewed in Pohnert, 2004). However, the mechanisms of induced transformation are poorly understood in sponges.

Luffariella variabilis produces a range of related natural products including the potent anti-inflammatory compound manoalide (de Silva and Scheuer, 1980, de Silva and Scheuer, 1981) the supply of which relies on wild harvest. Ultimately, the sustainable production of manoalide from natural sources, as for all marine natural products, will rely on an understanding of the culture potential of *L. variabilis* and a prerequsite knowledge of the variation in the production of its chemistry in time and space (Mendola, 2003). *Luffariella variabilis* also possesses an array of acetylated compounds and their corresponding alcohols (Chapter 2) with the potential that the acetylated compounds may hydrolyse to the corresponding alcohols after artificial wounding. Under this scenario, the amount of manoalide would be predicted to increase in contrast to the amount of manoalide monoacetate which would decrease.

In this study the variability in the production of the four major metabolites, manoalide monoacetate, manoalide, luffariellin A and seco-manoalide found within *L. variabilis* is examined. Specifically, seasonal patterns in the production of the four major compounds found within *L. variabilis* at three locations in the Palm Islands Group and at one time at Magnetic Island and Davies Reef are determined. Furthermore, temporal variation in the production of the major metabolites at Orpheus Island is determined and whether *L. variabilis* exhibits activated or induced production of metabolites.

Materials And Methods

Secondary metabolites of Luffariella variabilis

Luffariella variablis (Poléjaeff 1884) is a cryptic, coral reef sponge distributed widely through the Indo-Pacific (Bergquist, 1980, Bergquist, 1995) and produces a variety of bioactive sesterterpenes (de Silva and Scheuer, 1980, de Silva and Scheuer, 1981, Kernan et al., 1987) (Chapter 2). Manoalide monoacetate, manoalide, luffariellin A and seco-manoalide are the four major metabolites of *L. variabilis* from the central Great Barrier Reef (Chapter 2).

Study locations and design

To investigate spatial and temporal variation in the production of the major *L. variabilis* metabolites in the Palm Islands group of the central Great Barrier Reef (GBR), Queensland, Australia, adult sponges were sampled in Spring 2003, the Austral summer of 2003 and 2004, Autumn 2004 and Winter 2004. Twenty five adults were sampled on shallow (4 - 8 m) patches of reef at Orpheus Island (18º 35' 37"S 146º 29' 07"E) at each time. *Luffariella variabilis* is common within the central GBR and occurs in densities greater than $1m^{-2}$. Ten further sponges were collected at each time at similar depths at South Orpheus Island (18º 38' 35"S 146º 29' 32" E) and Fantôme Island (18º 41' 22"S 146º 31' 31" E). Samples were collected by removing a $4cm^3$ piece of mesohyl from near the top surface of the sponge. In addition, ten opportunistic samples were collected from Davies Reef (18º 49' 58"S 147º 38' 77" E) and five samples from Magnetic Island (19º 06' 09"S 146º 51' 46" E) in Spring 2003.

To determine finer scale monthly variation in the production of metabolites, a further 40 *L. variabilis* were sampled each month between July 2005 and June 2006 at Orpheus Island (18º 35' 37"S 146º 29' 07"E). Seventeen of the 40 sponges were tagged at the commencement of the study and repeatedly sampled by removing a $4cm^3$ piece of mesohyl from near the top surface of the sponge each month. The remaining 23 samples were collected from different sponges each

month. The same sponge was not sampled twice with clear cut mark defining previously sampled sponges.

Relationships of compound amount to sex of sponge

There was a decrease in the production of manoalide monoacetate and corresponding increase in the production of manoalide during the spawning period of *L. variabilis* (see Results section). To further investigate whether the amount of manoalide monoacetate and manoalide produced by individual sponges was associated with the sex of the sponge, seven reproductive females were identified as per the methods described in Chapter 4 in November 2005. The amounts of the metabolites were the compared with seven sponges which were previously tagged and known to be male (Chapter 4).

***Induced and activated defences of** Luffariella variabilis*

This experiment determined whether production of the major metabolites was activated (minutes to hours) or induced (hours to months) by artificial wounding of *L. variabilis*. Fifty *L. variabilis* individuals were marked in an area of ~200 x 10 m area at North Pioneer Bay, Orpheus Island. These were then randomly allocated to treatments and controls (n = 5 per treatment and control) where the sponges were artificially wounded by stabbing each individual or maintained as controls. At time = 0 all treatment sponges (n = 25 total) were stabbed over the entire sponge body (~ one stab 3 cm^{-2}) with a scalpel to depth of ~1 - 2 cm. Treatments (n =5) and controls (n =5) were then sampled at 0 mins, 3 hrs, 8 hrs, 24 hrs and 1 month after wounding and the amounts of the major metabolites measured for all samples.

Extraction, standard isolation and high performance liquid chromatography

The concentrations of the four major metabolites in *L variabilis* were determined using high performance liquid chromatography (HPLC) following the methods of

Chapter 2. Sponge samples were individually bagged underwater and on return to the surface, individually wrapped in aluminium foil and placed in liquid nitrogen. Pieces were subsequently freeze dried and 0.2 - 0.4 g dry weight of sponge extracted (3 x 10 ml) with dichloromethane (DCM). The three combined extracts were then filtered through glass wool and redissolved in 4 ml of dimethyl sulfoxide (DMSO) containing benzophenone 0.343 mg ml^{-1} as an internal standard prior to analysis by HPLC. Benzophenone was chosen as the internal standard as it is stable at room temperature and eluted with excellent baseline separation and peak shape prior to the compounds from the extracts. The manoalide group of compounds are stable at normal room temperature under normal light conditions and collection and handling is unlikely to have had any effect on the concentrations. HPLC analysis was performed using a Shimadzu LC10-AT pump coupled to a SPD-M10AVP PDA detector with a Phenomenex, Luna C18 (2), 5µm, 250 x 4.6 mm column. The HPLC was run on a gradient of acetonitrile: water. 73:27 + 0.1% trifluoroacetic acid (TFA) to 81:21+ 0.1% TFA over 35 minutes as eluent with a flow rate 1ml min^{-1} and with UV detection at 220 nm. Standards used in the HPLC analyses were isolated from DCM extracts of freeze dried *L. variabilis.* Metabolites were identified by comparison of ^{1}H and ^{13}C NMR (nuclear magnetic resonance) data (Chapter 2)

Amounts of seco-manoalide, luffariellin A, manoalide and manoalide monoacetate were quantified by measuring the peak areas for each compound and the internal standard. The ratio of peak areas (compound : internal standard) was calculated for each metabolite and converted to concentration by reference to standard curves. Standard curves were created at five concentrations for each of the four metabolites in DMSO containing an internal standard of 0.343 mg ml^{-1}. To obtain five concentrations of each metabolite, serial dilutions were performed to halve the highest starting concentration until the fifth and lowest concentration was reached. The highest starting concentrations for each standard curve were 10.65 mg ml^{-1} for manoalide monoacetate, 12.09 mg ml^{-1} for manoalide, 3.42 mg ml^{-1} for luffariellin A, 1.75 mg ml^{-1} for seco-manoalide. R^2 values for each standard curve were > 0.95 and all samples run were within the range of the standard curves.

Statistical analysis

Hypotheses were tested using repeated measures, multivariate or univariate analyses of variance (ANOVA). Assumption of normality and homogeneity of variance was checked graphically for each dataset prior to analysis by plotting residuals, and data were transformed where necessary (Quinn and Keough, 2002). Variance-covariance sphericity of the data used in any repeated measures general linear model (GLM) was estimated using the Greenhouse-Geisser ε (epsilon). Where MANOVAs were undertaken co-variance was tested for sphericity by the Mauchly criterion and where data was nonspherical the Huyn-Feldt adjusted degrees of freedom, exact *F*, and probabilities are presented. All analyses were done using SPSS (version 12). Logarithmic or sine transformations were performed as appropriate and are shown in the relevant table legends.

Spatial and temporal patterns of quantitative variation in the four main metabolites of *L. variabilis* in the sponges collected seasonally were analysed using a two factor multivariate general linear model (GLM) with time and location fixed factors. As samples were collected seasonally at South Orpheus and Fantôme Islands, only the corresponding seasonally collected Orpheus Island samples were used in this anaylsis.

Temporal patterns of quantitative variation of seco-manoalide, luffariellin A, manoalide and manoalide monoacetate in the repeatedly cut sponges were analysed using a repeated measures GLM with time and sponge (blocked factor) as the within subject factors, and sponge and compound as the between subject factors. Sponge was used as a blocked factor as it was possible that individual secondary metabolites were not independent of one another (Pennings and Paul, 1993, de Nys et al., 1996). To determine whether repeatedly sampling sponges had any effect on the amounts of secondary metabolites compared with controls, a one factor multivariate GLM with treatment as a fixed factor was run for every month. The production of compounds in repeatedly sampled sponges was never different from

controls so data are only presented for June 2006 after the sponges had been repeatedly sampled 12 times. At this point it would be most likely that any detectable change to the secondary metabolite composition or amounts would be evident.

Temporal patterns of quantitative variation of secondary metabolites in the monthly controls were analysed using a one factor multivariate GLM with month as a fixed factor.

Relationships of compound amounds to sex of sponge

A two factor GLM with sex (fixed) and compound (fixed) was used to determine whether the amounts of manoalide and manoalide monoacetate varied between female and male *L. variabilis* in November 2005.

Induced and activated production of metabolites in Luffariella variabilis

A two factor multivariate GLM with treatment (fixed) and time (fixed) was used to analyse the production of the major metabolites over time in treatment and control sponges.

Results

Spatial and temporal variation

There was no spatial and temporal variation in the amount of major secondary metabolites between individuals throughout all seasons at all locations. The rank order of the compounds was always consistent with manoalide monoacetate > manoalide > luffariellin A = seco-manoalide. Mean amounts of manoalide monoacetate were ~35 – 50 mg g^{-1} dry weight of sponge (range: 10 – 170 mg g^{-1}), manoalide ~15 - 20 mg g^{-1} (range: 5 – 45 mg g^{-1}) dry weight and luffariellin A and seco-manoalide were always between 0 - 3 mg g^{-1} dry weight (range: 0 – 13 mg g^{-1}). These results were demonstrated by non significant time ($F_{12,444} = 1.16$; $p = 0.30$), location ($F_{8,336} = 0.95$; $p = 0.46$) and time*location ($F_{24,587} = 0.82$; $p = 0.70$)

terms (Fig. 3.1.; Table 3.1). The one time opportunistic samples collected at Magnetic Island and Davies Reef (separated by >100 km) yielded similar results with mean ranges of between 25.08 - 27.93 mg g^{-1} of manoalide monoacetate; 17.30 - 21.53 mg g^{-1} of manoalide; 2.60 - 5.10 mg g^{-1} luffariellin A and 0.77 - 1.19 mg g^{-1} of seco-manoalide. This demonstrated that in September 2003 there was a generality of pattern in compound production across a range of spatial scales from metres to more than 100 km.

Finer scale temporal variability

To determine fine scale monthly variation in compound production, the amounts of the major compounds were measured in the same individuals and also in freshly collected individuals (controls) each month. Production of manoalide monoacetate and manoalide (the two most abundant compounds) was hardwired and did not vary significantly with time (Tables 3.2, 3.4; Fig. 3.2 a-b). This was in contrast to luffariellin A and seco-manoalide which did vary significantly over time. However, the scale of the variation was small (Table 3.2, 3.4; Fig. 3.2a-b). There was no difference in the amounts of compounds between repeatedly sampled and control sponges in June 06, the time at which the repeatedly sampled sponges had been sampled 12 times (Table 3.3).

Mean amounts of manoalide monoacetate were always between ~30 - 75 mg g^{-1} dry weight of sponge and manoalide between ~15 - 20 mg g^{-1} dry weight of sponge in both repeatedly sampled and control sponges (Fig. 3.2a-b). Luffariellin A and seco-manoalide amounts were always an order of magnitude less with between 0.1 and 3 mg g^{-1} dry weight of sponge (Fig. 3.2a-b). The amount of manoalide monoacetete decreased over November and December in both control and repeatedly sampled sponges while manoalide increased over this time period, however these effects were not significant. The amount of luffariellin A was approximately 2mg g^{-1} dry weight of sponge from June 2005 to January of 2006 before reducing to ~1mg g^{-1} dry weight of sponge. This was similar the amount of seco-manoalide over the duration of the study.

The variation in the production of metabolites in different among months was demonstrated by a significant time*compound term ($F_{26,392} = 1.78$; $p < 0.01$) term (Table 3.2). However, the monthly variation was driven by changes in the amount of luffariellin A (1- Factor repeated measures GLM $F_{6,78} = 3.69$; $p < 0.003$) when individual repeated measures GLMs were run for each compound. There were no effects of the blocked factor 'sponge'.

A non significant treatment term ($F_{4,35} = 1.09$; $p = 0.37$) demonstrated the lack of effect of repeated sampling on individual sponges (Fig. 3.2; Table 3.3).

In the control sponges, the significant month term in the MANOVA ($F_{44,992} = 1.70$; $p < 0.001$) (Fig. 3.2, Table 3.4) was driven by significant month terms for both seco-manoalide ($F_{11,273} = 3.29$; $p < 0.001$) and luffariellin A ($F_{11,273} = 2.70$; $p < 0.003$).

Relationships of compound amounds to sex of sponge

Female sponges did not contain differing amounts of manoalide monoacetate and manoalide compared with males (1-Factor ANOVA $F_{1,24} = 0.503$; $p = 0.485$). Amounts of manoalide monoacetate and manoalide found in both female and male *L. variabilis* were consistent with the amounts found in both the spatial and temporal studies with mean levels of manoalide monoacetate at ~35 - 50mg g^{-1} dry weight of sponge and manoalide at ~15 - 20 mg/g dry weight of sponge.

***Induced and activated production of metabolites in** Luffariella variabilis*

Wounding had no effect on the amount of any compound at any time reflecting the 'hardwiring' in the production of the major *L. variabilis* metabolites. This was demonstrated by a non-significant time, treatment and treatment*time terms (Fig. 3.3; Table 3.5). Furthermore, the mean compound levels were similar to levels in the both the long term spatial and temporal and temporal studies with amounts of

manoalide monoacetate between 35 - 65 mg g^{-1} dry weight of sponge, manoalide between 12 - 20 mg g^{-1} dry weight, luffariellin A and seco-manoalide between 0 - 3mg g^{-1} dry weight of sponge.

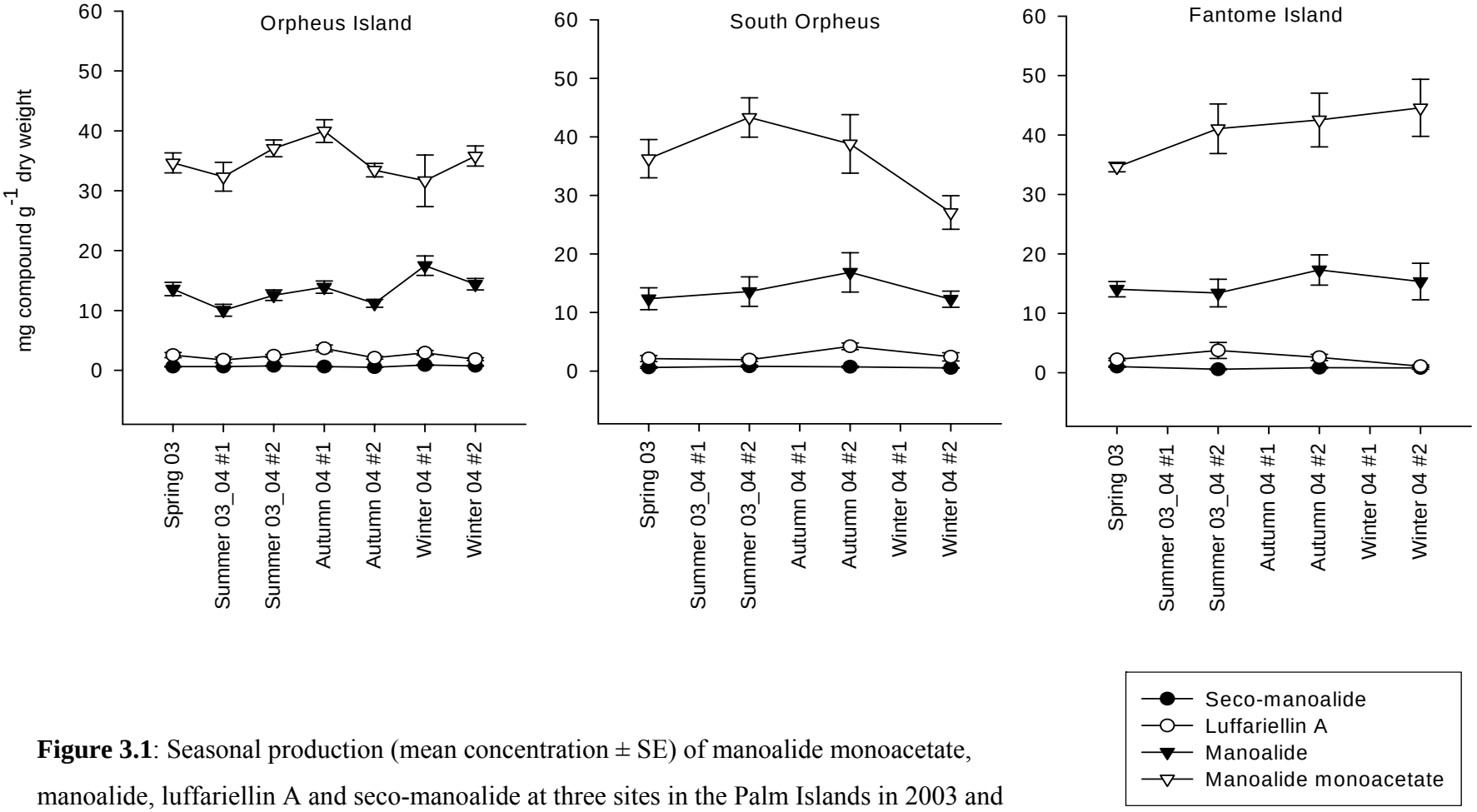


Figure 3.1: Seasonal production (mean concentration ± SE) of manoalide monoacetate, manoalide, luffariellin A and seco-manoalide at three sites in the Palm Islands in 2003 and 2004.

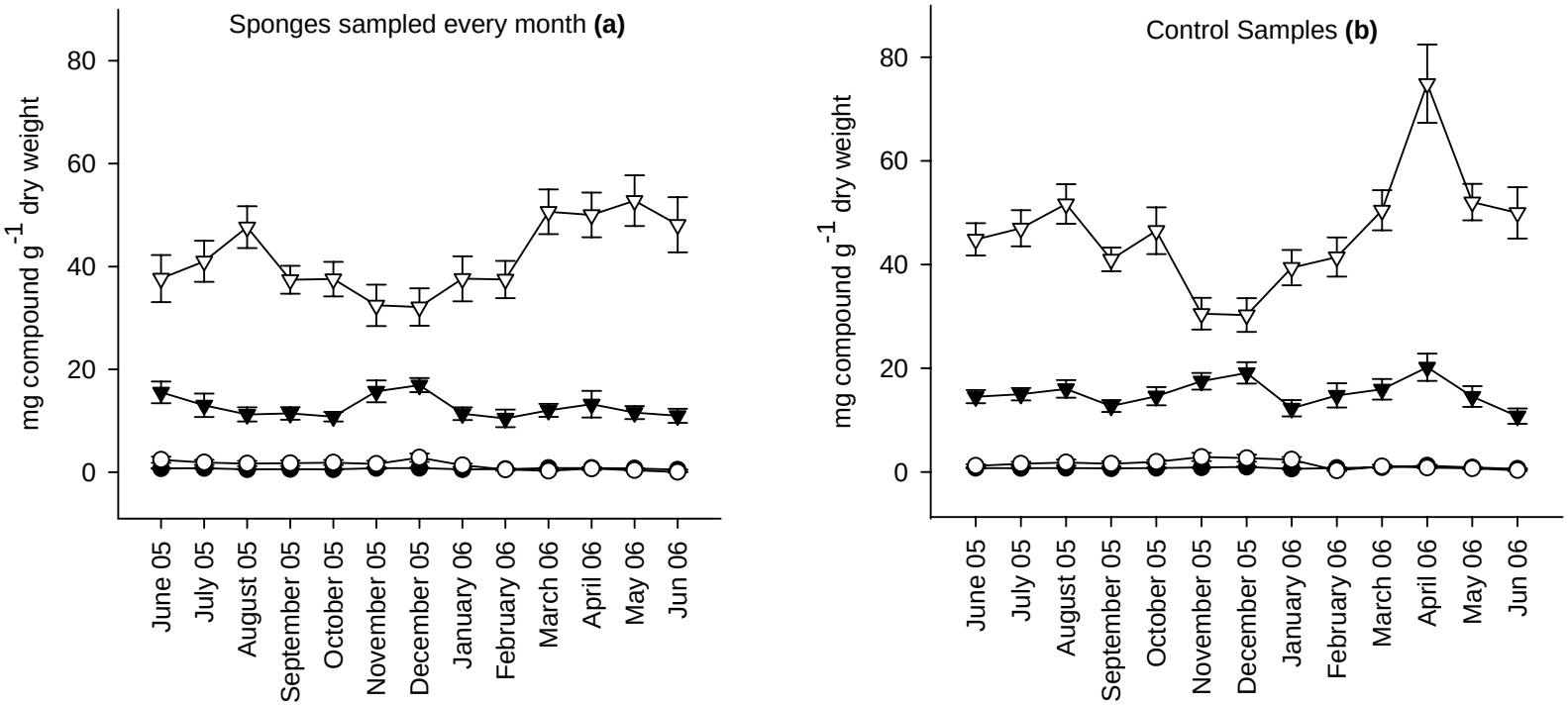


Figure 3.2a: Fine scale monthly production (mean concentration ± SE) of manoalide monoacetate, manoalide, luffariellin A and seco-manoalide in repeatedly sampled sponges.

Figure 3.2b: Fine scale monthly production (mean concentration ± SE) of compounds in sponges collected each month as 'controls'. These sponges had never previously been sampled.

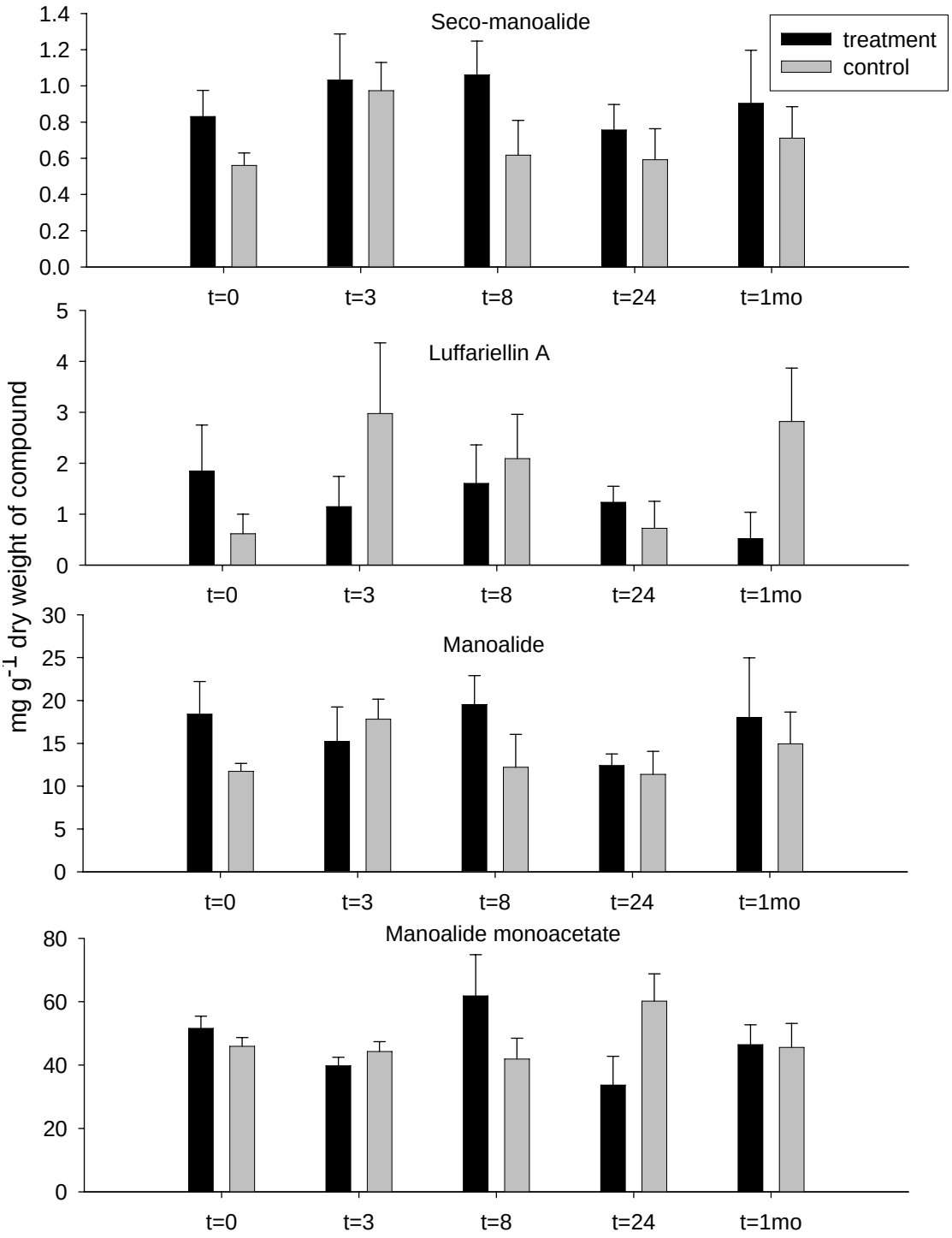


Figure 3.3: Mean concentration ± SE of seco-manoalide, luffariellin A, manoalide and manoalide monoacetate in sponges after wounding. Controls are unwounded sponges.

Table 3.1: MANOVA on sin (x) transformed data testing spatial and seasonal variation in the production of *L. variabilis* metabolites.

Effect	Value	***F***	Hypothesis df	Error df	p
Time	0.92	1.16	12.00	444.77	0.303
Location	0.95	0.95	8.00	336.00	0.462
Time * Location	0.89	0.82	24.00	587.29	0.706

Table 3.2: Repeated Measures GLM on sin $[(x+1)^2]$ transformed data testing monthly production of major metabolites. Sponge is included as a blocked factor.

	Source	df	MS	***F***	p
Within subjects	Time	8.71	0.65	1.16	0.314
	Time * Sponge	130.73	0.50	0.89	0.771
	Time * Compound	26.14	1.00	1.78	0.010
	Error (time)	392.20	0.56		
Between Subjects	Sponge	15	0.36	0.54	0.892
	Compound	3	8.72	12.93	<.001
	Error	45	0.67		

Table 3.3: MANOVA on sin $[(x+1)^2]$ transformed data comparing production of the major metabolites in cut vs. control *L. variabilis* in June 06. Note that at this time cut sponges had been repeatedly sampled 12 times.

Effect	Value	***F***	Hypothesis df	Error df	p
Treatment	0.89	1.09	4.00	35.00	0.374

Table 3.4: MANOVA on sin $[(x+1)^2]$ transformed data testing the monthly production of the major metabolites.

Effect	Value	***F***	Hypothesis df	Error df	p
Month	0.75	1.70	44.00	992.82	<.001

Table 3.5: MANOVA on ln (x+1) transformed data testing the effect of wounding on the production of the major metabolites.

Effect	Value	***F***	Hypothesis df	Error df	p
Treatment	0.80	2.26	4.00	37.00	0.082
Time	0.62	1.21	16.00	113.67	0.264
Treatment * Time	0.57	1.43	16.00	113.67	0.147

Discussion

Production of the four major sesterterpenes from *Luffariella variabilis*, manoalide monoacetate, manoalide, luffariellin A and seco-manoalide is 'hardwired'. Accordingly, the rank abundance of the four metabolites was always consistent with manoalide monoacetate and manoalide 10 to 70 times more abundant than luffariellin A and seco-manoalide. In addition, single collections made at Davies Reef and Magnetic Island yielded the same rank order and similar overall amounts of compounds demonstrating a generality of pattern over at least 100 km. At a finer scale, repeatedly sampled individual sponges also produced the four compounds in fixed ratios throughout the duration of the study demonstrating that individuals were also hardwired for compound production. However, the fixed ratios of the compounds were different between differing individuals. For example, one of the repeatedly sampled *L. variabilis* in the study produced small amounts of manoalide monoacetate, more luffariellin A compared with its analogue manoalide and small amounts seco-manoalide. This was in contrast to the remaining individuals, which always produced larger amounts of manoalide monoacetate, less luffariellin A vs. manoalide and larger amounts of seco-manoalide.

This variation in the ratio of major *L. variabilis* metabolites was also reported by Kernan et al., (1987) for three of the four major metabolites (via ^{1}HNMR) from 410 *L. variabilis* collected in Palau. Five percent of samples contained only the luffariellins A and B, eight percent contained a mixture of seco-manoalide, the luffariellins A and B and manoalide, and the remainder contained only seco-manoalide and manoalide. Sponges containing these three different ratios of metabolites were called 'chemotypes'. However, these data were in contrast to this quantitative study where there were no clearly definable chemotypes and *L. variabilis* produced a mix of all four major metabolites in consistent ratios in an individual over time.

The differing 'hardwired' ratios of metabolite production between *L. variabilis* individuals suggests a genetic contribution to metabolite production which is masked by sampling at the population level. Polymorphisms are well known within terpene synthase genes in plants and contribute to hardwired production of terpenes in these organisms (reviewed in Huber et al., 2004). Thus, the determination of the genotypes of *L. variabilis* may explain the fixed ratios of production of metabolites of *L. variabilis*. Futhermore, microbial symbionts are frequently postulated to be the producers of metabolites in marine organisms (Piel, 2004, Piel, 2006, Paul et al., 2006). Their contribution to metabolite production in *L. variabilis* is unknown and future studies will need to determine the eukaryote and prokaryote contribution to metabolite production.

From a production perspective, if terpene ratios are heritable in *L. variabilis* the ability to select and culture individual sponges with desirable ratios of metabolites may result in high yielding stocks. In this regard, the production of the major *L. variabilis* metabolites warrants further study given its large geographic range throughout the Indo-Pacific (Bergquist, 1995). It is possible that the major metabolite ratios vary geographically and that this information may support any wild harvest effort, in particular the use of higher yielding stocks or development of broodstock for closed lifecycle aquaculture for the production of metabolites.

In contrast to *L. variabilis* on the central Great Barrier Reef, marked seasonal, temporal and spatial variation in the production of secondary metabolites is described for other sponges. In the temperate sponge *Mycale hentschelli* in New Zealand, it is postulated that environmental variables lead to differential spatial and temporal production in the metabolites mycalamide A, pateamine, and peloruside A (Page et al., 2005a). Whilst no ecological hypotheses were directly tested, mycalamide A concentrations are greater in sponges found on deeper, high-energy reefs, whereas pateamine was only present in individuals in estuarine areas.

Concentrations of peloruside A varied significantly among estuarine individuals, but peloruside A was rarely present in samples from deep reef habitat. Furthermore, metabolite concentrations tended to increase in the spring as the water temperature increased, but declined before peak temperatures in the summer in the high-energy environment. Similar cyclical variations in metabolite production (e.g. increases during spring and summer) have been previously interpreted as preventing surface fouling for *Latrunculina wellingtonensis* (Duckworth and Battershill, 2001) and inhibiting competitors for space (allelopathy) on a seasonal basis in *Crambe crambe* (Turon et al., 1996).

Over the summer spawning season for *L. variabilis* (see Chapters 4 and 5) there was a decrease in manoalide monoacetate concentration with a concomitant increase in manoalide concentration. Whilst not significant (and there were no differences in manoalide monoacetate amounts between males and females), *L. variabilis* may sequester manoalide monoacetate within its larvae and further investigation is required. A similar scenario was postulated for the ascidian *Cystodytes* sp. where during seasonal larval release in summer, concentrations of two major metabolites, deacetylshermilamine B and shermilamine B decreased (whereas they displayed no variation at other times of the year) (Lopez-Legentil et al., 2006). Some *Cystodes* sp. zooids produced larvae almost equal to their bodyweight (Tarjuelo and Turon, 2004) and larval release (assuming sequestration) was proposed by the authors to drive the low concentrations of deacetylshermilamine B and shermilamine B observed after sexual reproduction.

As there was no variation in the amount of the major metabolites produced by *Luffariella variabilis* over time or in space and wounding caused no changes to the amounts of any of the major products present, the major *L. variabilis* metabolites are probably not costly to produce and most likely represent a hardwired constitutive defence.

Most theories for the evolution of chemical defences assume they are metabolically 'costly' (Herms and Mattson, 1992, Simms, 1992, Bergelson and Purrington, 1996, Tollrian and Harvell, 1999, Strauss et al., 2002, Stamp, 2003). That is, the presence of a defence decreases an individual's fitness when resources are limiting. Hence the amount of energy invested in defending tissue should correspond with the value of the tissue, and that activated/inducible defenses are more energy efficient than constitutive defenses (Zangerl and Rutledge, 1996, Toth et al., 2005). This is because there is low turnover of the costly defensive metabolites or production of defensive metabolites is only initiated in response to a stress factor (Dworjanyn et al., 2006). However, this does not appear to be the case for *L. variabilis.* Further work should be undertaken to determine whether the major metabolites of *L. varaibilis* are used for defence (Lindquist, 2002) and whether individual metabolite ratios are heritable (Wright et al., 2004).

In conclusion, the high level of production of the major metabolites of *L. variabilis* is hardwired at the population level. However, there is significant and consistent variation between individuals. Futher understanding of this variation and investigating the heitability of the trait will facilitate improved yield and act as a model for metabolite production in other species. The first step in this process is an understanding of the reproductive biology of *L. variabilis* (Chapter 4) and the processes affecting larval settlement and metamorphosis (Chapter 5).

CHAPTER FOUR - THE REPRODUCTIVE BIOLOGY OF *Luffariella variabilis*

Introduction

Elucidation of the reproductive and sexual strategies used by marine organisms is central to understanding broader ecological processes driving population distributions, abundances, dynamics, and gene flows. This is particularly true for sessile marine invertebrates where reproductive strategies are wide ranging and result in a complex array of life histories (Strathmann, 1985, Jackson, 1986, Ward, 1995). Strategies employed by marine invertebrates to optimise recruitment demonstrate a strong link between the number and size of propagules and subsequent survivorship of offspring (Caley et al., 1996, Hall and Hughes, 1996, Fautin, 2002, Underwood and Keough, 2001). An additional trade-off involves the minimum size at which sexual reproduction can commence (Hall and Hughes, 1996, Kapela and Lasker, 1999, Smith and Hughes, 1999). Reproductive strategies to maximise the arrival of recruits into populations include the variation of sex ratios (Kramarsky-Winter and Loya, 1998), mechanisms of fertilisation (i.e. external vs. internal) (Ward, 1995), and the mode of larval feeding (planktotrophy vs. lecithotropy) (Strathmann, 1985, Fautin, 2002).

This variability in the strategies to optimise recruitment is displayed broadly in the Phylum Porifera. Sponges employ an impressive variety of both asexual and sexual reproductive capability (Battershill and Bergquist, 1990) despite lacking specialised reproductive tissues and organs (Knox et al., 1994). Viviparity (Ereskovsky et al., 2005, Whalan et al., 2005), oviparity (Corriero et al., 1998, Mariani et al., 2001), gonochorism (Fell, 1983, Witte and Barthel, 1994) and forms of hermaphroditism (Fromont, 1999) have been reported. Furthermore, changes in sexual strategies, in particular the switch from female to male (and vice versa), occurs for some species between successive reproductive seasons (Gilbert and Simpson, 1976, Fell, 1983).

While some fundamentally physiological aspects of sponge reproduction such as gametogenesis, cytodifferentiation of gametes, and larval development are well studied (see reviewed in Fell, 1983, Simpson, 1984) less is known about the spatial and temporal variability of reproduction and reproductive output. Quantification of the relationships between body size, sexual maturity, fecundity, recruitment, and subsequent adult distribution are rare in sponges. Furthermore, associations of patterns of sponge reproduction with key biological and environmental drivers influencing sexual strategies, reproductive output, and measures of fecundity, show no clear trends (e.g. Ayling, 1980, Fromont, 1994a, Uriz et al., 1995).

Among the various environmental factors influencing reproduction in marine invertebrates, water temperature is frequently invoked to have the most significant effect (Simpson, 1984, Fell, 1983, Witte and Barthel, 1994, Corriero et al., 1996). Temperature dependent regulation of reproduction is common in corals (see reviews in Fautin, 2002), sea cucumbers (Morgan, 1995), sea urchins (Muthiga, 2003), and barnacles and oysters (Ruwa and Polk, 1994). Temperature is also an important parameter affecting sponge reproduction for sponges in tropical and temperate systems. Fromont (1999) demonstrated the initiation of gamete development for five temperate demosponges when water temperatures were increasing or reaching their maxima in Western Australia. Similar patterns have also been shown for sponges in the Mediterranean (Lepore et al., 1995) and Japan (Watanabe, 1978). Despite tropical systems generally exhibiting less seasonal variation in environmental parameters some tropical sponges also display similar seasonal patterns of gametogenesis (Ilan and Loya, 1990b). In contrast, some sponges reproduce year round (e.g. Ilan and Loya, 1990a) in both tropical and temperate environments suggesting that not all reproduction is controlled by exogenous (environmental) cues.

The sexual reproduction of the coral reef associated sponge *Luffariella variabilis* (Polejaeff 1884) was examined in the context of a broader study of its biology, chemical ecology, and aquaculture potential, and determined the relationship between gametogenesis, larval release, and temperature, over two

reproductive cycles. The specific aims were to determine seasonality, modes and potential cues for reproduction of *L. variabilis* and describe sex ratios and the process of gametogenesis, documenting size at sexual maturity, and the relationships between size and levels of fecundity.

Materials And Methods

Study Site and Sampling Design

Luffariella variabilis is a cryptic, coral reef sponge distributed widely through the Indo-Pacific (Bergquist, 1980). All samples were collected on a shallow area of coral reef (4 - 8m) at Orpheus Island (18º 35' 37" S 146º 29' 07" E) in the Palm Islands group, of the northern Great Barrier Reef, Queensland, Australia. Water temperature was measured in 6 m of water in the vicinity of 18º 36' 768 S; 146º 28'.986 E (Orpheus Island Research Station - James Cook University) by a data logger. *Luffariella variabilis* is common at the site and occurs in densities greater than 1 m^{-2}. The length, width and height of each sponge from which samples were taken was measured *in situ* to estimate sponge volume.

To investigate sexual reproduction in *L. variabilis*, 40 adult sponges were sampled monthly between January 2004 and February 2006 (two reproductive seasons). Seventeen of the 40 sponges were repeatedly sampled by removing a 4 cm^3 piece of mesohyl from different areas of the sponge each month (these are referred to as 'tagged' individuals). The remaining 23 samples were collected from different sponges each month. There was no effect of repeated sampling of individual sponges on any of the variables measured (see Results section) and all data presented are combined (n = 40).

Histological Analysis

All samples for reproductive analysis were histologically prepared by first fixing a 4 cm^3 piece of each sponge in formalin, acetic acid calcium chloride mixture (FACC) for at least 24 hrs. This was followed by a dehydration series (Windsor, 1994) and embedding in paraffin. Thin sections (5 μm) were made

using a rotary microtome (Swift) and subsequently stained with haemotoxylin and eosin to highlight reproductive propagules (spermatic cysts, eggs, embryos, and larvae).

One section for each sponge was observed on a Leica DM-LB microscope with attached camera using IM50 image manager software. Three representative fields of view of 1 mm^2 were then taken of each section. Sponges were identified as male or female based on the presence of eggs, embryos, or larvae (female) or spermatic cysts (male). Non-reproductive sponges were classified by the absence of reproductive propagules.

To quantify patterns and timing of reproduction, the number of eggs, embryos and larvae (females), or spermatic cysts (males), were counted in each of the three fields of view. The numbers of propagules were then adjusted to give a mean value $1mm^{-2}$ as the 'reproductive index - RI' for each section (sponge). In addition, the outline of each propagule was traced using the IM50 software to determine its surface area and to give a mean area of propagules within a section. This was multiplied by the reproductive index to give a mean percentage area occupied by reproductive propagules for each sponge. Sizes of individual reproductive propagules, and larvae were also compared from year to year to determine whether patterns of sizes were consistent. Furthermore, the volume of each adult sponge was estimated (from the measured length, width and height *in situ*) to determine any relationship with number of reproductive propagules.

Statistical analysis

Sex ratios were analysed by comparing observed versus expected ratios of 50 : 50 in *L. variabilis* by performing chi-squared goodness of fit tests run in October 2004 and 2005, the months where most reproductive *L. variabilis* were found.

Three factor ANOVAs were used to elucidate differences in reproductive index, percentage area of section occupied by reproductive propagules and size of reproductive propagules amongst months (fixed factor) and between

years (fixed factor). This design also determined whether repeatedly sampled (fixed factor) sponges and control sponges were different (Underwood 1981).

ANOVAs were only performed for males in September and October of 2004 and 2005 as there was no male reproduction in August of 2004. ANOVAs for females were run in August to December of 2004 and 2005 as females were most reproductive over this period.

Assumptions of normality and homogeneity of variance were checked graphically by plotting residuals for each dataset prior to analysis, and data were transformed where necessary (Quinn and Keough, 2002).

Results

Patterns of Reproduction

Luffariella variabilis is gonochoristic, viviparous and an asynchronous developer of eggs, embryos and larvae for females (Fig. 4.1a-c) and spermatocytes for males (Fig. 4.1d-e). There were clearly identifiable reproductive propagules visible in histology sections enabling a comprehensive examination of reproduction by both males and females over time.

The overall reproductive pattern of *L. variabilis* was consistent between years but reproductive individuals were detected later in 2005 for both females (July 2004 and August 2005) and males (August 2004 and September 2005). In both years only females were initially identified with the identification of males and females in the subsequent months (Fig. 4.2).

Oogensis commenced in July 2004 and in August 2005 and corresponded with the lowest water temperatures for each year (~21 ºC). Spermatogenesis commenced when water temperatures began to rise above 22.5 ºC in both years and reproductive males were first detected in August 2004 and September 2005. The later detection of both female (August 2005) and male

reproduction (September 2005) corresponded with the delayed minimum temperature (~21 ºC) and the subsequent water temperature increase. The timing of larval release (November-December) prior to the maximum water temperature (~30 ºC) remained unchanged between years (see Reproductive Output- Females section below). This resulted in a shorter period for embryogenesis in 2005 (one month) with a concomitant decrease in overall reproductive output for females of 30 %.

There was no difference between the reproductive status in all tagged individuals and control individuals at any time, with repeated sampling having no effect on reproductive measurements. Furthermore, in tagged sponges, eggs, embryos, larvae and sperm were detected from month to month in unison with controls demonstrating that this sampling strategy reliably detected reproductive products in the mesohyl.

Sex Ratios

L. variabilis is gonochoristic (Fig. 4.2). The sex ratio (male : female) of *L. variabilis*, was assessed in October 2004 and 2005, the period when greatest numbers of both male and female reproductive individuals were detected. The sex ratio in *L. variabilis* was not significantly different from 50:50 (ratio of male to female 20:13; $\chi 2 = 1.96$ $p = 0.223$; ns in 2004 and ratio of male to female 16:8; $\chi 2 = 2.60$ $p = 0.102$; ns in 2005).

Reproductive output - Females

To construct a reproductive index (RI) for *L. variabilis* females the numbers of eggs, embryos and larvae were quantified mm^{-2} of each section. Development of embryos occurred asynchronously. Only eggs (Fig. 4.1a) were detected in July 2004 (mean of 0.33 mm^{-2}) or August 2005 (mean of 0.5 mm^{-2}) and increased in September 2004 (mean of 1.16 mm^{-2}) and September 2005 (mean of 0.75 mm^{-2}). Eggs then declined in both October 2004 (0.40 mm^{-2}) and 2005 (0.30 mm^{-2}) as fertilisation occurred and embryogenesis commenced. Embryos (Fig. 4.1b) were subsequently detected in very small numbers in September 2004 (0.01 mm^{-2}), increasing in both October 2004 (mean of 1.33 mm^{-2}) and 2005 (mean of 1.19 mm^{-2}), before declining in November 2004 (mean of 0.45

mm^{-2}) and November 2005 (mean of 0.19 mm^{-2}) as fully formed larvae were detected. Larvae (Fig. 4.1c) were first detected in November 2004 (0.96 mm^{-2}) and 2005 (0.59 mm^{-2}) as they differentiated from embryos, before declining (as females became spent) to 0.50 mm^{-2} in December in both years (Fig. 4.3 a,b). No larvae were detected after December.

There was a significant decrease in overall RI between 2004 and 2005 with females exhibiting a 30 % lower mean RI in 2005 than 2004 (2005 - 0.91 ± 0.14 mm^{-2}; 2004 - 1.27 ± 0.11 mm^{-2}) indicated by a significant year term ($F_{1,81}$ = 7.41; $p < 0.008$) (Table 4.1a). The overall pattern of RI was consistent between years but the magnitude of the RI varied amongst sampling periods indicated by a significant sample period x year term ($F_{4,81}$ = 2.85; $p < 0.029$) (Fig. 4.3a-b; Table 4.1a).

Concomitant with a lower RI, was a significant 33 % lower overall percentage section area occupied by female propagules in 2005 (3.44 %) compared with 2004 (5.09 %). This was indicated by a significant year term ($F_{1,81}$ = 4.54; $p < 0.036$) (Fig. 4.4; Table 4.1b). However, the pattern of mean percentage female section area remained the same in both years with values increasing in July, August, and September, reaching maximum values in October as the number of embryos peaked. The percentage occupation of the choanoaderm then began to decline in both years as larvae were released in November and December (Fig. 4.4).

Cross sectional areas of individual female propagules (eggs, embryos and larvae) were not different between years despite the shortened development time available for embryos in 2005. This demonstrated that whilst minimum temperature onset delayed the production of eggs and led to a reduced number of reproductive propagules, the sizes of individual propagules did not vary over both 2004 and 2005 indicated by a non-significant year term ($F_{1,81}$ = 1.10; $p = 0.297$) (Table 4.1c). Cross sectional areas of female propagules (Fig. 4.3c) ranged from 310 μm^2 (eggs) to 124789 μm^2 (larvae) and there was also no relationship between the number of female propagules and sponge size for any month.

Larvae were parenchymellae (Fig. 4.1c), ciliated, hollow and spheroid, ~300 – 400 μm in length and ~200 μm wide with a band of cilia at the aboral pole as is typical of larvae of the Dictoceratida (Subclass Ceractinomorpha, Order Dictyoceratida) (Maldonado and Bergquist 2002).

To determine fecundity of reproductive females correlations between RI and size of female were made in October, November and December (2004 and 2005) when the most females were detected (total n = 67). No correlations were found for any month, however, there was a significant positive correlation between size and total fecundity ($r^2 = 0.82$; $p < 0.001$).

Reproductive output – Males

To construct a reproductive index (RI) for males the numbers of spermatic cysts were quantified mm^{-2} of each section. Development occurred asynchronously (Fig. 4.1d, e, f) and numbers of spermatic cysts reached a maximum in September 2004 (mean of 28 mm^{-2}) and 2005 (mean of 25 mm^{-2}). Spermatic cyst number then decreased in October 2004 (mean of 5 mm^{-2}) and 2005 (mean of 8 mm^{-2}) as sperm were released. Males were detected a month later in 2005 (Fig. 4.5a) as the mean temperature (22.5 ºC) correlated with spermatogenesis was not reached until September 2005 (compared with August of 2004).

Males exhibited no difference in overall mean RI between years (15.17 ± 1.92 mm^{-2} in 2004 vs. 16.64 ± 3.04 mm^{-2} in 2005) indicated by a non-significant year term ($F_{1,67} = 1.65$; $p = 0.203$) (Table 4.2a). Similarly, there was no difference in mean male percentage section area between years indicated by a non significant year term ($F_{1,67} = 3.40$; $p = 0.07$) (Table 4.2b) and the pattern of percentage section area was also similar (range of 3.9 – 4.9 % in September and 0.6 - 1.2 % in October). Section percentage area was 1.8 % for August 2004 only (Fig. 4.4).

Cross sectional areas of spermatic cysts were different between years (Fig. 4.5b) indicated by a significant year term ($F_{1,67} = 5.86$; $p < 0.018$) (Table 4.2c). Areas of spermatic cysts ranged from 1051 μm^2 to 1675 μm^2 and there was also no relationship between the number of spermatic cysts and sponge size for any month.

To determine fecundity of reproductive males, correlations between RI and size of males were made in September and October, the months where the most males were detected (total n = 91). No correlations were found for any month, however, there was a significant positive correlation between size and total fecundity ($r^2 = 0.21$; $p < 0.001$).

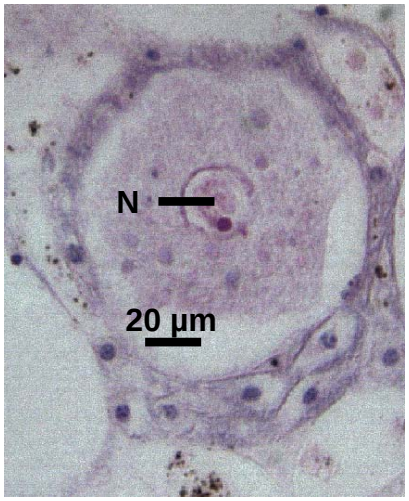


Figure 4.1a

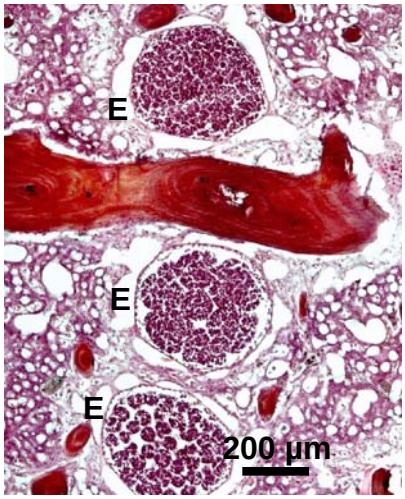


Figure 4.1b

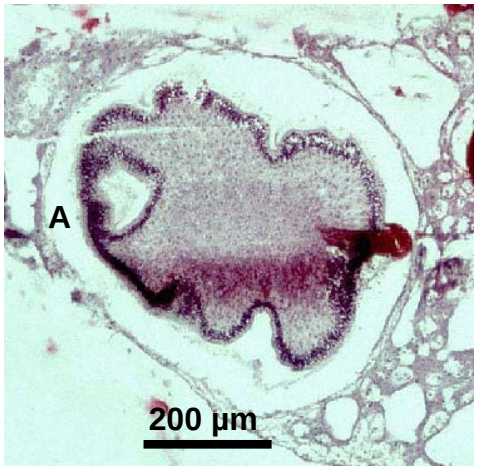


Figure 4.1c

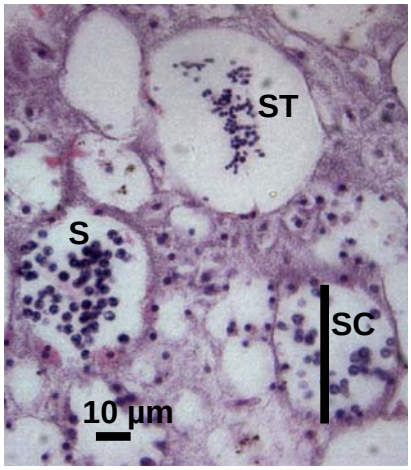


Figure 4.1d

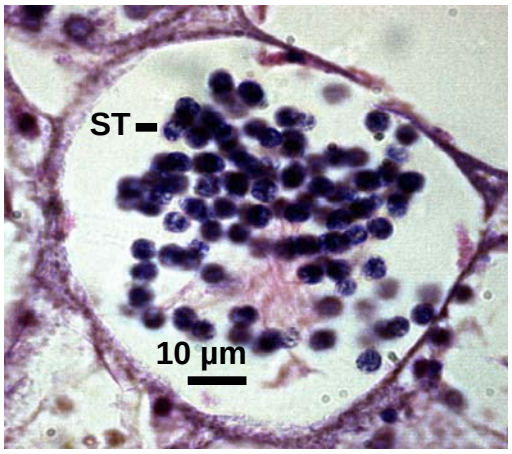


Figure 4.1e

Figure 4.1. Histology images of reproductive structures in *L. variablis*. **(4.1a)** Early egg with nucleus (N) and yolk granules. **(4.1b)** Asynchronous development of embryos (E). The youngest embryo is in the centre. A primary collagen fibre is also visible (C). **(4.1c)** Fully formed larva showing ciliated surface and ring of cilia (A) at the aboral pole. **(4.1d)** Spermatic cyst (SC), spermatocytes (S) and spermatids (ST). **(4.1e)** Spermatids (ST) within a single spermatic cyst. Scale bars; A = 20 µm, B,C = 200 µm, D,E = 10 µm

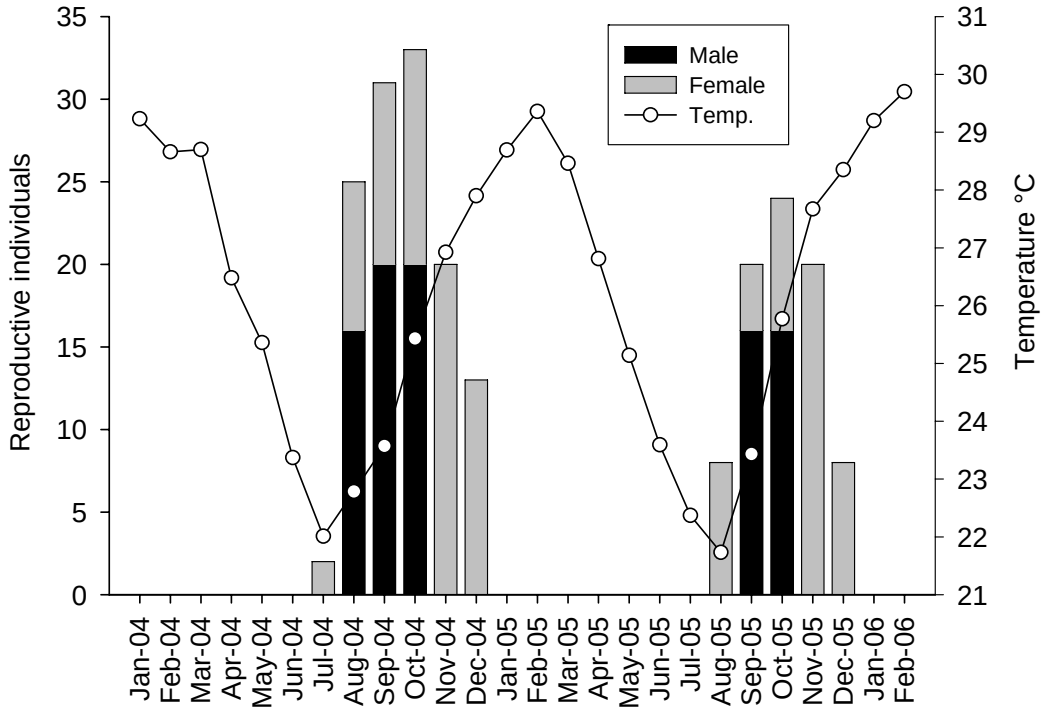


Figure 4.2: Numbers of reproductive males and females (out of n=40 total collected each month) and mean monthly water temperature over 2004 and 2005.

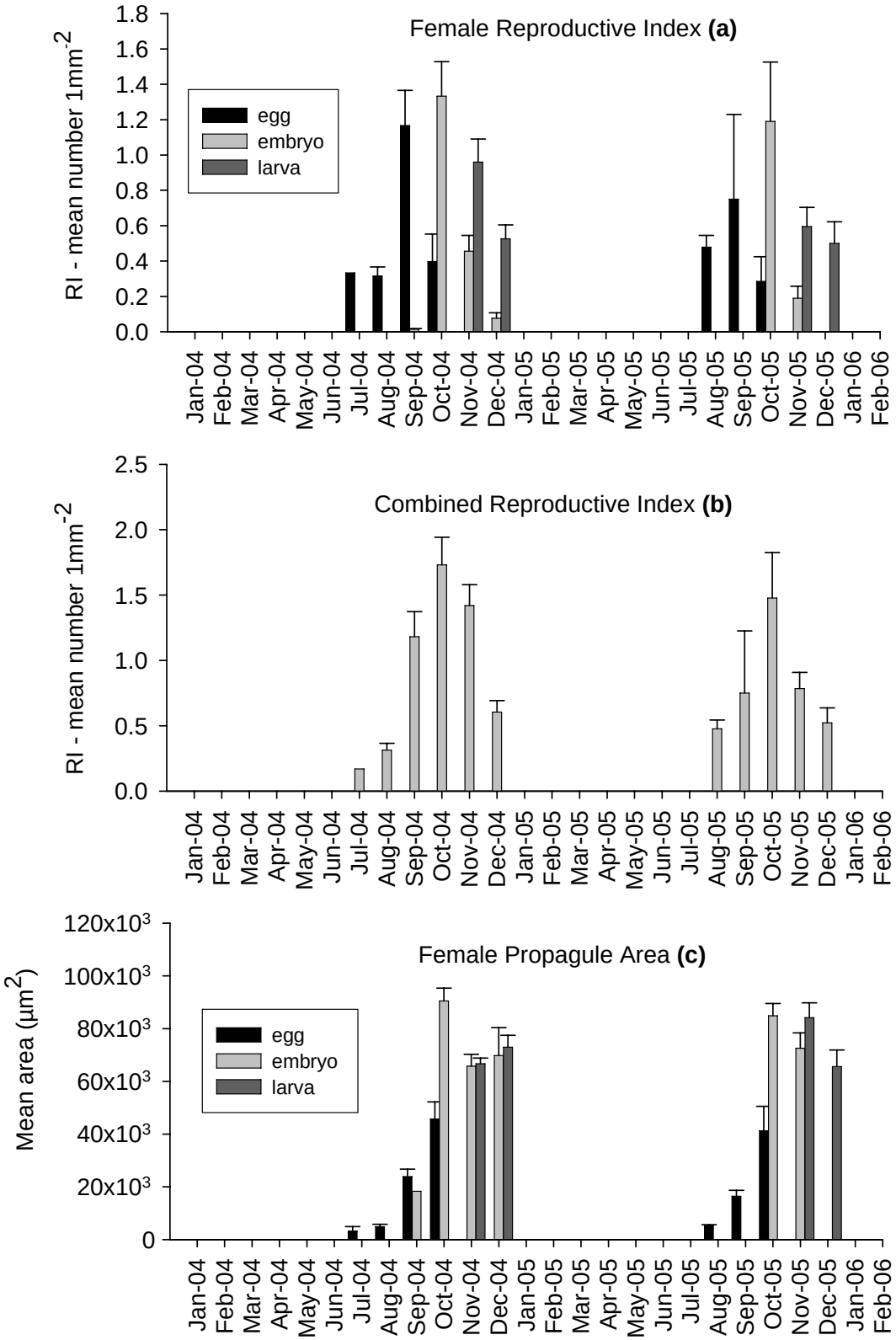


Figure 4.3a: Mean female reproductive index ± SE showing numbers of eggs, embryos and larvae over 2004 and 2005.

Figure 4.3b: Mean overall reproductive index ± SE for females including all stages of development of reproductive propagules.

Figure 4.3c: Mean female propagule areas ± SE over 2004 and 2005.

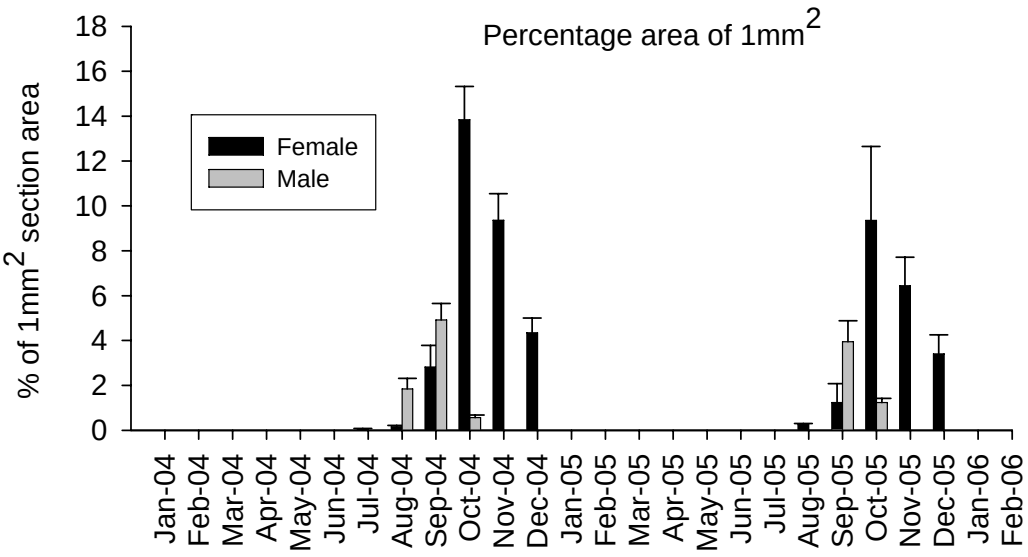


Figure 4.4: Mean percent tissue area occupation of females ± SE (combined egg, embryo and larva areas) and males ± SE (combined areas of spermatic cysts).

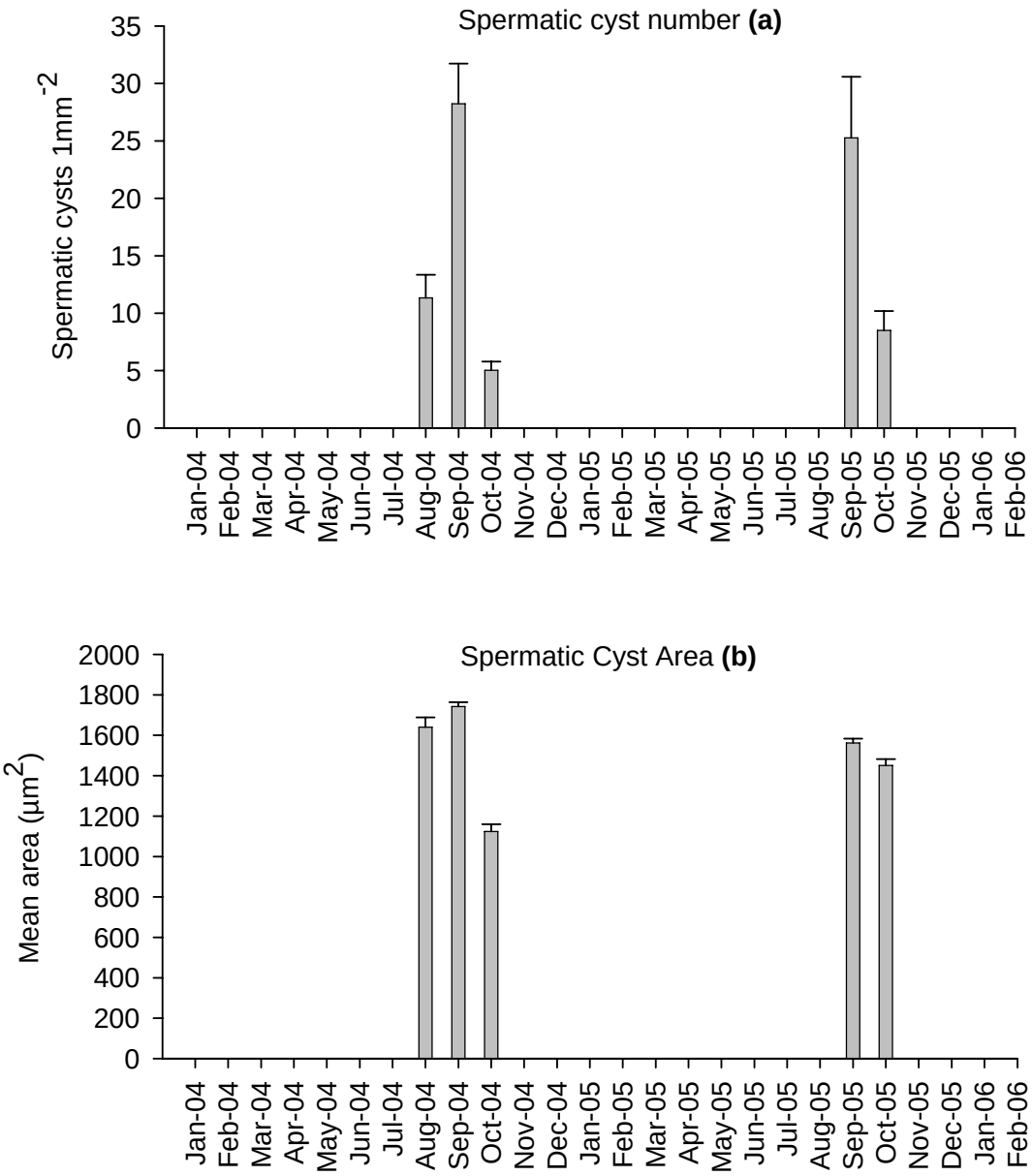


Figure 4.5a: Male RI ± SE (i.e. number of spermatic cysts) over 2004 and 2005.

Figure 4.5b: The means of the areas ± SE of individual spermatic cysts over 2004 and 2005.

Table 4.1: Selected output for a 3-factor ANOVA testing the effects of year, sample period (August to December 2004 and 2005) and any effects of repeated sampling (tagged sponges) on (1) female reproductive index (RI) (2) female percent tissue area occupied by reproductive propagules (3) propagule areas (eggs, embryos and larvae). As repeatedly sampling individuals had no significant effect ($p > 0.1$) in all cases, 'tag' terms and interaction terms have not been included. Data are log transformed.

Source	df	MS	***F***	p
a) Reproductive Index (RI)				
Year	1	0.53	7.41	0.008
Sample Period	4	0.81	11.23	<.001
Sample Period * Year	4	0.21	2.85	0.029
Error	81	0.07		
b) Percent tissue area				
Year	1	0.75	4.54	0.036
Sample Period	4	6.25	37.70	<.001
Sample Period * Year	4	0.26	1.60	0.183
Error	81	0.17		
c) Female propagule area				
Year	1	0.07	1.10	0.297
Sample Period	4	3.48	59.16	<.001
Sample Period * Year	4	0.09	1.44	0.230
Error	81	0.06		

Table 4.2: Selected output for a 3-factor ANOVA testing the effects of year, sample period (September to October 2004 and 2005) and any effects of repeated sampling (tagged sponges) on (1) male reproductive index (RI) (2) male percent tissue area occupied by reproductive propagules (3) spermatic cyst areas areas. As repeatedly sampling individuals had no significant effect ($p > 0.1$) in all cases, 'tag' terms and interactions not been included. Data are log transformed.

Source	df	MS	***F***	p
a) Reproductive index (RI)				
Year	1	0.31	1.65	0.203
Sample Period	1	7.95	42.91	<.001
Sample Period * Year	1	0.44	2.38	0.128
Error	67	0.19		
b) Percent tissue area				
Year	1	0.75	3.40	0.070
Sample Period	1	10.80	49.03	<.001
Sample Period * Year	1	1.41	6.40	0.014
Error	67	0.22		
c) Spermatic cyst area				
Year	1	0.11	5.86	0.018
Sample Period	1	0.23	12.69	0.001
Sample Period * Year	1	0.29	15.87	<.001
Error	67	0.02		

Discussion

Luffariella variabilis is gonochoristic and has an annual reproductive cycle. Females are reproductive for five to six months and males for two to three months. Females with eggs were first detected when water temperatures reached their lowest point for the year (21 ºC) in July 2004 and August 2005. Subsequently, males were detected as water temperature rose above 22.5 ºC. The release of sperm, fertilisation of eggs, and development of embryos occurred asynchronously, and larvae were progressively released through November and December in 2004 and 2005. Reproduction ceased before the highest water temperatures (~30 ºC) of the year were reached in January.

The seasonal pattern for *L. variabilis* reproduction corresponds with minimum, rising and maximum water temperatures. Oogenesis co-occurs with the coldest water temperatures of the year (~21 ºC) and spermatogenesis by rising water temperatures increasing above 22.5 ºC. The mean water temperature was approximately one degree lower in August in 2005 compared with August of 2004 and corresponds with a shift in reproduction in both females and males. This small change in water temperature appears to have important consequences for the dynamics of gamete production by both sexes in *L. variabilis* and may also affect the synchronicity in gamete production. In the case of delayed oogenesis and spermatogenesis, a lower subsequent reproductive output occurs as the period in which embryogenesis can take place is likely constrained by the release of larvae which appears to be regulated by water temperatures rising to their maximum. Climate models for the GBR predict average annual sea surface temperatures (SST) on the GBR to increase over the coming century by between 1 - 3^{o} C, with a greater relative contribution to winter warming and a shift towards more warm SST extremes and a reduction in cold SST extremes (Lough, in press, Hoegh-Guldberg, 2004). This scenario may severely limit the reproductive capability of *L. variabilis* given the influence of minimum and maximum temperature cues initiating gametogenesis and determining the end of the larval release period.

Patterns of reproduction in marine invertebrates are frequently correlated with a variety of environmental factors including temperature, photoperiod, salinity and lunar cycles (see reviews in Harrison and Wallace, 1990, Fautin, 2002, Maldonado, 2006). In sponges (and other invertebrates) water temperature is most frequently invoked to have the most significant influence on reproduction (Simpson, 1984, Fell, 1993). Rising water temperatures cue oogenesis and spermatogenesis in both gonochoristic and viviparous sponges from tropical (Fromont, 1994a, Fromont, 1999), temperate (Usher et al., 2004) and polar (Witte and Barthel, 1994) regions. Furthermore, for some species larval release occurs before maximum water temperatures are reached (Mariani et al. 2005), while for others the release of eggs (for oviparous species) occurs as temperatures begin to fall (Fromont, 1994a, 1999, Usher 2004). The reproductive period of *L. variabilis* females was similar to other viviparous sponges in temperate (Ayling, 1980) and tropical regions (Hoppe, 1988). Extended larval release periods are the result of asynchronous development of embryos (Young, 1995). Mortality risks of larvae are presumably mitigated through time and space via extended spawning periods and widely dispersed offspring may experience different environmental conditions (Young, 1995). At the organism level, maximum female reproductive outputs of 9 – 13 % occupation of the choanoderm for *L. variabilis* is within the same order of magnitude as a number of other sponge species (Reiswig, 1973, Hoppe, 1988, Corriero et al., 1998, Ereskovsky, 2000). However, values of up to 70 % have been recorded (Ereskovsky, 2000).

The reproductive index of male *L. variabilis* is also similar to other viviparous sponges (Ayling, 1980, Fromont, 1994b) which all show numbers of spermatic cysts between 0.6 and 100 mm^{-2}. However, spermatic cysts of 1000 mm^{-2} have been recorded (Ayling, 1980). At the individual level, male percentage occupation of the choanoderm varied between 0.6 – 4.9 % compared with areas ranging from 1 - 20 % in other oviparous and viviparous sponges (Reiswig, 1976, Fromont, 1994b).

*Luffariella variabili*s is reproductive at a very small size. This suggests that initial reproductive investment by *L. variabilis* is not significantly delayed by

production of biomass with the investment of resources in sexual reproduction not being affected by somatic growth. Theoretically reproduction should commence on balancing generation time, the fecundity benefits of delaying reproduction, and the increasing risk of mortality through time (Schaffer and Gadgil, 1975, Harvell and Grosberg, 1988, Roff, 1992). Invertebrates employ strategies such as delaying reproduction to reach a sufficient size to mitigate mortality risks due to factors such as incidental grazing (in sponges, sensu Maldonado and Uriz, 1998). Since *L. variabilis* has a cryptic distribution and produces an array of secondary metabolites (Chapter 2) this may offer protection from incidental grazers and permit reproduction at a small size.

In agreement with an early investment in reproduction there was no relationship between size (in cm^3) of *L. variabilis* and gametes mm^{-2} for females and males with smaller sponges producing as many reproductive propagules as larger sponges per unit area. However, total fecundity, when extrapolated to take into account for body mass, yielded positive correlations for both male and female fecundity with size. This supports the generalised paradigm of a relationship between fecundity to body size reported for other invertebrates (e.g. Hall and Hughes, 1996, Hughes et al., 2000). Reports vary as to the generality of the pattern, however limited studies in sponges found reproductive investment was correlated with size for *Crambe crambe* (i.e. there was a higher larval output unit $area^{-1}$ with increasing size) (Uriz et al., 1995), whilst Fell and Lewandroski (1981) and Fromont (1994a) found no patterns in *Halichondria* spp.

In conclusion, temperature is likely to be critical factor in the reproductive biology of *L. variabilis.* Oogenesis appears to be cued by the coldest water temperatures of the year of 21 ºC, spermatogenesis commences above 22.5 ºC, and reproduction ceases before the highest temperatures of the year (30 ºC) are reached. A delay in the lowest temperature by one month in 2005 most likely delayed oogenesis and spermatogenesis. However, termination of reproduction occurred at the same time (December) constraining the total time available for fertilisation and embryogenesis resulting in a concomitant decrease in female reproductive output of 30 %. Temperature shifts associated with climate

change models therefore have important implications for the viability of *L. variabilis* populations and presumably other invertebrates as higher winter temperatures may reduce the time for embryogenesis, or cause a failure in oogensis and/or asynchronicity in gamete formation. Whilst some marine invertebrates shift their reproductive cycles in relation to temperature changes (Velazquez, 2003), it is unknown how broadly applicable this phenomenon is, and how resilient their reproductive physiology is to temperature changes. Given the importance of temperature cues on spermatogenesis, oogenesis, embryogenesis, and larval release in sessile invertebrates the effect of an increase in minimum SST may have implications as broad as those of an increase in maximum SST.

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CHAPTER FIVE – A HIERARCHY OF CUES INFLUENCES THE SETTLEMENT OF *Luffariella variabilis* LARVAE

Introduction

Most marine benthic invertebrates have complex life histories characterised by sessile and planktonic phases (Sly et al., 2003), and in most cases planktonic larvae are the primary mechanism of dispersal in sessile invertebrates (Caley et al., 1996). The length of the planktonic larval phase can vary from minutes to months and this is usually predicated by the mode of development of the larva (lecithotrophic or planktotrophic) (Pawlik, 1992, Hadfield and Paul, 2001). The subsequent transition from a planktonic to benthic existence, where the future of metamorphosed larvae is dependent on an appropriate habitat choice, is a crucial stage in the life history of organisms where the adult is sessile or has low mobility (Keough and Raimondi, 1995, Raimondi and Morse, 2000).

The processes governing the release of larvae and their ability to locate, settle, and metamorphose within a suitable habitat is central to understanding the community dynamics of benthic marine invertebrates (Harrington et al., 2004, Huggett et al., 2006). Flow (Metaxas, 2001), light (Maida et al., 1994) and gravity (Young, 1995) all play important roles guiding larval behaviour, settlement and metamorphosis at large scales (kilometres to 100s kilometres), while biotic and abiotic interactions predominantly guide larvae at smaller scales (millimetres to metres) (Steinberg et al., 2001). These smaller scale factors include surface texture (Berntsson et al., 2000), chemical cues from biofilms (reviewed in Fusetani, 2004), conspecifics (Dreanno et al., 2006a, Huggett et al., 2006), and other biological sources (Zimmerfaust and Tamburri, 1994, Raimondi and Morse, 2000, Swanson et al., 2006).

In contrast to other organisms such as polychaetes (Butman et al., 1988); (Minchinton, 1997), bivalves (Butman et al., 1988), barnacles (Clare and Matsumura, 2000, Dreanno et al., 2006b), oysters (Zimmerfaust and Tamburri,

1994), ascidians (Stoner, 1992) and bryozoans (Keough, 1998), there is little information on the response of sponge larvae to environmental, biological and chemical stimuli (reviewed in Maldonado, 2006). Of the environmental factors investigated for sponges, light plays a key role in influencing the release, behaviour, and settlement of larvae. For example, photoperiodicity determines larval release in *Callyspongia* sp. (Amano, 1988), and *Halichondria panicea* releases larvae after being artificially shocked by intense illumination (Amano, 1986, Maldonado and Young, 1996). After release, the parenchymellae of demosponges can display photopositive (Mariani et al., 2005), photonegative (Maldonado et al., 1997, Leys and Degnan, 2001, Maldonado et al., 2003)) or photoneutral behaviour (Uriz et al., 1998). Photopositive behaviour is associated with dispersal by currents (reviewed in Maldonado, 2006)whilst photonegative behaviour is proposed to guide competent larvae to dark benthic microhabitats (Maldonado et al., 1997).

Similarly, the selection of surfaces by sponges on which to settle and metamorphose varies. Larvae of some species settle in the presence of geniculate coralline algae (Jackson et al., 2002), while others preferentially settle on biofilms (Woollacott and Hadfield, 1996). In contrast, the larvae of some species indiscriminately select glass, basalt rock and porcelain (Bergquist and Sinclair, 1968, Bergquist, 1978). However, it is unclear from these studies how chemically mediated settlement is decoupled from surface textures or the presence biofilms. Furthermore, few studes link the plethora of cues that facilitate settlement processes, from larval release to metamorphosis that are critical for successful recruitment.

In this study, the hierarchy of responses by the larvae of the Indo-Pacific dictyoceratid sponge *Luffariella variabilis* are elucidated to physical, biological, and chemical cues to determine the processes affecting their dispersal and habitat selection: specifically (1) Larval release by *L. variabilis*; (2) the larval response to light on release from the parent sponge; (3) the further influence of light on settlement and; (4) the responses of *L. variabilis* larvae to common invertebrate settlement cues including newly settled and adult conspecifics.

Materials And Methods

General

L. variabilis is a cryptic, coral reef sponge distributed widely through the Indo-Pacific (Bergquist 1980, 1995). *Luffariella variabilis* adults are found in aggregations in areas of low illumination and occur in high abundance (> 1 m^{-2}) on the Great Barrier Reef. All sponges were collected on a shallow area of coral reef (4 - 8m) at Orpheus Island (18° 35' 37"S 146° 29' 07" E) in the Palm Islands group, Queensland, Australia. *Luffariella variabilis* larvae are ciliated, hollow, and spheroid (Fig. 5.1a) and are approximately 400µm x 200µm with a band of cilia at the aboral pole (Fig. 5.1b) which is typical of the larvae of the Subclass Ceractinomorpha, Order Dictyoceratida. The term 'settlement' used here describes the permanent attachment of larvae to the substratum by the anterior pole and the completion of metamorphosis (Fig. 5.1c). Metamorphosis involves the flattening of the posterior half of the larva to form a mauve coloured disc. The larvae from between four and seventeen sponges were pooled for use in all experiments.

Larval Release

To determine patterns of release and the number of larvae released in the field and *in vitro*, 20 gravid *L. variabilis* were collected at Orpheus Island (18° 35' 37"S 146° 29' 07" E) Queensland, Australia on 16 November 2005 and placed in flow through aquaria until 19 December 2005. A further 11 gravid *L. variabilis* were marked *in situ*. Gravid sponges were identified by removing a ~1 cm^3 piece of mesohyl and visually checking for the presence of white larvae (~400 µm). Mesh traps were placed over gravid sponges to collect released larvae. Larvae swam up on release and were collected in an inverted container. To determine the time of spawning, traps on sponges *in vitro* were checked at dusk (1815 hrs), midnight (0000), dawn (0500), morning (0730), mid morning (1000), midday (1200) and late afternoon (1600) for five sequential days. Larvae were also collected and counted for sponges *in situ* on the same days and at the same times except midnight (due to the high number of tiger sharks observed feeding at this time). Larvae were only released during the day (see Results section). Subsequently larvae were collected *in*

vitro mid morning and mid afternoon. Larvae were also collected and counted at least once per day *in situ* to determine whether larval release patterns in the field were the same as those *in vitro*.

Behaviour of larvae on release

Larval release (commencing at ~ 0700 hrs) in *L. variabilis* is cued by daylight (see Results section) and the response of larvae to natural light directly on release was quantified. To determine the direction in which newly released larvae swam in response to light over time, larvae were obtained immediately on release from parent sponges *in vitro,* and subjected to the light treatment experiments described below. To ensure only newly released larvae were used, traps were replaced on spawning sponges at 0600 hrs. Low numbers of larvae were available directly on release and as such, experiments were repeated over 3 replicate days.

For each experiment, groups of 10 larvae were introduced into 1 litre measuring cylinders containing 0.2µm filtered sea water (hereafter FSW) which were (1) totally covered with black plastic, (2) top half covered (3), lower half covered, or (4) uncovered. All experiments were conducted under natural light and photoperiod. The positions of larvae within the cylinders were recorded every 20 minutes for the first 120 minutes and at 240 minutes. Because larvae swam upwards on release, ten newly released larvae were killed with a 5 % formalin solution at this time to determine whether they are positively, negatively or neutrally buoyant.

Behaviour of larvae after 2 hours (post release behaviour)

Larvae initially swam upwards on release (t = 0 minutes), but exhibited a strong negative phototaxis after 20-40 minutes with settlement commencing after two hours in the dark (see Results section). Only small numbers of larvae (single to tens) were available immediately on release. Therefore the response of larvae 2 – 4 hours after release to light was quantified. This allowed for improved replication with hundreds to thousands of larvae. Two experiments were conducted which decoupled the effect of depth on larval behaviour in response to light.

In the first experiment, three replicate groups of 100 larvae were placed in 1 litre measuring cylinders with the (1) top half covered, (2) lower half covered or, (3) cylinder totally uncovered. The position of swimming and settled larvae within the cylinders was recorded at 0.5, 1, 2, 3, 6, 8, 11 and 18 hours. The experiment was conducted under natural light and photoperiod.

The second experiment was used to decouple the influence of depth on the phototactic response. In the experiment, 300 larvae were placed in a 40 x 20 cm aquarium half covered with black plastic and with a 3 cm water depth (in the horizontal direction). The experiment was conducted under natural light and photoperiod. The cover created a high light area of 449 μmol m^{-2} s^{-1} outside the cover, and a low light area of 36.2 μmol m^{-2} s^{-1} under the cover. The position of swimming and settled larvae was noted at 0.5, 1, 2, 3, 6, 8, 11 and 18 hours and was replicated three times over three days.

Light levels and settlement

Given the behavioural response of larvae to light and that settlement only occurred in the dark (see Results section) the effect of light on settlement was quantified. Four replicate containers (containing 10 ml of 0.2 μm FSW), each holding 20 larvae were placed at 5, 10, 15, 20, 25, 30 and 35 cm from an overhead cold light source (Leica CLS 150X). Larvae maintained in containers in the dark were controls. Containers were configured to prevent shading (Fig. 5.2a). Light levels were measured using a LI-COR LI250 light meter and were consistent between containers equidistant from the light source (Fig. 5.2b). The proportion of larvae settled in each container was measured at 0.5, 1, 2, 3, 6, 8, 11 and 18 hours. Two replicate experiments were conducted over two consecutive days. A repeated measures general linear model (GLM) was run on arcsine square root transformed data with time as the within subject factor. Distance and day were between subject factors.

Settlement in the presence of conspecifics

Given the aggregated pattern of adult *L. variabilis* in the field, the potential gregarious nature of larval settlement was quantified. Densities of 1, 2, 5, 10

and 50 larvae were placed in containers containing 10 ml of 0.2 µm FSW and the proportion of larvae settled was measured at 0.5, 1, 2, 3, 6, 8, 11 and 18 hours. Five replicates of all densities were used in the dark and natural light at the beginning (mid November 2005) of the spawning season. Settlement only occurred in darkness (see Results section) and experiments were repeated in the dark at the end of the spawning season (mid December 2005). This was to determine whether larvae changed their behaviour over time. A repeated measures GLM with time as the within subject factor and density as the between subject factor was used to analyse the on arcsine square root transformed settlement data. Single larvae were excluded from the analysis.

Settlement cues

The effect of common invertebrate settlement cues on larval settlement was determined. Experiments were conducted early in the spawning season (mid November 2005) and late in the spawning season (mid December 2005) to determine whether larvae altered their response to settlement cues over time.

In the first experiment (mid-November 2005) three replicate groups of 10 larvae were introduced into treatments containing 10ml of 0.2µm FSW plus a settlement cue and time to settlement was measured at 0.5, 1, 2, 5, 8, 11 and 18 hours. As larvae do not settle in the light (see Results section) the experiment was conducted in both the light and dark to determine whether light in combination with settlement cues induced settlement. Treatments contained (a) a biofilm on a polyethylene container left in flowing unfiltered seawater for 24 hrs after which the water was removed and replaced with FSW; (b) 20 settled and metamorphosed live larvae settled on the base of a polyethylene container with the water removed and replaced with FSW; (c) a 0.5 mm^2 piece of an undetermined crustose coralline alga collected at Orpheus Island; (d) 20 µl of crustose coralline extract (Harrington et al. 2004). In the extract treatment 100 grams of the surface of *Neogoniolithon fosliei* was extracted twice in 300ml of methanol and the extracts dried under rotary evaporation and nitrogen. The extract was then redissolved in DMSO and made up in methanol to give 5 g extract L^{-1} methanol in 10 % DMSO, equivalent to 0.01 mg ml^{-1} DMSO; (e) 20 µl of a 10 % DMSO blank control

(equivalent to 0.01 mg ml^{-1} DMSO); (f) a sterile container with FSW as a control. A repeated measures GLM was run with time as the within subject factor and cue as the between subject factor was used to analyse the on arcsine square root transformed settlement data. The experiment was repeated each day for three days.

In the second experiment (mid-December 2005) a modified design incorporating the same cues as above was repeated in the dark as larvae did not settle in the light (see Results section). A single experiment was run with three replicates of each of the treatments above and time to settlement measured at the same times of 0.5, 1, 2, 5, 8, 11 and 18 hours. Two additional treatments using a 1 mm^2 piece of sponge skeleton, or a 1mm^2 piece of fresh *L. variabilis* pinacoderm/mesohyl, were also incorporated to determine the effects of adults on larval settlement. A repeated measures GLM was run with time as the within subject factor and cue as the between subject factor was used to analyse the on arcsine square root transformed settlement data.

In a final experiment, the effects of the settlement cues described above on settlement of single larvae were also tested in mid-December 2005 with three replicate treatments for each cue, each with a single larva. This was to determine the response of single larvae to settlement cues. These data were not formally analysed.

Conspecific Settlement Cues

Larvae settled more rapidly with increasing density (see Results section) suggesting larvae released a cue as they settled. To determine whether there was a settlement cue associated with conspecifics, groups of 200 larvae were settled overnight in 60 ml aliquots of 0.2 µm FSW (to produce 'conditioned' water). This 'conditioned' water was subsequently removed and filtered to 0.2 µm. The water (10ml aliquots) were then placed in petri dishes and individual larvae (n = 85) or groups of ten larvae (n = 17) were added. Controls were larvae placed in one day old aerated FSW. All experiments were conducted in the dark and the proportion of larvae settled was measured at 0.5, 1, 2, 5, 8, 11 and 18 hours. A repeated measures GLM with time as the within subject factor

and ‘conditioned water’ as the between subject factor was used to analyse the arcsine square-root transformed settlement data. Single larvae were excluded from the analysis.

Statistical analyses

Hypotheses were tested using either repeated measures or univariate analyses of variance (ANOVA). Assumption of normality and homogeneity of variance was checked graphically for each dataset prior to analysis by plotting residuals, and data were transformed where necessary (Quinn and Keough, 2002). Any experiments counting single larvae were excluded from formal analyses. If heterogeneous variances were still encountered after transformation ANOVA was still used as it is resilient to heterogeneous data (Underwood 1981). Variance-covariance sphericity of the data used in any repeated measures general linear models was estimated using the Greenhouse-Geisser ε (epsilon) and significances of within-subjects F ratios adjusted accordingly. Tukey’s post-hoc tests were used to determine experimental groupings. Gaines-Howell post-hoc tests were used on repeated measures data if unequal variances were encountered (e.g. from proportional settlement data). All analyses were done using SPSS (version 12).

Results

Larval release

The larval release period for *L. variabilis* was between 0700 hrs and 1600 hrs *in situ* and *in vitro* at all times (~5 weeks) with the maximum release of larvae (up to 500 larvae sponge^{-1}) occurring mid-morning. No larvae were found in traps emptied at dusk (1815 hrs), midnight (0000 hrs) and at dawn (0500 hrs) *in vitro* demonstrating that there was no larval release after 1600hrs. Similarly, no larvae were released in the field between 1815 and 0500. The maximum release of larvae was 830 larvae sponge^{-1} day^{-1} and the 30 sponges in this study released 45,283 larvae over the spawning period.

The number of larvae released by individual sponges was not consistent over time with some sponges releasing small numbers (< 100) larvae sponge^{-1} day^{-1} with occasional large pulses (> 400) (Fig. 5.3a-c). In contrast, others released large pulses of larvae (> 400) on most days (Fig. 5.3a-c). The duration of the time of spawning varied between sponges with sponges not spawning for a period of time and then recommencing spawning. However, sponges *in vitro* had the same release patterns to those in the field and larval release almost ceased *in vitro* and *in situ* during a period of rough, overcast weather. Spawning ended abruptly in all sponges in mid December (Fig. 5.3a-c).

Behaviour of larvae on release

This experiment determined the directional response to light of larvae at the time of release from parent sponges. All larvae swim upward after release regardless of treatment (Fig. 5.4 a-d). However, there was a profound reversal of behaviour after 20 - 40 minutes with larvae exhibiting a negative phototaxis and moving to the darkest parts of all treatments regardless of orientation (Fig. 5.4 b-c). In the uncovered treatment, half the larvae were at the surface (4.3 ± 1.4) and the other half (5.3 ± 1.2) at the bottom (Fig. 5.4a). Larvae did not move from the top of the totally covered (all dark) cylinder (Fig. 5.4d). Larvae were negatively buoyant on release and therefore actively maintained their position within the water column.

Behaviour of larvae between 2 and 4 hours old

This experiment determined the directional response of larvae to light at 2 - 4 hours after release. Larvae at 2 – 4 hours after release maintained the negative phototaxis first exhibited after 20 minutes and moved directly to the darkest areas of all treatments. After 30 minutes, 95.0 ± 5.0 larvae were at the top of the half black top cylinder (Fig. 5.5a) and 98.3 ± 1.7 at the bottom of the cylinder with the base half covered (Fig. 5.5b). Settlement occurred almost exclusively in the dark. Fifty percent settlement was reached at ~ five hours in the cylinder half covered at the top and 50 % settlement was reached in the cylinder half covered at the bottom at ~ nine hours. This was attributed to higher incidental irradiance reaching the bottom of the cylinder with light inhibiting settlement (see Results section: Lights levels and settlement). In natural light (the uncovered cylinder), larvae were more broadly distributed. After 30 minutes, 64.0 ± 32.0 larvae were on the bottom and 34.0 ± 32.0 at the surface (Fig. 5.5c). After two hours, 80.0 ± 9.0 larvae were on the bottom and 16.0 ± 9.0 at the surface. Larvae did not begin to settle until natural light ceased after six hours (Fig. 5.5c).

In the second experiment to determine the effect of light on 2 - 4 hour old larvae without the potential bias of depth inherent with measuring cylinders replicate groups of 300 larvae were placed in a half shaded aquarium. All larvae rapidly moved to the dark area within 30 minutes. No larvae moved away from the dark area, and all larvae settled when natural light ceased (~ 6 hours).

Light levels and settlement

Light has a significant effect on the settlement of larvae with the proportion and rate of larval settlement decreasing with increasing light intensity (Fig. 5.6). Light levels of 56.00 ± 2.78 μmol m^{-2} s^{-1} and 14.23 ± 0.88 μmol m^{-2} s^{-1} slowed the settlement rate of larvae and inhibited overall settlement after 18 hours by ~ 60 % and 35 % respectively compared with controls, which showed > 95 % settlement after 18 hours. Light levels of 3.49 ± 0.11 μmol m^{-2} s^{-1} to 1.26 ± 0.03 μmol m^{-2} s^{-1} slowed the rate of settlement but had the same overall settlement as controls after 18 hours (Fig. 5.6). Settlement of larvae in

all other light treatments did not differ from that of the controls. Therefore, larvae were able to settle at similar overall proportions to dark controls when subjected to light levels less than 3.49 μmol m^{-2} s^{-1} (15 cm from the light source) (Fig. 5.6).

A repeated measures GLM on arcsine square root transformed data found no significant effect of replicate days on which the experiment was run. All replicates were thus combined and the analysis rerun with time and distance as within subjects and distance as the between subjects factors (Table 5.1). The variation in total settlement between treatments was indicated by a significant time term ($F_{4,203} = 176.51$; $p < 0.001$). Furthermore, the differing overall rates of settlement were indicated by a significant time x distance term ($F_{25, 203} = 3.54$; $p < 0.001$). A Games-Howell post hoc test ($p = 0.05$) on distance determined four groups of treatments: 5 cm & 10 cm **(a)**; 15 cm **(bc)**; 20 cm, 25 cm, 30 cm **(cd)**; 35 cm & control (dark) **(d)**.

Settlement in the presence of conspecifics

Larvae of *L. variabilis* exhibit gregarious settlement with increasing densities of larvae (Fig. 5.7a-b). Groups of 50 and 25 larvae settled the fastest and achieved a higher total settlement compared with groups of 10, 5, 2 and single larvae. Single larvae had the slowest and lowest overall settlement. Settlement proceeded in the same overall pattern but more slowly in mid December with groups of 50 and 25 larvae reaching maximum settlement at five to eight hours vs. three hours in November (Fig. 5.7b). Overall settlement was ~ 95 % at both times.

A repeated measures three factor GLM was run on arcsine transformed data with time, density and season as within subjects factors and density and season as between subjects factors. Significant within subjects factors were only found for density ($F_{4,40} = 3.94$; $p < 0.009$) and season ($F_{1,40} = 12.37$; $p < 0.001$) demonstrating the overall difference between the densities and timing of the experiments (Table 5.2).

A two factor GLM on settlement data run at t = 5 hours, where settlement began to plateau, gave significant density ($F_{4,40} = 3.46$; $p < 0.016$) and season ($F_{1,40} = 5.39$; $p < 0.025$) terms (both fixed). Density*season was not significant ($F_{4,40} = 0.72$; $p = 0.585$). A Tukey's post hoc test for density (i.e. both seasons combined) at $p < 0.05$ resulted in three groups of 2 larvae **(a)**, 5 &10 larvae **(ab)**; 25 & 50 larvae **(b)**.

Settlement cues

This experiment determined whether common invertebrates settlement cues affected the settlement of *L. variabilis* larvae and whether any effects changed across the period of spawning. No cue had any significant effect on settlement at any time or density of larvae although larvae settling in the presence of a piece of live adult *L. variabilis* displayed a faster settlement rate (Fig. 5.8a-b; Fig. 5.9a-b; Table 5.3).

Settlement assays using conditioned water

While there was no effect of common invertebrate settlement cues on settlement of larvae, both single and groups of 10 larvae placed in 'conditioned' water reached 55 % settlement after only 20 minutes, in contrast to < 10 % settlement in controls. Moreover, single larvae never reached more than 20 % settlement in controls while settlement after 18 hours was 80 % for single larvae in 'conditioned' water (Fig. 5.10a). In contrast, groups of ten larvae reached ~ 80 % settlement for both treatments and controls, however settlement was more rapid for larvae in 'conditioned' water (Fig. 5.10b). A repeated measures GLM run on untransformed data for ten larvae found no significant effect of replicate days in the experiment. All replicates were combined and the analysis rerun with time and conditioned water as within subjects and conditioned water as the between subject factors. The more rapid rate of settlement of those larvae in conditioned water was demonstrated by a significant time x conditioned water term ($F_{4,121} = 35.75$; $p < 0.001$) (Table 5.4).

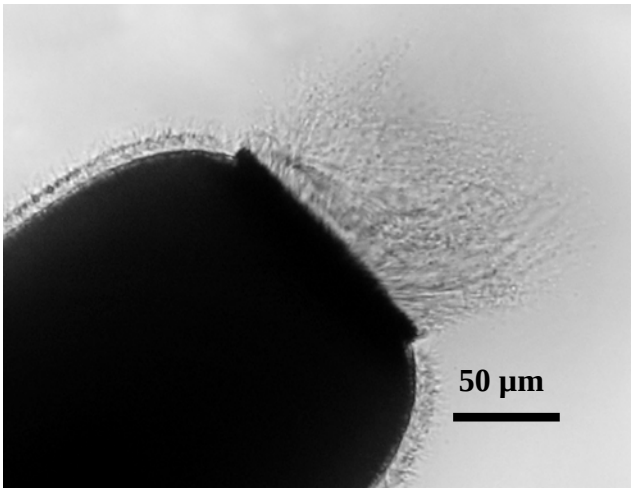

Figure 5.1a: Close up of cilia at aboral pole of an *L. variabilis* larva whole larva.

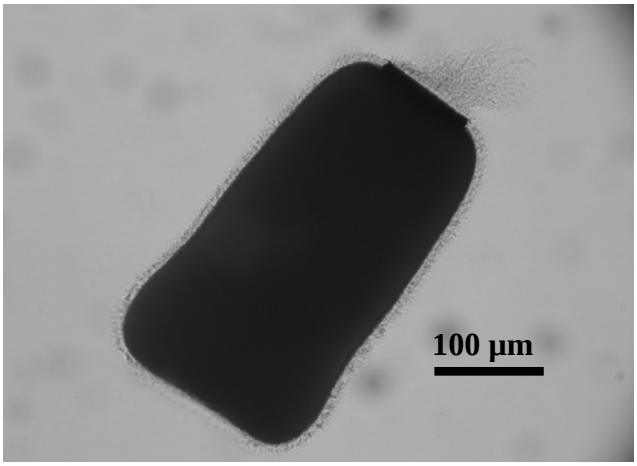

Figure 5.1b: *L. variabilis* larva. The band of cilia at the aboral pole are bent in response to a light and maintain directional control of the larva.

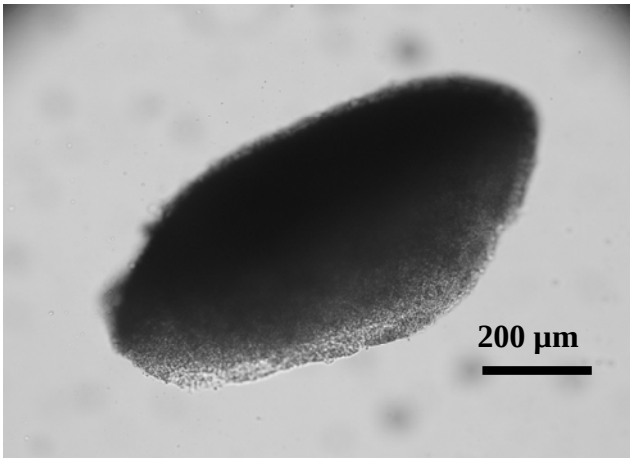

Figure 5.1c: Metamorphosed *L. variabilis* larva approximately 8 hours old.

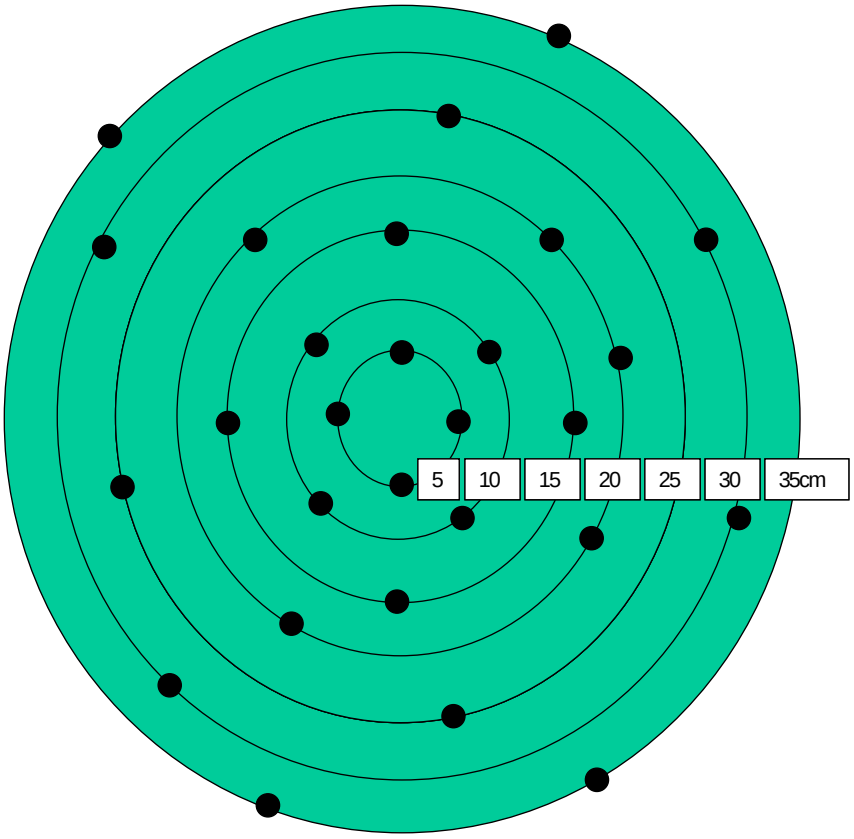

Figure 5.2a: Schematic diagram of the arrangement of containers containing larvae around a cold light source. The light source is in the centre of the arrangement and the arrangement of jars minimises shading.

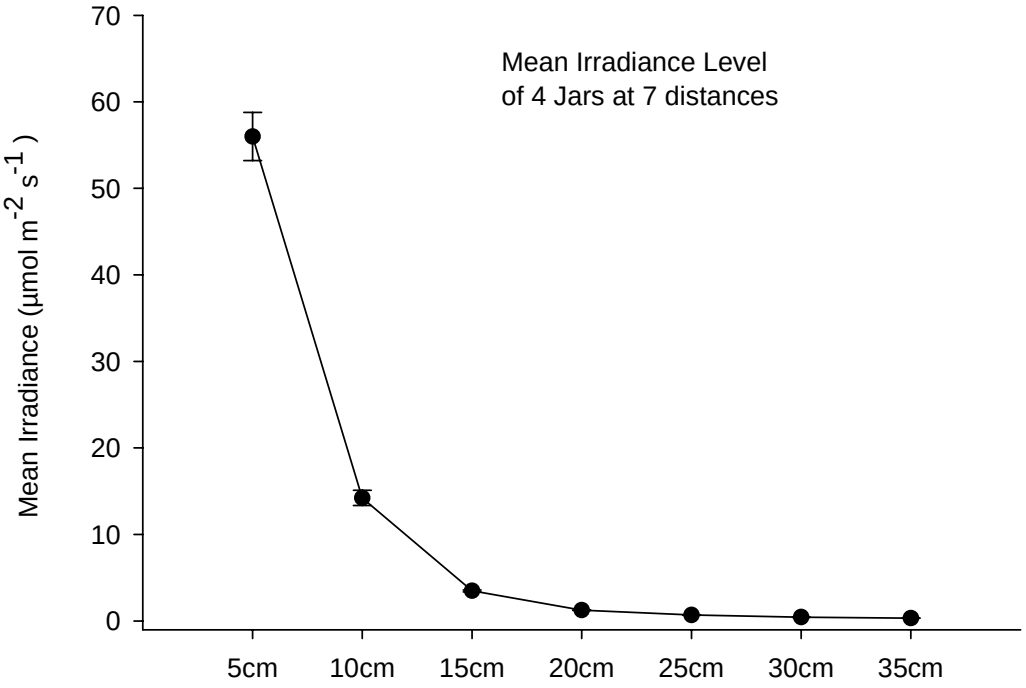

Figure 5.2b: Irradiance levels (mean ± SE) at different distances from the cold light source.

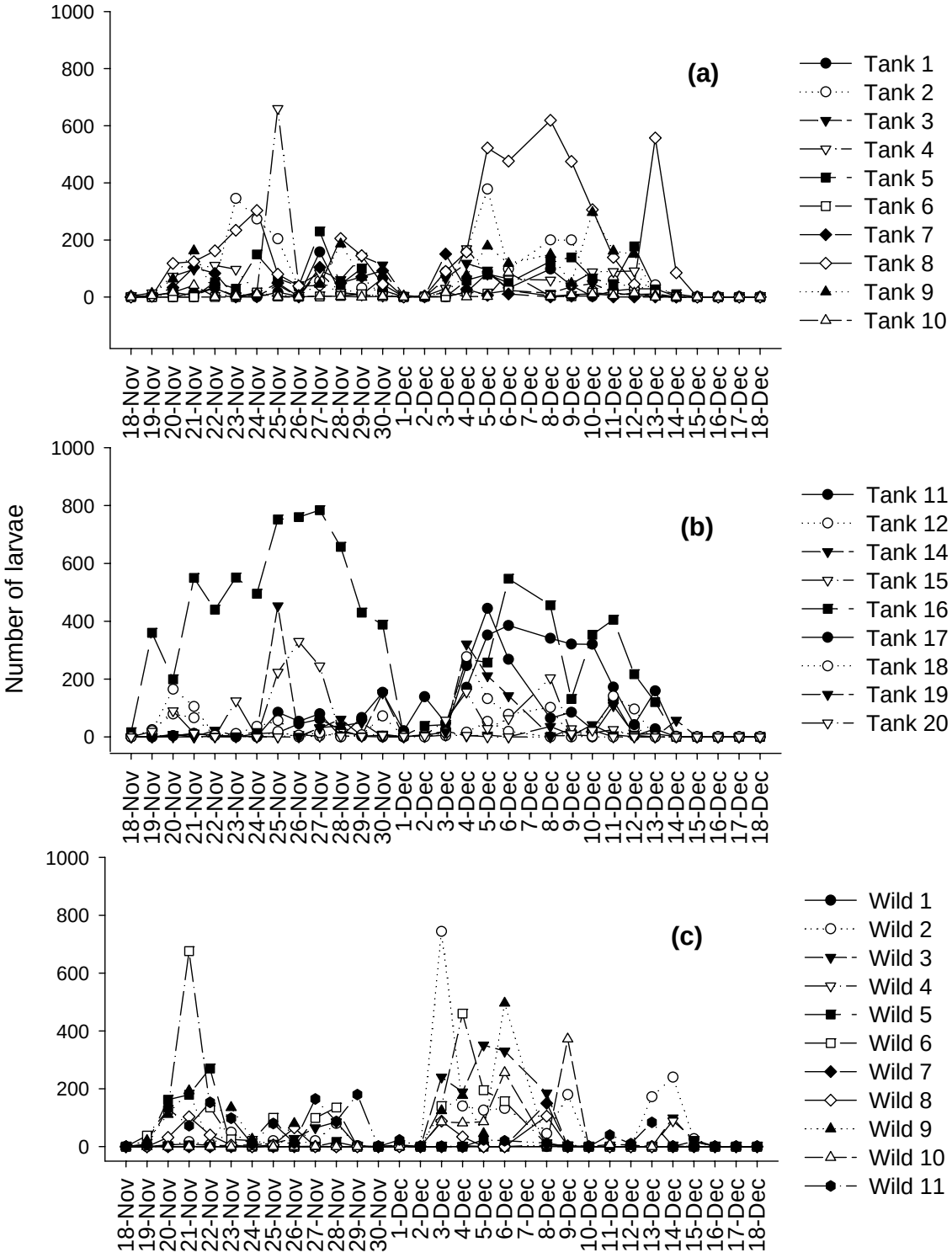

Figure 5.3: Larval release by individual sponges *in vitro* (**Fig. 5.3a,b**) and in the field over time (**Fig. 5.3c**).

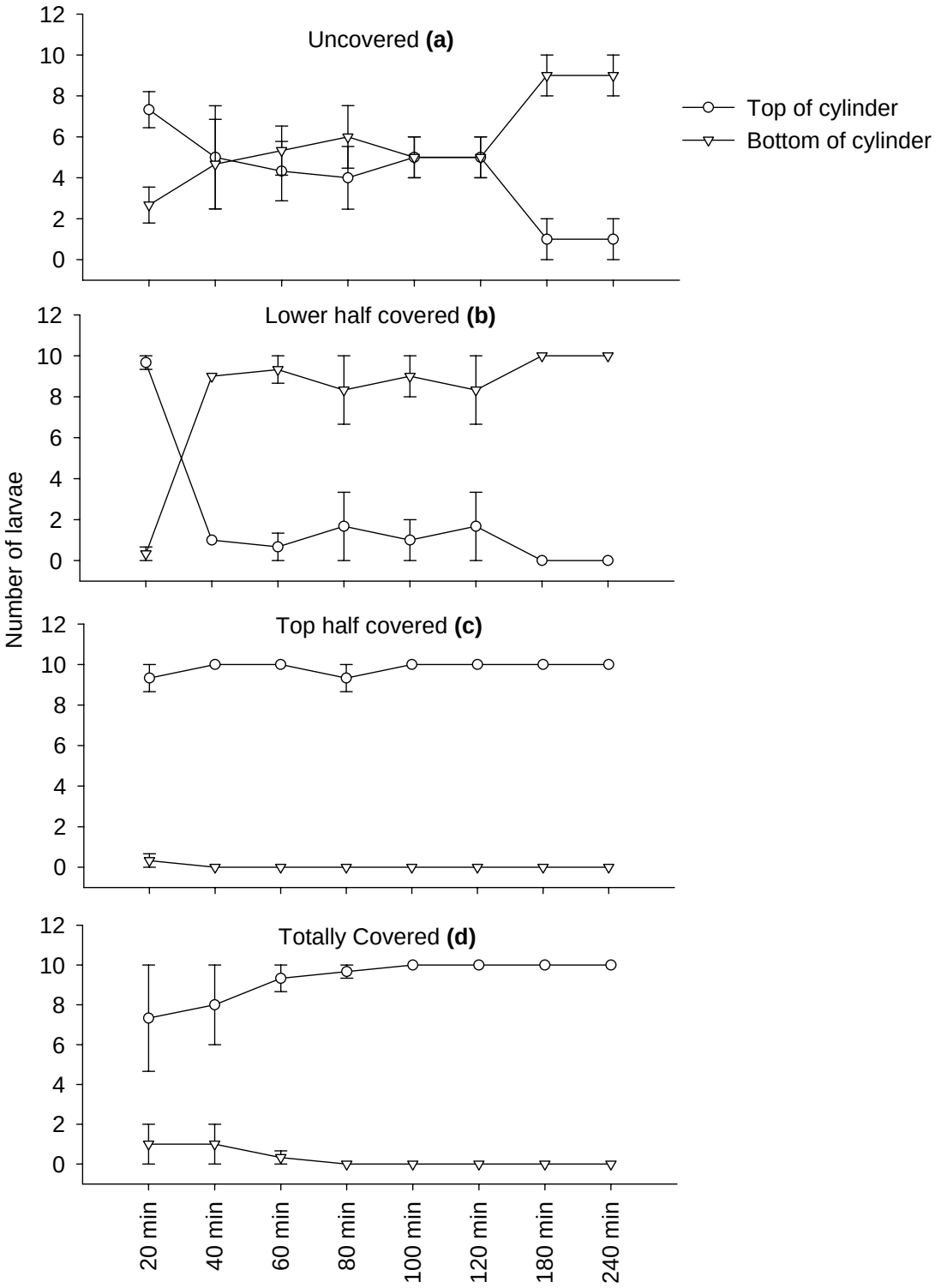

Figure 5.4: Position of newly released larvae (mean number ± SE) in the uncovered cylinder (**Figure 5.4a**); Lower half covered cylinder (**Fig. 5.4b**); Half covered top cylinder (**Figure 5.4c**); and Totally covered cylinder (**Figure 5.4d**).

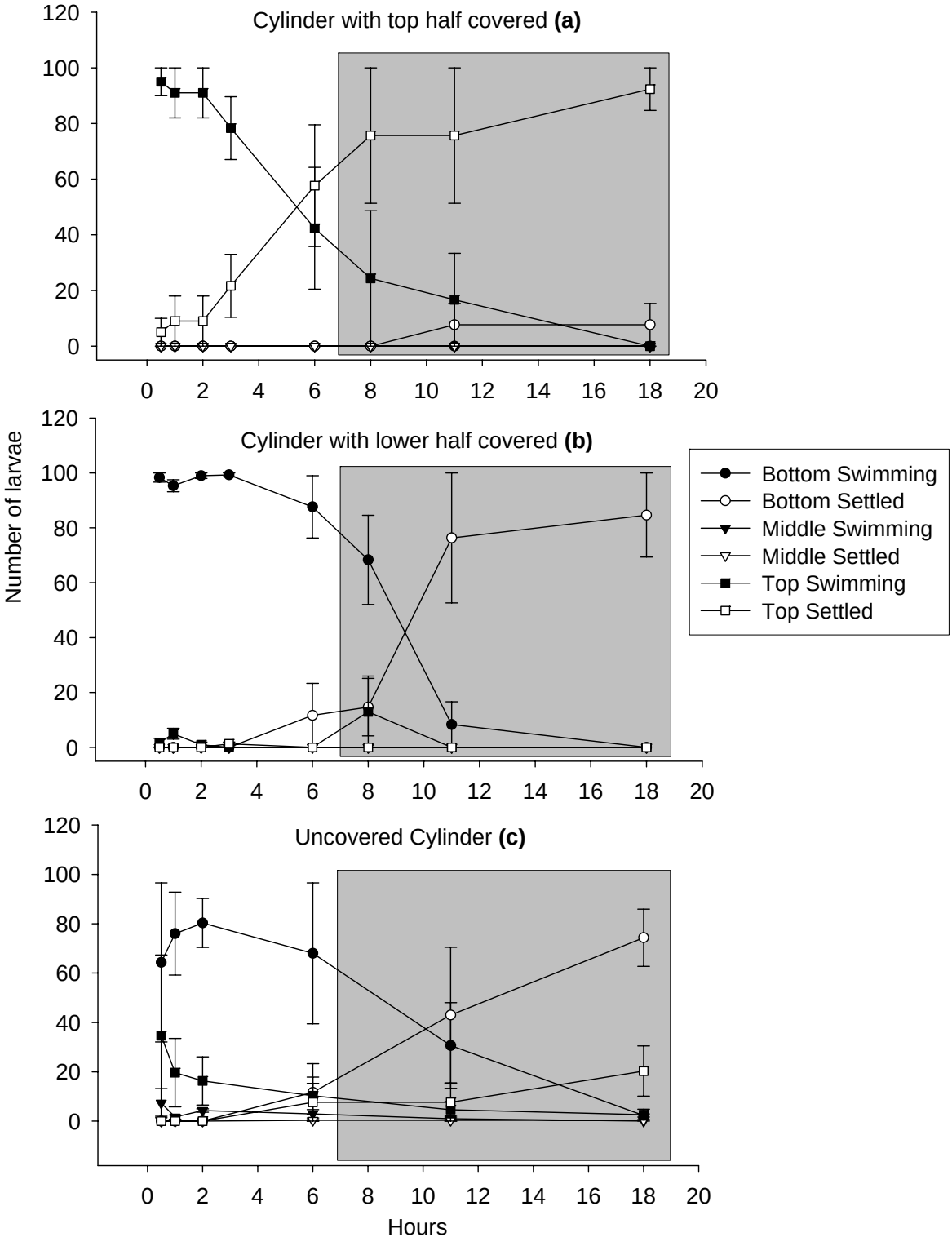

Figure 5.5: Position of 2 - 4 hour old larvae (mean number ± SE) in the half covered top cylinder (**Figure 5.5a**); Lower half covered (**Figure 5.5b**); Uncovered (**Figure 5.5c**). The grey boxes represent the fall of darkness.

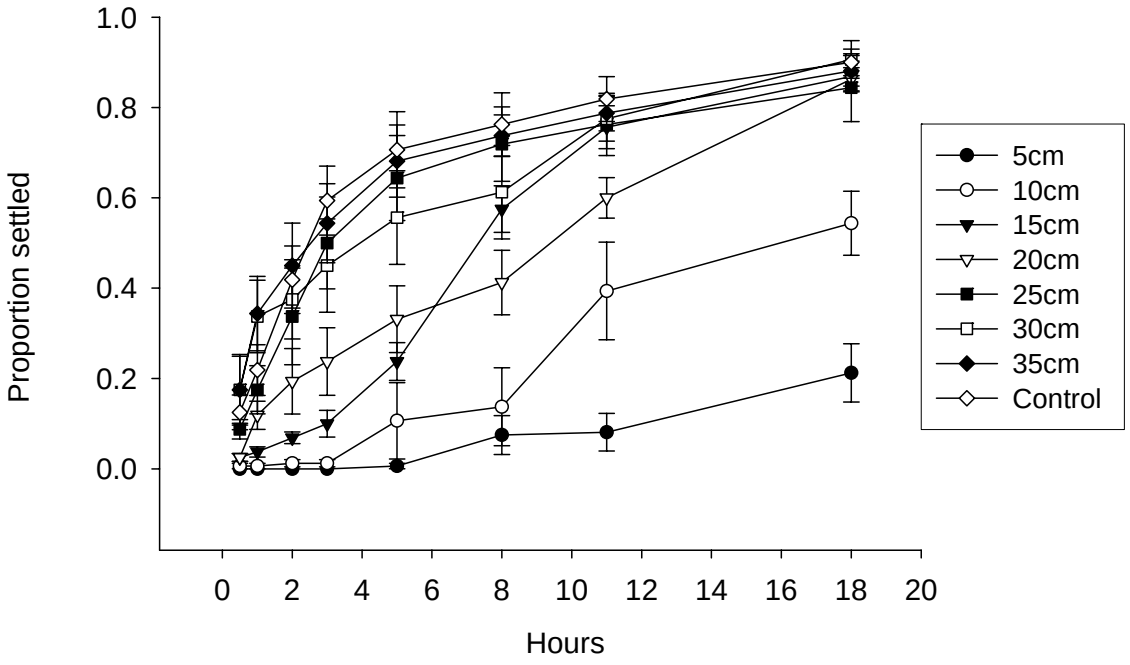

Figure 5.6: Mean proportion of larvae settled ± SE at different distances from the cold light source.

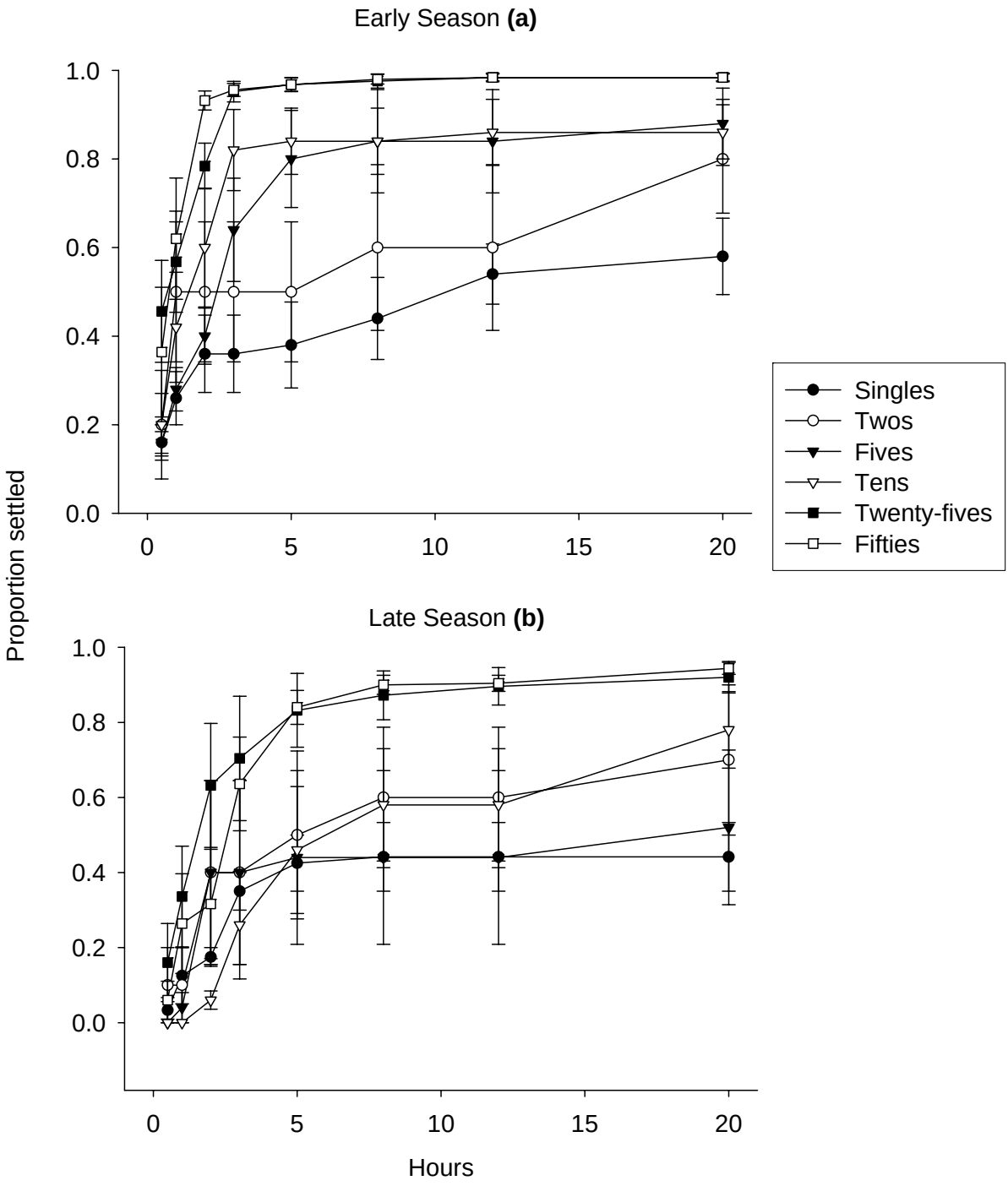

Figure 5.7a: Mean proportion of larvae settled ± SE at different densities (in mid November) in the dark; **Figure 5.7b**: Mean proportion of larvae settled ± SE at different densities (in mid December) in the dark.

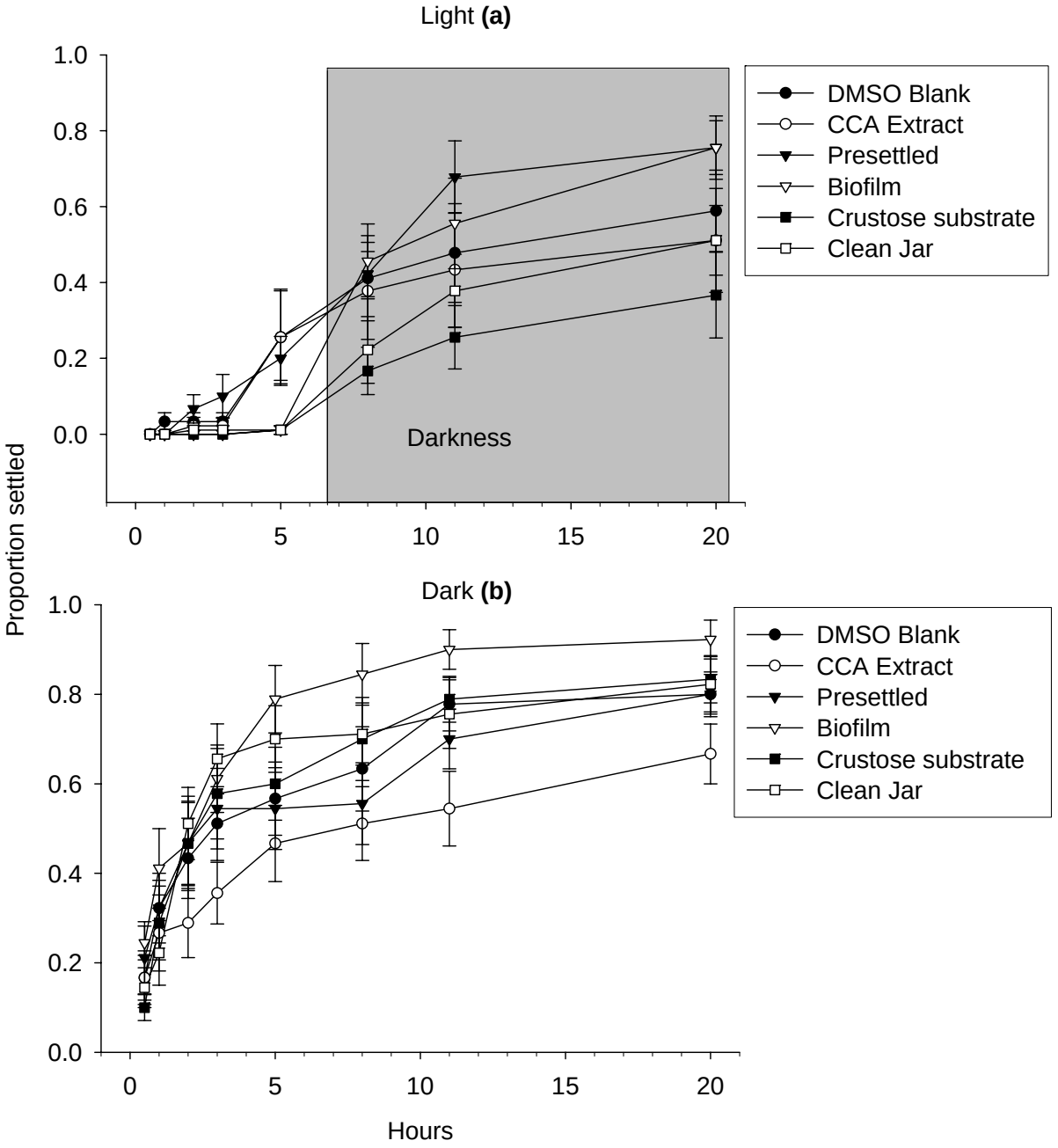

Figure 5.8a: Mean proportion of larvae settled in the light ± SE in response to common invertebrate larval settlement cues (mid November).

Figure 5.8b: Mean proportion of larvae settled in the dark ± SE in response to common invertebrate larval settlement cues (mid November).

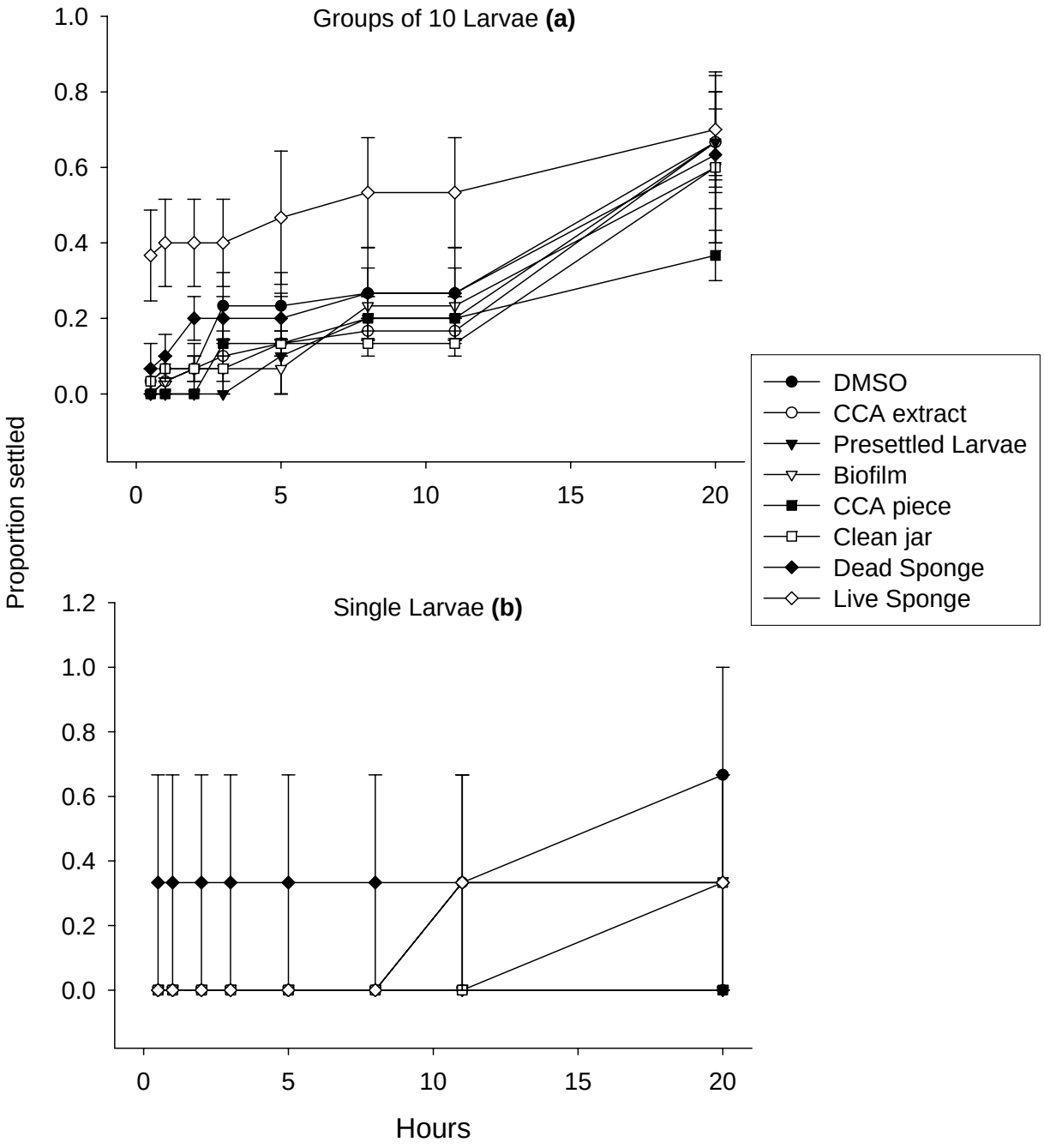

Figure 5.9a: Mean proportion of groups of ten larvae settled in the dark ± SE in response to common invertebrate larval settlement cues (mid December).

Figure 5.9b: Mean proportion of single larvae settled in the dark ± SE in response to common invertebrate larval settlement cues (mid December).

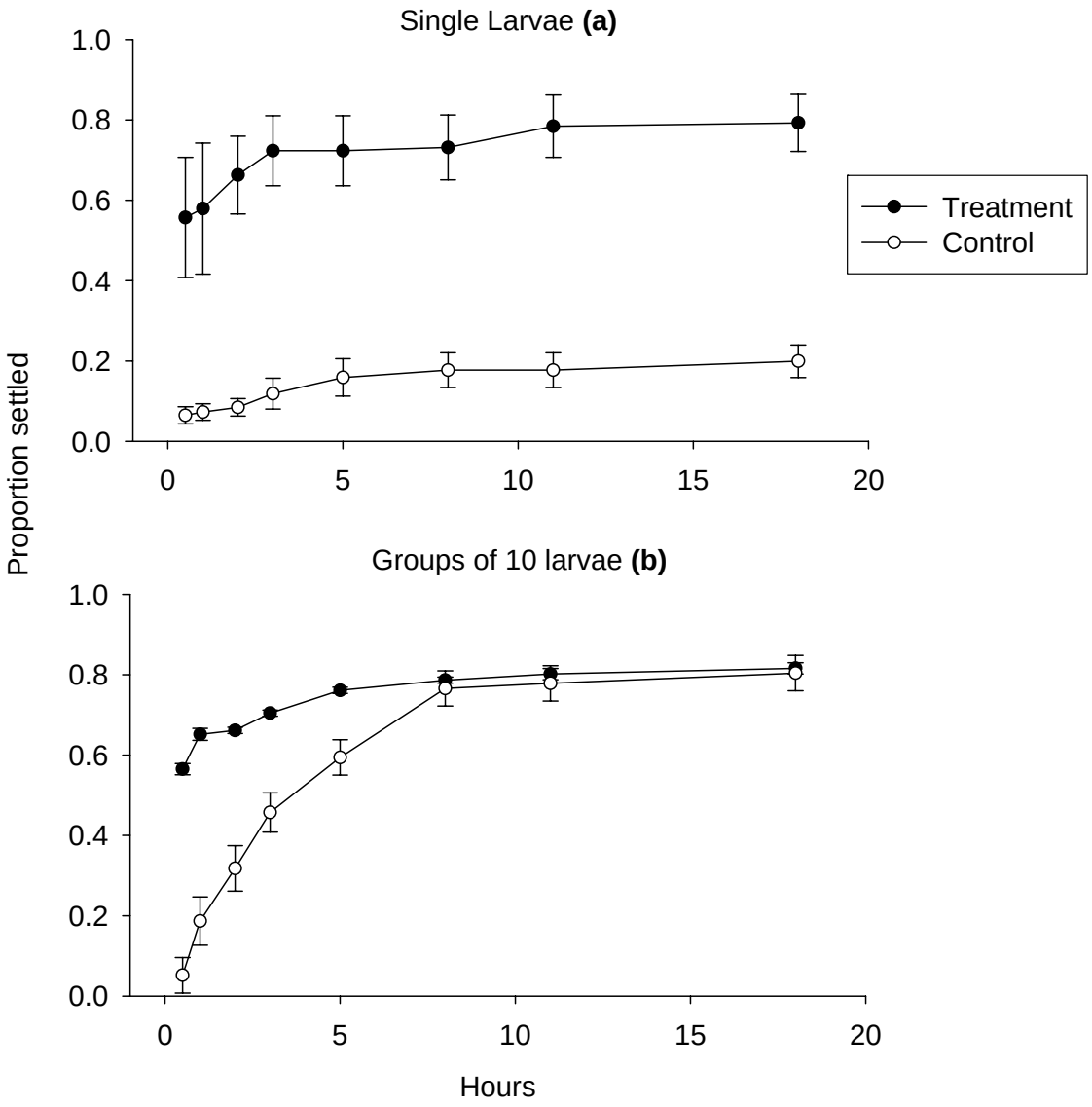

Figure 5.10a: Mean proportion ± SE of single larvae settled exposed to 'conditioned' water and controls.

Figure 5.10b: Mean proportion ± SE of groups of ten larvae settled exposed to 'conditioned' water and controls.

Table 5.1: Results of a repeated measures ANOVA testing settlement at different distances from a cold light source. Data are arcsine square root transformed.

	Source	df	MS	***F***	p
Within-Subjects	Time	3.62	8.35	176.51	<.001
	Time * Distance	25.38	0.17	3.54	<.001
	Error (Time)	203.10	0.05		
Between-Subjects	Distance	7	4.08	22.41	<.001
	Error	56	.18		

Table 5.2: Results of a repeated measures ANOVA testing settlement at different densities at the beginning and end of the spawning season. Data are arcsine square root transformed.

	Source	df	MS	***F***	p
Within-Subjects	Time	2.94	6.81	80.96	<.001
	Time * Season	2.94	0.14	1.77	0.158
	Time * Density	11.75	0.07	0.89	0.589
	Time * Season* Density	11.75	0.12	1.50	0.136
	Error (Time)	117.52	0.09		
Between-Subjects	Season	1	4.97	12.37	0.001
	Density	4	1.58	3.93	0.009
	Season * Density	4	0.17	0.43	0.785
	Error	40	0.40		

Table 5.3: Results of repeated measures ANOVA testing settlement influenced by common invertebrate settlement early in the spawning season. Data are arcsine square root transformed.

	Source	df	MS	***F***	p
Within-Subjects	Time	3.16	11.01	124.00	<.001
	Time * Day	6.32	0.270	3.03	0.008
	Time * Cue	15.80	0.19	2.12	0.012
	Time * Day *Cue	31.59	0.08	0.87	0.661
	Error (Time)	113.71	0.09		
Between-Subjects	Day	2	5.22	8.35	0.001
	Cue	5	1.34	2.14	0.083
	Day * Cue	10	0.76	1.22	0.314
	Error	36	0.62		

Table 5.4: Results of repeated measures ANOVA testing settlement in the presence of a conspecific settlement cue. Data are arcsine square root transformed.

	Source	df	MS	***F***	p
Within-Subjects	Time	3.78	2.70	81.40	<.001
	Time * Condwater	3.78	1.19	35.75	<.001
	Error (Time)	121.04	0.03		
Between-Subjects	Condwater	1	4.71	29.46	<.001
	Error	32	0.16		

Discussion

A hierarchy of cues drives dispersal and habitat choice in *L. variabilis* larvae. Light cues the release of larvae by adult sponges and larvae swim upwards at the time of release. Subsequently, larvae become strongly photonegative and only settle at the same rate as dark controls when subjected to light levels lower than ~3 μmol m^{-2} s^{-1}. There are strong gregarious settlement effects with increasing densities of larvae leading to higher overall settlement. Accordingly, a cue released by settling *L. variabilis* larvae significantly increases the rate of settlement and overall settlement, with the strongest effect on single larvae providing a mechanism for gregarious settlement. However, common invertebrate settlement cues have no effect on the settlement of *L. variabilis* larvae.

This is one of few studies to demonstrate that light cues the release of brooding demosponge larvae and quantifies an entire season's larval release from a large sample size of sponges. The dynamics of the release of *L. variabilis* larvae appear similar to other brooding demosponges with the release of larvae in one or two annual peaks for weeks or months, usually during summer (Maldonado and Young, 1996, Lindquist et al., 1997, Mariani et al., 2005). Some brooding demosponges also release small amounts of larvae throughout the year in addition to large outputs once or twice a year (Zea, 1993, Lindquist et al., 1997) while others release larvae all year round (Leys and Degnan, 2002). Furthermore, the rate of release of larvae by sponges ranges from several larvae per individual over a few hours to the release of the entire brood at one time (reviewed in Maldonado, 2006) and the release rate of between 10s and 1000s larvae day^{-1} for *L. variabilis* corresponded with other demosponges (Meroz and Ilan, 1995, Lindquist et al., 1997).

A light cue for larval release is proposed to ensure the daytime release of some demosponge larvae (Amano, 1986, 1988). Newly released *L. variabilis* initially swim upwards indicating either a positive phototaxis or negative

geotaxis and this upward movement may facilitate dispersal (Bergquist and Sinclair, 1968, Wapstra and Van Soest, 1987, Maldonado et al., 1997). In contrast, larvae became negatively phototactic after 40 minutes. This change in phototaxis was confirmed by the same negative phototactic response by two to four hour old larvae which actively swim either up, down or sideways to access the darkest areas of the vessels in which they were held. The change in phototaxis of sponge larvae from positive to negative is suggested to facilitate dispersal and increase the chance of intercepting settlement cues (*sensu* Wapstra and Van Soest, 1987, Harrison and Wallace, 1990, Raimondi and Morse, 2000).

Light dependent settlement of *L. variabilis* larvae corroborates with the distribution of adult *L. variabilis* in the field which are almost always found in areas of low irradiance (i.e. crevices, caves and between rubble). Other demosponge larvae show strong responses to light. For example, the parenchymellae of *Halichondria caerulea* stop swimming at a given distance from a light source suggesting that a photonegative response was only displayed below a given irradiance level (Maldonado et al., 1997). As *L. variabilis* larvae were released during the day but even very low amounts of light delayed their settlement, daytime release is probably required to provide a gradient of light to guide larvae to dark microhabitats. The selection of dark habitats potentially provides protection against grazers, silt and ultraviolet radiation, or mitigates their competition with photoautotrops (Maldonado and Uriz, 1998). Accordingly, dispersal potential of *L. variabilis* larvae is likely to be short due to the short time they swim upwards and the rapid onset of a negative phototaxis. This may result in genetically structured local populations (Goffredo et al., 2004).

While light cues the release of larvae and guides their behaviour and settlement at large scales, smaller scale environmental variables such chemical cues and surfaces may explain some sponge settlement patterns. *Luffariella variabilis* larvae did not respond to a variety of common invertebrate settlement cues. However, despite no settlement response to settled, attached and metamorphosed conspecifics, motile *L. variabilis* larvae settled

gregariously. This also corroborates with the aggregated distribution of *L. variabilis* adults *in situ* and gregarious settlement by larvae leads to aggregations of conspecific adults in other invertebrates (Burke, 1986, Gotelli, 1990). Gregarious settlement is thought to increase protection from predation (Sebens, 1983, Keough, 1984), enhance competitive abilities (Maldonado and Uriz, 1998), and increase filter-feeding efficiency (Bologna et al., 2005), while also reducing juvenile and adult mortality (Osman and Whitlatch, 1995). Furthermore, gregarious settlement is usually linked to substratum associated compounds and adult conspecifics (Burke, 1986, Toonen and Pawlik, 1994).

In the case of *L. variabilis*, gregarious settlement is associated with conspecific larvae rather than adults, although there was an increased settlement response to adults. When *L. variabilis* larvae were placed in conditioned water, initial settlement rates were six fold faster compared with controls demonstrating that larvae released a waterborne settlement cue. Furthermore, the effect was highest on single larvae with a four fold increase in overall settlement. This is one of the few studies to unequivocally demonstrate that a conspecific cue not related to adult conspecifics or other biotic or abiotic factors induces settlement in larvae. However it is likely that dilution of the cue under natural conditions may render it effective at only small spatial scales. A similar model is found in barnacles where gregarious settlement is mediated by pheromones released by larvae (Matsumura et al., 1998, Dreanno et al., 2006) and these are either waterborne or surface bound in larval footprints (Clare et al., 1994). Experiments to determine the ecological benefits from these effects given the ability to manipulate behaviour and settle *L. variabilis* larvae would contribute to an understanding of how gregarious behaviour guides larvae to find appropriate habitats in the restricted time they have for dispersal, and how cues affect post larval distribution and survivorship of *L. variabilis.*

In conclusion, a hierarchy of cues influences the settlement of *L. variabilis* larvae. Light cues the release of larvae and they swim upwards immediately after release. Subsequently, larvae become strongly photonegative and only settle in low light levels. At smaller scales, there are strong gregarious

settlement effects and a waterborne cue released by settling *L. variabilis* larvae significantly increases their proportion and rate of settlement. This corroborates with the clumped distribution of adults in the field in dark habitats and is one of the few studies for a marine invertebrate which integrate factors affecting the larvae from release from parents through to settlement and metamorphosis.

CHAPTER SIX – GENERAL DISCUSSION

In this thesis the key processes for the development of aquaculture of the sponge *Luffariella variabilis* (Porifera: Demospongiae) for the supply of the high value marine natural product, manoalide are examined. At present aquaculture has the best track record in the production of high value marine natural products. However, the knowledge capital critical to developing aquaculture production of organisms producing marine natural products is lacking and there are several critical prerequisites to ensure the sustainability and productivity of such ventures. They include quantifying variability in natural product production of the target organism (Chapters 2 & 3); elucidating reproductive strategies (Chapter 4); and quantifying the processes affecting recruitment (Chapters 5).

Spatial and Temporal Production of Manoalide by *Luffariella variabilis*

Rigorous studies quantifying natural spatial and temporal variation in metabolite production by sponges are rare and this is an obvious weakness when attempting to optimise yields of natural products. This investigation of the natural products chemistry of *L. variabilis* yielded four known major metabolites, manoalide monoacetate, manoalide, luffariellin A and seco-manoalide in addition to three novel natural products, luffariellin A acetate, luffariellin B acetate and seco-manoalide acetate (Chapter Two). The production of the four major metabolites is hardwired in space and time at the population level in the Palm Islands (Chapter Three). Manoalide monoacetate was always the most abundant compound, followed by manoalide, luffariellin A and seco-manoalide. Accordingly, manoalide monoacetate and manoalide were always 10 to 70 times more abundant than seco-manoalide and luffariellin A which varied over time. However, the scale of this variation was small. Collections made at Davies Reef and Magnetic Island yielded the same rank order and yields of compounds as samples taken in the Palm Islands. This demonstrated a generality of pattern across a range of spatial scales from metres to more than 100 km. Opportunity for understanding the factors driving

variability in the production of these metabolites over larger scales warrants further study given the large geographic range of *L. variabilis* throughout the Indo-Pacific (Bergquist, 1980, 1995). This may support any wild harvest effort, in particular the selection of high yielding stocks and broodstock for the closed lifecycle aquaculture production of metabolites.

In this study, the 'hardwiring' of metabolite production at the population level by *L. variabilis* was also reflected in the lack of any inductive effect on metabolite production. In addition, repeatedly sampled *L. variabilis* individuals produced the four major metabolites, in differing ratios, throughout the duration of the study. However these ratios were fixed over time within individuals. The stability of the *L. variabilis* metabolite ratios over time, and the sizeable quantities in which the individual terpenes are produced suggests a significant genetic component underlying their production.

While the knowledge of genetic control over the production of secondary metabolites in sponges has not been reported, heritable production of secondary metabolites occurs in terrestrial (Zangerl and Berenbaum, 1990, Berenbaum and Zangerl, 1992) and marine plants (Wright et al., 2004). The exploitation of the variable production of metabolites by land plants has been utilised in agriculture in developing pest and pathogen resistant cultivars (reviewed in Kennedy and Barbour, 1992) and these studies will provide models for optimisation of metabolite production from marine organisms.

In the marine environment, descriptions of quantitative variation in secondary metabolite production mostly focus on phenotypic change in the brown algal response to herbivores (Van Alstyne, 1988, Cronin and Hay, 1996b, Targett and Arnold, 1998), pathogens (Dixon, 2001) and environmental factors such as nutrients, temperature, light, desiccation and salinity (Yates and Peckol, 1993, Arnold et al., 1995, Cronin and Hay, 1996a, Pavia et al., 1997, Van Alstyne and Pelletreau, 2000, Jormalainen et al., 2001). However, there is only one study quantifying the extent to which algal secondary metabolites are heritable and this is for a group of halogenated furanones from a red alga (Wright et al. 2004). The only other quantitative study on the heritability of a

trait in a marine organism is that for spine length in a bryozoan (Harvell 1998).

While there are no examples from the marine environment on the heritability of the production of terpenoid metabolites, these compounds are also produced by terrestrial plants and are known in some cases to be highly heritable (Doran and Matheson, 1994, Huber et al., 2004, Byun-McKay et al., 2006, King et al., 2006). Both environmental and genetic effects contribute to qualitative and quantitative variation in terpenoid metabolite contents suggesting the strong potential for the interaction of genotype and the environment (Krischick and Denno, 1983, Kennedy and Barbour, 1992, Laitinen et al., 2005). This provides a platform for examining whether terpene production is heritable in *L. variabilis*. Consequently, selective breeding analogous to that used in the land plants could be engaged if the production of the compounds is heritable. This would allow the selection of high yielding stocks and the determination of the effects of environmental influences on compound production in *L. variabilis*.

Another aspect to understanding the heritability of metabolite production in marine invertebrates begins with the determination of the origin of a metabolite, either from microbial symbiont, or a combination of host and symbiont (Unson et al., 1994, Bewley et al., 1996). The best cases of important drug leads, which are unequivocally known to be produced by a symbiont are the byrostatin group of compounds from *Bugula neritina*. The larvae of *B. neritina* contain the highest concentrations of the bryostatins which are not present in adult tissue (Lopanik et al., 2004). Furthermore, the compounds are produced by a γ-proteobacterium *Endobugula sertula* (Lopanik et al., 2004) and the symbionts are vertically transferred to the larvae from the parent (Haygood et al., 1999). However, the field of vertical transfer of symbionts and symbiont production of sponge metabolites is still in its infancy (Maldonado et al., 2005, Oren et al., 2005, Enticknap et al., 2006) but will be critical for compounds which are partly or totally produced by endosymbionts. If a compound is symbiont produced, molecular, and

microbial manipulations could then be engaged to optimise metabolite production (Haygood et al., 1999).

Reproduction And Larval Recruitment Of *Luffariella variabilis*

As for all aquaculture industries, the closure of lifecycles is essential. This allows for the sustainable production of target organisms especially in cases where wild harvest cannot not provide enough biomass or there are regulatory issues preventing the collection of biomass (Hart et al., 2000, Simmons et al., 2005). Furthermore, closed lifecycle aquaculture allows the selection of strains with heritable traits including the production of secondary metabolites for the development of high yielding broodstock.

Reproduction

Luffariella variabilis is gonochoristic and has a predictable annual reproductive cycle. Furthermore, the seasonal pattern of *L. variabilis* reproduction corresponds with minimum, rising and maximum water temperatures. Oogenesis is cued by the coldest water temperatures of the year of 21 ºC, spermatogenesis commences above 22.5 ºC, and reproduction ceases before the highest temperatures of the year (30 ºC) are reached. Temperature had an important effect on reproductive onset and development in *L. variabilis* between 2004 and 2005 and a delay in the lowest temperature by one month in 2005, delayed both oogenesis and spermatogenesis. However, termination of reproduction occurred at the same time (December) constraining the total time available for fertilisation and embryogenesis resulting in a concomitant decrease in female reproductive output of 30 %.

Rising water temperatures cue oogenesis and spermatogenesis in both gonochoristic and viviparous sponges from the tropics to the poles (Fromont, 1994a, Fromont, 1999, Usher et al., 2004, Witte and Barthel, 1994). Moreover, larval release occurs before maximum water temperatures are reached in some species (Mariani et al., 2005), while in other viviparous species, the release of eggs occurs as temperatures begin to fall (Fromont, 1994a,1999, Usher, 2004).

Therefore, temperature shifts associated with climate change models have important implications for the viability of sponge populations as higher winter temperatures may reduce the time for embryogenesis, or cause a failure in oogensis and/or asynchronicity in gamete formation. Whilst some marine invertebrates shift their reproductive cycles in relation to temperature changes (Velazquez, 2003), it is unknown how broadly applicable this phenomenon is and how resilient their reproductive physiology is to changes in temperature. Therefore there is a need to understand the affects of both minimum and maximum temperature on invertebrate reproductive ecology.

Recruitment and larval biology

Following an understanding of the reproductive biology of *L. variabilis*, the recruitment process for larvae is the subsequent stage in understanding the fundamental biology of *L. variabilis*. A hierarchy of cues drives habitat choice in *L. variabilis* larvae with light playing important roles in larval behaviour, settlement and metamorphosis. Initially, light cues the day release of upward swimming larvae and this behaviour by larvae is generally associated with dispersal (Maldonado et al., 1997). In contrast, after 20 minutes, *L. variabilis* larvae display a strong negative phototaxis and only settle in the dark. This behaviour is hypothesised to guide competent larvae to dark benthic microhabitats (Wapstra and Van Soest, 1987). Furthermore, the rate of settlement and overall settlement of *L. variabilis* larvae is reduced in light levels above ~3 μmol m^{-2} s^{-1} which obligates their choice of dark microhabitats. This information strongly corroborates with the distribution of adults in the field. The choice of dark microhabitats by sponge larvae is hypothesised to provide protection from grazers, silt and ultraviolet radiation, or mitigate their competition with photoautotrophs (Maldonado and Uriz, 1998).

***Settlement in** Luffariella variabilis*

Larvae of *L. variabilis* settled gregariously at higher rates and overall totals in unison with increasing densities of larvae. These data corroborate with the aggregated distribution of *L. variabilis* adults in the field. Gregarious settlement is thought to increase protection from predation (Sebens, 1983,

Keough, 1984), enhance competitive abilities (Maldonado and Uriz, 1998) and increase filter-feeding efficiency (Bologna et al., 2005), while also reducing juvenile and adult mortality (Osman and Whitlatch, 1995). Gregarious settlement is usually linked to substratum associated compounds and adult conspecifics (Burke, 1986, Toonen and Pawlik, 2001). In contrast, increased rates of settlement were due to the release of a cue by larvae as they settle. This is the first report of a waterborne cue mediating settlement of a sponge larvae and the process appears to be analgous to the cyprid settlement of some barnacles which is mediated by larval pheromones (Matsumura et al., 1998, Dreanno et al., 2006a).

In conclusion, a hierarchy of cues influence the behaviour and settlement of *L. variabilis* larvae with light having dual roles in cueing larval release and influencing larval settlement. Settlement and metamorphosis are facilitated in *L. variabilis* by a waterborne cue released by larval, but not adult conspecifics.

Future Directions

In this section a series of future directions to optimise the production of manoalide by *L. variabilis* are proposed. They will also contribute to the understanding of the chemical ecology of *L. variabilis*.

The ecological roles of the manoalide group of compounds needs to be understood given their high levels of production and as terpenoid metabolites are known to have defensive functions in other demosponges (Epifanio et al., 1999).

While the manoalide group of compounds are found in relatively high amounts and in fixed ratios in individuals over time suggesting the compounds are produced by *L. variabilis*, sponges can have up to half their wet weight represented by endosymbionts which produce some secondary metabolites (Becerro and Paul, 2004). Therefore, the true producer of the manoalide group of compounds needs to be determined. Furthermore, if manoalide is produced

by endosymbionts, the vertical transmission of any symbionts needs to be assessed.

If manoalide is produced by *L. variabilis* and not symbionts, the heritability of compound production in *L. variabilis* should be assessed, and if applicable, selective breeding processes employed to produce high yielding stocks via larval ongrowth. The tradeoffs between growth and metabolite synthesis should then be examined as terpenoid metabolite synthesis has been shown to have costs ranging from high to neutral in other taxa (Koricheva, 2002). However, this study suggests a low cost of production of secondary metabolites in *L. variabilis*. In addition, the interactions between both environment and genotype affect qualitative and quantitative variation in terpenoid metabolite synthesis in other taxa (Gershenzon, 1994, Laitinen et al., 2005, King et al., 2006) and studies should be undertaken to elucidate the interactions between any genotypes, the environment and metabolite synthesis in *L. variabilis.*

The formation of *L. variabilis* chimeras was noted during larval settlement experiments *in vitro*. This was not always when larval densities were high. Sibling and non-sibling demosponge larvae are known to fuse at settlement (Ilan and Loya, 1990b, Maldonado and Uriz, 1998) but there is no evidence that the formation of chimeras is chemically mediated (Maldonado, 2006). This requires further examination considering settling *L. variabilis* conspecifics release a yet uncharacterised waterborne settlement cue. Furthermore, the ecological and genetic implications of the formation of chimeras requires investigation in *L variabilis* as the initial larger size of the recruit may favour growth and may also reduce the time in which they reach a 'size refuge' from grazing and other physical disturbances (Reiswig, 1973, Ayling, 1980, Fell, 1993).

In addition to the work focused on this study (natural products chemistry, reproductive biology and settlement), understanding a suite of post recruitment processes, including the optimisation of on-growth protocols, is required.

Conclusions

This work targets the critical links between the fundamental biology and chemical ecology of *L. variabilis*, providing the knowledge base for any future aquaculture effort. Furthermore, the platform developed for *L. variabilis* will be transferable to other sessile marine invertebrates producing secondary metabolites of interest to medicine and industry.

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