EFFECT OF SPATIAL AND ENVIRONMENTAL FACTORS ON THE REPRODUCTIVE CYCLE OF THE BRITTLE STAR, *Ophiopholis aculeata*, BASED ON A NEW MATURITY STAGE INDEX

GINA DOYLE

Effect of spatial and environmental factors on the reproductive cycle of the brittle star, *Ophiopholis aculeata*, based on a new maturity stage index

By

©Gina Doyle

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Ocean Sciences Centre and Department of Biology,

Memorial University,

St. John's (Newfoundland and Labrador) Canada

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Abstract

Monthly collections of the brittle star *Ophiopholis aculeata* were made and individuals were analyzed using traditional methods (gonad indices, gametogenic stages and oocyte diameters), along with a newly developed quantitative method coined the 'maturity stage index' (MSI). The MSI was statistically related to conventional methods of studying reproduction, but provided greater sensitivity for fine-scale and inter-individual comparisons. This allowed the study of reproductive synchrony in *O. aculeata* over several scales: (1) within a given habitat, (2) between adjoining habitats, (3) among individuals in each habitat, and (4) within and among natural clusters of individuals. Complementary laboratory trials were conducted to further examine the influence of temperature and spatial distribution on gametogenesis. *Ophiopholis aculeata* exhibited an annual gametogenic cycle with spawning in late summer. Significant differences were seen between habitats and among individuals in certain months, and synchrony was generally greater around the time of spawning and in nearby/grouped individuals. Laboratory experiments indicated that temperature is unlikely to act as a proximate reproductive cue, and phytoplankton is probably the major environmental factor mediating the annual reproduction of *O. aculeata*. Together, field and laboratory findings further evidenced the synergistic roles of exogenous factors and inter-individual communication in coordinating the reproductive cycle of marine invertebrates.

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List of abbreviations and symbols

EG – early growth (gametogenic cycle)

EV – early vitellogenic oocytes (oogenesis)

G – growth (gametogenic cycle)

GE – germinal epithelium (oogenesis)

GI – gonad index

KWA – Kruskal-Wallis ANOVA on Ranks

M – mature (gametogenic cycle)

MSI – maturity stage index

O – oogonia (oogenesis)

OSC – Ocean Sciences Centre

OWA – one-way ANOVA

R – residual oocytes (oogenesis)

S – spent (gametogenic cycle)

SC – spermatocytes (spermatogenesis)

SG – spermatogonia (spermatogenesis)

SZ – spermatozoa (spermatogenesis)

TWA – two-way ANOVA

V – vitellogenic oocytes (oogenesis)

Co-authorship statement

The research described in this thesis was carried out by the author, Gina Doyle, who was responsible for data collection and analysis. Guidance for experimental designs and data analysis was provided by Annie Mercier and Jean-François Hamel, who also made significant editorial contributions during the preparation of the chapters/manuscripts.

Chapter 1 - Introduction

1.1 Reproductive biology of benthic invertebrates

Sexual reproduction occurs in marine invertebrates through several different methods. Copulation is common in some groups, like nudibranchs, cephalopods and crustaceans, while pseudo-copulation occurs in some nemerteans and polychaetes. Brooding is another reproductive strategy, whereby fertilized eggs are maintained within or underneath a parent, or in protective capsules, until release at a more mature stage. The ancestral and most common reproductive method is broadcast spawning (also known as free spawning), in which oocytes and spermatozoa are released into the water column, where fertilization occurs (Thorson 1950; Giese 1959; Giese and Kanatani 1987). When a broadcast-spawning adult releases gametes into the water, the likelihood of spermatozoa successfully fertilizing oocytes is presumed to be low, even considering the large number of gametes released, based on the fast diffusion rate and short life of gametes (Levitan 1995; Metaxas et al. 2002). It is thought that the influence of exogenous (environmental) and endogenous (internal) rhythms enable marine invertebrates to temporally synchronize aspects of their reproductive cycle, theoretically increasing the likelihood of fertilization and larval survival. Exogenous factors include photoperiod, temperature, lunar cycle, tides, pressure changes, exposure during low tides (dessication, increased temperatures, low oxygen, and wave action), salinity changes, upwelling,

plankton levels and current, among others. Endogenous factors are mostly hormonal, as the reproductive cycle is largely under tight endocrinal control (Giese and Kanatani 1987). In his study on fertilization success, Levitan (1995) described four levels of factors which affect the probability of fertilization: gamete, individual, population and environment. Several factors at the individual level (aggregation, synchrony in gamete production and release) and at the population level (density, distribution) are especially important. These factors are well studied in many marine invertebrate species, particularly in the context of maturation and spawning (Giese and Kanatani 1987). One of the phyla most commonly used in experiments on reproduction and spawning are the echinoderms (reviewed by Mercier and Hamel 2009).

1.2 The study of reproductive periodicity and synchrony in echinoderms

Most temperate and boreal echinoderms display an annual reproductive cycle, which represents a circannual rhythm. Many different exogenous and endogenous factors have been implicated in mediating circannual rhythms, though most studies to date have largely focused on exogenous factors. In particular, gametogenesis and spawning in echinoderms and other marine invertebrates have been linked with temperature, lunar cycle, photoperiod, salinity, phytoplankton, food availability and tides (see reviews by Giese and Kanatani 1987; Himmelman 1999; Mercier & Hamel 2009; and references therein). Photoperiod and temperature in particular have long been studied to

determine their influence on reproductive cycles in echinoderms, although few studies have uncoupled these factors to study the influence of one without the other. This is particularly important in species from climes with large temperature and light variation throughout the year.

Echinoderms are important members of marine benthic communities in oceans worldwide, occurring at all depths, from the littoral zone to the abyssal plain. The reproductive system of echinoderms varies throughout the different classes, but a majority of them have separate sexes and are broadcast spawners. Many factors are thought to affect reproductive cycles in echinoderms, one such factor being spatial distribution. Studies on the influence of distribution on reproduction have examined a variety of scales, normally focusing on broad-scale population comparisons from different locations (e.g. Lessios 1981; King et al. 1994; Lefebvre et al. 1999; Guettaf et al. 2000; Lamare et al. 2002), though a few have focused on smaller scales (i.e. Kelly 2000). Most studies of reproductive periodicity in echinoderms use the same analytical methods, generally some combination of gonad indices, somatic indices, internal and external biometrics, histology, oocyte size frequency distributions, gametogenic 'staging', and maturity indices. Each of these has its own benefits and drawbacks, but a problem with many of them is the difficulty to quantitatively compare individuals at finer spatial scales.

Within the realm of echinoderm reproduction, echinoids are seemingly the most studied (e.g. Gonor 1973; Byrne 1990; King et al. 1994; Byrne et al. 1998; Meidel and Scheibling 1998; Seward 2002; Kelly 2000). Gonor (1973) found a

high degree of synchrony between three populations of the sea urchin *Strongylocentrotus purpuratus* along the Oregon coast, with spawning generally around the same time. Byrne (1990) studied the sea urchin *Paracentrotus lividus* from two different habitats on the west coast of Ireland (exposed intertidal and sheltered subtidal), and found that the overall reproductive trend was the same between habitats, but subtidal individuals had larger gonads and were reproductively mature for longer than intertidal individuals (likely because they were in a better nutritional state). King et al. (1994) studied the sea urchin *Centrostephanus rodgersii* in two separate populations in Australia and found gametogenesis to be generally synchronous, with some variations in duration of maturation and spawning. Byrne et al. (1998) also studied reproduction in *C. rodgersii* in two habitats in Australia (algae-less urchin barrens and algae-dominated fringe) at four different locations spanning between the northern and southern distribution limits, with the fringe habitat generally having higher reproductive output (thought to be caused by greater food availability). This study found synchronous reproduction at all locations, demonstrating that gametogenesis and spawning were likely influenced by similar environmental cues across the entire range of this species. Meidel and Scheibling (1998) studied reproduction in the green sea urchin *Strongylocentrotus droebachiensis* in three habitats in Nova Scotia (kelp beds, barren grounds and grazing fronts) at two different sites (wave-exposed and sheltered). The overall reproductive cycle was found to be synchronous between habitats, sites and sexes, with some small discrepancies. For example, urchins from barren grounds generally had lower

gonad indices for most of the year (likely due to lower quality/quantity of food) than those from sheltered areas. Kelly (2000) studied reproduction in the boreal echinoid *Psammechinus militaris* using samples collected from contrasting habitats 200 m apart at two different locations in Scotland. The habitats were a fucoid and laminarian-dominated littoral zone with relatively fine sediment and small boulders, and an algal-dominated subtidal zone with smaller boulders. Gonad index was significantly different between sites, between locations, and between years; however, it was difficult to truly compare the habitats between sites, based on some variations in physical conditions at each site.

Asteroidea have also been studied extensively and are often used as model organisms, while the remaining classes are less well known. This is interesting when you consider a class such as Ophiuroidea (the brittle stars), as its members are numerically dominant in many macrofaunal assemblages and are the most diverse of the extant echinoderms, with at least 16 families and 2000 species (Hendler 1991).

1.3 The reproductive biology of ophiuroids

Brittle stars (class Ophiuroidea), are prominent members of many marine benthic communities around the world. They have adapted strategies to live in a wide range of marine habitats, trophic levels and ecological niches (Fell 1966; Hendler 1991).

Despite this, ophiuroids are not as well studied as other classes of echinoderms, and important aspects of their reproductive biology are poorly

understood. Reproduction can occur through asexual or sexual means, with fissiparity being the most common method of asexual reproduction (Hendler 1991). Species demonstrating asexual reproduction via fission or cloning are typically tropical or subtropical in distribution, with small sizes at sexual maturity (e.g. Mladenov and Emson 1988; Chao and Tsai 1995; McGovern 2002). There is significant variation within sex ratios of various ophiuroids, with some species showing further differences among populations or size classes (reviewed by Hendler 1991). The reproductive system of ophiuroids is unique in having bursae – sacs in the coelom that have roles in reproduction, respiration, and a suspected role in excretion. Each bursa has an opening to the external environment, known as the bursal (or genital) slit. Each slit is protected by genital plates and scales (Hendler 1991). Ophiuroid gonads are usually found in association with the bursae, in the body cavity underneath the stomach (Hendler 1991).

Descriptions of ophiuroid reproductive cycles vary between species, but share many of the same general characteristics. For both males and females, the gametogenic cycle begins with the gonad in a shrunken state, following a spawning event (Bowmer 1982; Hendler 1991; Falkner and Byrne 2003). In females, residual (unspent) oocytes may be present in the gonadal lumen following spawning, often in the process of being lysed by degenerative phagocytes (Fenaux 1970; Hendler and Tyler 1986; Hendler 1991; Falkner and Byrne 2003). Oogonia proliferate from the germinal epithelium and/or the genital rachis (Hendler and Tyler 1986; Hendler 1991; Falkner and Byrne 2003). Cellular division of the oogonia leads to increasingly smaller cells: from oogonia, to

primary oocytes and then secondary oocytes (Patent 1969; Fenaux 1970; Tyler 1977; Bowmer 1982; Hendler and Tyler 1986; Hendler 1991). Female ophiuroids only have secondary oocytes in their ovaries shortly before spawning. Some experimental results suggest that this is because maturation divisions only occur just before spawning (Patent 1969; Fenaux 1970; Tyler 1977; Bowmer 1982; Hendler 1991). Regardless, as meiotic divisions occur, the oocytes grow in number and size, undergo vitellogenesis, and begin to fill the lumen. As the oocytes proliferate and grow, the ovarian walls become swollen, causing the gonad to appear larger. When enough oocytes are mature and the ophiuroid receives the correct endogenous and/or exogenous triggers, spawning occurs (Hendler 1991).

Spermatogenesis occurs similarly to oogenesis in ophiuroids. Spermatogonia begin to generate from the germinal epithelium and/or the genital rachis (Hendler 1991; Falkner and Byrne 2003). Cellular division leads to increasingly smaller cells: from spermatogonia, to primary spermatocytes; secondary spermatocytes; spermatids; and spermatozoa. Maturing testes tend to display the developing germ cells in a layer in order of maturity, beginning with spermatogonia near the germinal epithelium, ending with free spermatozoa in the lumen. This layer becomes thicker as spermatogenesis progresses, more spermatids are produced and spermatozoa begin to fill the centre of the lumen. Close to the spawning event, the free spermatozoa in the lumen form a separate mass (Fenaux 1970; Bowmer 1982; Hendler and Tyler 1986; Hendler 1991; Falkner and Byrne 2003). Spent gonads may possess germ cells in various

stages of degradation (Fenaux 1970; Hendler and Tyler 1986; Hendler 1991; Falkner and Byrne 2003). Male specimens typically have active sperm through most or all of the year, not just during the reproductive cycle (Taylor 1958; Lønning 1976; Blake 1978; Hendler 1991).

Gametogenesis and spawning in ophiuroids varies among species. Bowmer (1982) reviewed the available literature and suggested that there appear to be three trends in ophiuroid breeding strategies. Many species showed discrete, short, annual reproductive cycles, some showed extended breeding periods (some having peaks of spawning), and some species showed year-round asynchronous cycles (with or without peaks of spawning). Even within those species reported to have short, annual reproductive cycles, variations exist; some species having been reported to spawn in a single event, while others require multiple spawning events to completely empty the gonads (i.e. *Amphiura filiformis*; Bowmer 1982).

Although studies have looked at the relationship between reproduction and exogenous factors in ophiuroids (i.e. Tyler 1977; Hendler 1979; Bowmer 1982; Yamashita and Iwata 1983; Stancyk 1974; Rumrill and Pearse 1985; Hendler and Tyler 1986; Valentine 1991; McClintock et al. 1993; Stewart and Mladenov 1995; Lefebvre et al. 1999; Morgan and Jangoux 2002; Grange et al. 2004; Borges at el. 2009), we still know relatively little about how environmental conditions control the reproductive cycle in most species (Bowmer 1982).

1.4 The biology and reproduction of *Ophiopholis aculeata*

The daisy brittle star, *Ophiopholis aculeata* (Ophiuroidea; Ophiurida), is a ubiquitous subtidal species, often found in tide pools and in rocky substrates of the upper sublittoral zone (Falk-Petersen 1982; Gosner 1978). The species has a circumpolar distribution, and is common in arctic and boreal waters on both sides of the Atlantic, living as far south as the British south coast in the east and Cape Cod in the west. *Ophiopholis aculeata* is also common in the Pacific, living as far south as California in the east and Japan in the west. Daisy brittle stars are rarely found deeper than 300 m, although some have reportedly been found as deep as 1500 m (Mortensen 1927; Fell 1966; Gosner 1978). Few studies have focused on population dynamics of this species, but Himmelman et al. (2008) found population density at 7-12 m depth in the Gulf of St. Lawrence (eastern Canada) to be >80 individuals · m^{-2}. The species is known to prefer living under stones and shells, and to contort its body to fit into irregular crevices. Its habitat choice is influenced by many factors, with a particular preference for low light intensity and high substrate complexity. This may help *O. aculeata* avoid predators (Fell 1966; Drolet et al. 2004).

Preliminary studies in eastern Newfoundland have found *O. aculeata* living in two main habitats – under rocks, and inside rhodoliths, also known as maërl (non-genticulate coralline red algae; pers. obs.). Anatomically, *O. aculeata* is similar to most ophiuroids. It is primarily red or purple, and its arms are often banded. The central disc can grow to approximately 20 mm in diameter, while the

arms can reach up to 80-90 mm in length (Mortensen 1927; Serafy 1971; Gosner 1978). The central disc is covered in small granules, with radial shields concealed and primary plates completely exposed. *Ophiopholis* species are separated from other genera in the family Ophiactidae by their ovular dorsal plates, which are all surrounded by a circle of small plates, separating the dorsal plates from one another. They have an ovular mouth shield, with 2-6 oral papillae on either side of the jaw, infradental papillae on top of the jaw and a vertical row of teeth on top of that. Their ventral plates are rectangular, and are also separated from one another, but by a small pit instead of plates. They have 4-8 arm spines per segment, with tentacle scales covering their tube foot pores near proximal spines (Mortensen 1927; Serafy 1971).

Ophiopholis aculeata has two bursae in each interambulacral area, one on either side of each arm, and one gonad per bursa, for a total of ten (Taylor 1958; Hendler 1991). Falk-Petersen's (1982) study of echinoderms in a Norwegian fjord found that all ophiuroids studied, including *O. aculeata*, had planktotrophic (feeding) larvae. Falk-Petersen (1982) did not make an estimate of oocyte production, but suggested that it was similar to other planktotrophic echinoderms in the area, ranging from tens of thousands to millions. The average oocyte size was found to be 10 µm. A combination of sources reviewed by Hendler (1991) indicate that the maximum oocyte diameter of *O. aculeata* is 120 µm and that the number of oocytes per individual is approximately 4.4×10^5. In *O. aculeata*, testes are almost always white, while the oocytes have been reported as greenish, red, orange, yellow and light pink (Hendler 1991).

There have been some preliminary studies on the reproductive cycle of *O. aculeata*, but there is only one known study of the complete reproductive cycle, the results of which were never published outside a doctoral thesis (Blake 1978). This investigator did a histological study of the reproductive cycle of *O. aculeata* in the northwest Atlantic, with study sites in Blue Hill Falls, Maine (USA) and the eastern Avalon Peninsula, Newfoundland (Canada). Blake's (1978) histological analysis of the reproductive cycle was based on a small sample size of specimens collected from unspecified habitats over a loosely structured sampling period. In his review, Hendler (1991) concluded that Blake's findings "exemplify the drawbacks of correlative, non-experimental studies".

Blake's (1978) results remain useful for comparison with other accounts of reproduction in *O. aculeata*. Several studies conducted between 1886 and 1942 found that the timing of the spawning seasons varied between years and locations, but always lasted for 1-5 months, and always fell between April and August (Boolootian 1966). A more recent study by Falk-Petersen (1982) found that the spawning season stretched from April to July. The study by Blake (1978) contrasted with these early studies, as he concluded that *O. aculeata* spawned in the fall. Despite the fact that his results differed from the early studies, he supported his findings by continuing his study in two locations over two spawning seasons. He found that the spawning season may fluctuate annually, and may vary between different populations. He also suggested that *O. aculeata* in Newfoundland spawned earlier than conspecifics in Maine during the first year of his study, but that both populations spawned around the same time the year

after. Blake (1978) concluded that the gonad index underwent the same pattern every year, but that the timing of index changes varied annually.

1.5 Research objectives

Because of their gregarious nature and widespread ubiquitous distribution, ophiuroids provide excellent opportunities for the study of fine-scale determinants of reproductive synchrony. The current study plans to:

I. Develop an easy, reliable, quantitative method of measuring gametogenic development, independent of traditional methods such as gonad indices and gametogenic 'stages' (Chapter 2);

II. Use this method in Chapter 3 to:

a) Determine the timing of reproduction in *O. aculeata*.

b) Investigate gametogenic synchrony in *O. aculeata* at various scales (among individuals, among discrete groups, between two habitats) over the annual cycle;

c) Experimentally probe the underlying exogenous controls of reproduction in brittle stars.

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Chapter 2 - A quantitative study of ovarian development in an ophiuroid and a holothuroid using a newly developed maturity index

2.1 Abstract

It was hypothesized that an accurate quantitative method of studying gametogenesis could be developed that would allow fine statistical comparisons among individuals and between sampling dates. Monthly collections of the brittle star *Ophiopholis aculeata* were made to determine gonad indices as well as mean oocyte density and diameter using histological techniques. A maturity stage index (MSI) was developed that integrates disc diameter, oocyte density, and oocyte surface area. Comparative examination showed that the MSI was significantly related to the conventional gametogenic staging system, and that it provided greater sensitivity for fine-scale studies including statistical analysis. The use of the MSI was also tested in a holothuroid with a different reproductive strategy (*Mesothuria lactea*), demonstrating that it can readily be adapted by modifying the parameters to account for mode of reproduction and average oocyte size.

2.2 Introduction

Marine invertebrates are a diverse group of animals distributed in at least 15 phyla that present a wide array of reproductive strategies. Studies of marine invertebrate reproduction traditionally focus on the spawning event, considered to be the climax of the reproductive cycle (Giese and Kanatani 1987). Therefore, our knowledge of reproduction relies heavily on gamete release or incidence of larvae, from which the rest of the reproductive cycle is often extrapolated (Tyler 1977; Hendler 1991).

Over the years, techniques for studying reproduction have improved to increasingly encompass different aspects of the reproductive cycle. These techniques include gonad indices (GI), scanning electron microscopy (SEM), and histology (Giese and Pearse 1974; Mercier and Hamel 2009). Methods involving GI and other indices typically compare the fluctuation in a chosen index (e.g. ratio of gonad weight on body wall or shell weight) across the reproductive period, generally assuming that changes in the index are attributable to reproduction. Standard histology and more sophisticated SEM methods are typically used to visually compare the reproductive organs as they proceed through the reproductive cycle. Many histological studies base their analysis on methods developed for various echinoderms by Yoshida (1952), Fuji (1960), Patent (1969), Fenaux (1970) and others, who divided both the male and female gametogenic cycles into distinct stages based on characteristics such as size, abundance, and organization of gametes, staining properties, thickness of

reproductive tissues (e.g. gonad wall) and presence of phagocytes. This system has been modified to fit many species by different researchers. Results are often presented in tables or graphics showing proportions of individuals in the different stages at any given time, and are commonly supplemented by gonado-somatic indices and oocyte size frequency distributions. Most recent studies on reproduction in ophiuroids focus on the use of gametogenic staging, often in conjunction with other methods (Table 2-1).

Conventional methods generally work well for studies trying to provide broad information about reproductive cycles, such as determining approximate onset of gametogenesis and spawning period, especially for species with synchronous gametogenic cycles. However, when studying species that do not exhibit such 'clear cut' reproductive cycles, some of these methods can be less effective, particularly for fine comparisons within and between samples (pers. obs.). In their review of the endogenous and exogenous controls of echinoderm reproduction, Mercier and Hamel (2009) state that: "the current (lack) of knowledge (about reproductive cycles) may simply reflect our inability to evaluate the reproductive cycles properly, most studies being of insufficient duration and/or detail to measure reproductive variability in space and time". Furthermore, they provide a discussion of the various issues with gonad indices in different echinoderms, all of which can be used to form a single conclusion: the GI alone is not always a reliable proxy of gametogenic development; it is best used to supplement other methods, particularly gametogenic stages established with histology. Histology and associated analytical techniques provide a stronger tool,

and have been used extensively in echinoderms and other marine invertebrates. These methods are quite effective for many studies, but their qualitative nature makes it hard to standardize measurements among different researchers, studies and species. This also makes statistical analysis more difficult.

Relatively few studies on marine invertebrates have tried to circumvent difficulties associated with the qualitative methods and GI by developing other quantitative methods. Many of the most notable examples come from studies of commercial bivalves. One method commonly used to quantify reproductive condition of bivalves over time is gamete volume fraction (GVF). This represents the proportion of germinal tissue containing follicles with developing and/or mature gametes, and can also demonstrate proportions of connective tissue and lumen space. This is determined by performing histology and analysing the tissues using stereology (Bayne et al. 1978; Lowe et al. 1982; Newell et al. 1982; MacDonald and Thompson 1986). The GVF is most often estimated visually, with methods such as a point-count technique (Newell et al. 1982; MacDonald and Thompson 1986), though more recent studies used imaging software (Toro et al. 2002). Some use a calculation of the GVF that depends on the volume of the gonad composed of gametes in different stages of development, such as previtellogenic oocytes, vitellogenic oocytes and mature oocytes (Jaramillo 2001). Many use the GVF to compare reproductive condition and synchrony within and between sexes and/or populations (Newell et al. 1982; MacDonald and Thompson 1988; Toro et al. 2002). A derivation of the GVF is seen in research by Buchanan (2001), who measured three indices in a commercial

mussel species, including (1) proportion of histological sections comprised of follicles, (2) proportion of section comprised of gametes, and (3) proportion of follicles comprised of gametes. The first two indices provided reliable descriptions of the reproductive cycle, though the second was most robust. The third index was not a good indicator of gametogenic changes, as the proportion of gametes in follicles did not vary enough following spawning. Gómez-Robles et al. (2005) used digital imagery to measure two indices (lipid index and protein index) to indicate oocyte quality in a commercial oyster. Osada et al. (2007) and Enríquez-Díaz et al. (2009) measured mitotically active gonial cells and stereologically-measured gonad biomass, respectively. These methods are quite useful, but the approach of Osada et al. (2007) entails extensive manipulation of live animals (injection and later detection of an incorporated chemical compound), and that of Enríquez-Díaz et al. (2009) is still largely based on analysis of whole gonad size/weight using histology and image analysis.

Most quantitative studies still focus on oocyte size frequencies to define gametogenic development, although these can be problematic when trying to distinguish between mature and spent individuals, which often exhibit the same size range of oocytes due to partial spawning. In 1952, Yoshida developed a maturity index (MI) which was adapted by Patent (1969), Tyler (1977) and Bowmer (1982), among others. The MI, based on the numbers of individuals in each of the pre-defined gametogenic stages (usually numbered as 1-6, from recovery to spent), was meant to define some average stage-based maturity in a given month. This was calculated using the formula:

$$MI = \frac{\sum [1(\text{\# of animals in Stage 1}) + 2(\text{\# in Stage 2}) \ldots + n(\text{\# in Stage n})]}{\text{Total number of animals in a given month}}$$

This type of maturity index works well when the gametogenic stages are already determined, and is valuable when trying to compare male and female specimens or samples from different locations or years. However, it comes with the disadvantage of being calculated using the qualitative gametogenic stages that rely on personal interpretation.

The present study was undertaken in an attempt to develop a straightforward and reliable quantitative determination of gametogenic development that would lend itself to more robust statistical analysis and be useful for both assessment of breeding periods and fine-scale studies of reproductive synchrony at various levels. It was carried out during an investigation of the reproductive cycle of the brittle star *Ophiopholis aculeata* using monthly gonadal samples collected in females from a rocky habitat (see Chapter 3). First, gonadal development was established in the sample series using conventional methods (GI, histological stages, oocyte sizes). Then, the various stereological measures (oocyte diameter, area and density) were tested in a number of different Maturity Stage Index (MSI) formulas. Initial assessment of female gonadal sections of *O. aculeata* indicated that there is an inverse relationship between oocyte density (initially high and decreasing as gametogenesis progresses) and oocyte diameter (initially low and increasing as gametogenesis progresses) in this species, but that each phase of growth (as

defined by qualitatively determined stages of maturity) was difficult to solely characterize based on either of these variables alone. It was hypothesized that a functional formula would yield a numerical value that would significantly correlate with the qualitatively determined stage of maturity, thus becoming a quantitative proxy for gametogenic development. The reliability of this new quantitative index was subsequently compared with that of traditional indices and singular parameters of the formula (oocyte density, oocyte diameter). Finally, the use of the MSI was tested in a second species that exhibits an entirely different reproductive strategy to preliminarily assess if/how the MSI formula can readily be adapted.

2.3 Materials and methods

2.3.1 Sample collection

This study was conducted concurrently with an investigation of the reproduction of *O. aculeata* (see Chapter 3). Samples were collected off the municipality of St. Philip's located on Conception Bay, in south-east Newfoundland (N 47° 35.5, W 52° 53.5). Sample collection was undertaken via mid-monthly dives from June 2008 to June 2009, as well as an extra dive at the end of August 2008 in the midst of the anticipated spawning period. Samples were collected from an area of small rocks on a slight low-grade slope extending from 10-15 m deep and measuring approximately 100 m by 100 m across. A minimum of 20-30 individuals were collected during each collection period. Only

sexually mature females (disc diameter ≥ 8.5 mm) were used for the present analysis.

2.3.2 Biometrical analysis

Photographs were taken of each specimen under a Nikon SMZ1500 stereomicroscope attached to a Nikon DXM1200F digital camera. Photos were used to take two perpendicular measurements of disc diameter using the imaging software Simple PCI® (v. 6.0). Each individual was then preserved in 4% formaldehyde for a minimum of seven days. Initial assessment found that all gonads in an individual were in the same developmental state. Gonads were removed using forceps to extract both gonadal lobes in each bursa (including the genital rachis).

2.3.3 Traditional methods

For comparative purposes, all samples were analyzed using two traditional methods of studying gametogenesis: gonad index and stage determination from histological sections (Mercier and Hamel 2009).

Gonad index (GI)

Gonad indices were calculated for a monthly minimum of 10 sexually mature females. All ten gonadal lobes were removed, blotted to remove excess liquid, and weighed together. The GI of each individual was calculated as the percent wet weight of all gonads to wet weight of the central disc (with arms removed).

Histology and gametogenic stages

Two gonadal lobes (from the same bursa) were used for histological analysis. They were dehydrated in a Leica TP1020 Semi-enclosed Benchtop Tissue Processor® that moved the samples through a graded series of ethanol baths (70% ethanol, Flex 80 and 95 and 100% ethanol), two baths of Neo-Clear Clarifier®, followed by paraffin infiltration and embedding in two vacuum-baths of Paraplast®. Three 12-14 µm sections were made from each paraffin block (2 gonads x 3 histological sections each = 6 sections per individual), and were then mounted and stained using the periodic acid-Schiff (PAS) method (Junqueira et al. 1986).

Four oogenic stages were defined: early growth, growth, mature and spawning/spent, as described below, based on the work of Patent (1969), Fenaux (1970) and Falkner and Byrne (2003). Some studies devise systems using five or six stages, typically including an indeterminant stage (often a post-spawning or recovery stage) or two separate stages for spawning (i.e. partially spent and post-spawning). However, a four stage system was thought to work best for the current study. The 'indeterminant' stage was seen in so few samples that they were not considered useful for quantitative analysis and were simply discarded. In addition, the population of *O. aculeata* used in the current study appears to exhibit partial spawning, so that fully spent individuals were rare. All samples in any state of spawning were considered in the 'spent' category.

The stages used were as follows:

Stage 1 – Early Growth (Figure 2-1A): The ovaries are very small, though the gonad walls and germinal epithelium are at their thickest. Oocytes are generally tear-drop shaped, measuring 10-30 µm at the longest axis. Small, relatively infrequent pre-vitellogenic oocytes are growing from the germinal epithelium. They are basophilic, staining dark blue/purple with PAS.

Stage 2 – Growth (Figure 2-1B): Early on, oocytes are still closely linked to the germinal epithelium, but they are larger (30-50 µm) and more abundant. Most are tear-drop shaped, but some are elongated and spindle-shaped. In the later part of the growth stage, the ovaries become larger, and the gonad wall thinner and less prominent. Some oocytes are still closely associated with the germinal epithelium, but most are spread throughout the ovarian lumen. The sizes of oocytes range between 30 and 70 µm. Most large oocytes are spindle-shaped or polygonal. Most have begun vitellogenesis, have a clearly visible germinal vesicle and single nucleoli, and are much less basophilic, staining light purple with PAS.

Stage 3 – Mature (Figure 2-1C): The ovaries are swollen, and the gonad wall and germinal epithelium are very thin. The lumen of the ovary is packed with large vitellogenic oocytes, with almost no connective tissue visible. Virtually all oocytes are fully grown, with a uniformly polygonal or ovular shape and diameters ranging from 70 to 110 µm. Very few small oocytes (10-50 µm) may occur along the germinal epithelium. PAS staining imparts a light pink shade to mature oocytes. This stage is very brief and only seen in few individuals.

Stage 4 – Spent (Figure 2-1D): In the early part of this stage, the ovary is still swollen, but smaller in appearance than when mature. The ovary contains residual vitellogenic oocytes (80-120 µm) in the process of being lysed by phagocytes, which are less densely packed than in the mature stage, exhibiting significant gaps between them. As this stage progresses, the ovary is no longer swollen, and has returned to a relatively small size, and the gonad wall remains thin. By the late spent stage, the gonad wall shows signs of thickening with occasional presence of few very small residual oocytes.

2.3.4 Maturity stage index (MSI)

A quantitative measure of oogenesis was developed and tested using digital photographs of the histological sections obtained with a Nikon Eclipse 80i microscope attached to a Nikon DXM1200F® digital camera. The surface area of each digital micrograph analyzed with Simple PCI (v. 6.0) depended on the size and maturity of the gonad: 0.834 mm^2 (at 10x), 0.208 mm^2 (20x) or 0.005 mm^2 (40x). All visible oocytes present in the studied surface were counted and measured at the longest axis. This process was repeated for each of the six histological sections from each individual (from three separate slides).

As a quantitative measurement of gametogenic development, several formulas were compared which incorporate different variables that change throughout the gametogenic cycle and which can be quantified easily (as per the classical methods). As previously mentioned, there is an inverse relationship between oocyte density and diameter, and it was hypothesized that these factors

could only provide a clear view of the reproductive cycle if used in combination. Initial assessment of population structure and size at sexual maturity suggested a wide size range (disc diameter) for mature individuals of this species, and therefore this factor was also considered in several of the formulas. Coefficient of variation (CV; as the proportion of standard deviation of oocyte diameter to the mean) was also a potential variable, because of the possibility of variation in oocyte size at different gametogenic stages (high variation would lead to high CV). Also considered were oocyte diameter^2 and oocyte surface area instead of oocyte diameter in some of the formulas, again to test for the potential effect of oocyte size variation (to determine which representation of oocyte size is most effective for statistical analysis). These variables were arranged in multiple formulations:

(1) Oocyte density · oocyte diameter · CV · 0.001

(2) Oocyte density · $\frac{\text{oocyte surface area}}{100}$ · CV · 0.001

(3) $\frac{\text{Oocyte density}}{\text{Size of individual}}$ · oocyte diameter · CV · 0.01

(4) $\frac{\text{Oocyte density}}{\text{Size of individual}}$ · oocyte surface area · 0.01

(5) $\frac{\text{Oocyte density}}{\text{Size of individual}}$ · oocyte diameter^2 · 0.01

Oocyte density is the number of oocytes present per mm^2 of ovarian tissue, oocyte diameter is the mean diameter of all oocytes present, size of individual is the disc diameter, oocyte surface area = ¼ · π · oocyte diameter^2,

and CV (coefficient of variation) is the standard deviation (of oocyte diameter) over the mean multiplied by 100. Each of these maturity stage indices (MSI) was calculated using all samples throughout the sampling period to determine their relationship with traditional methods of studying gametogenesis.

2.3.5 Applicability using another species

Ophiopholis aculeata possesses small transparent oocytes (up to 120 µm) with limited lipid reserves typical of planktotrophic larval development, which will likely affect which of the formulas works best to describe gametogenic development in this species. For comparison with a species displaying a different reproductive strategy, data was obtained on the deep-sea holothuroid *Mesothuria (=Zygothuria) lactea*, which has larger opaque lipid-rich oocytes (up to 700 µm) typical of lecithotrophic larval development. This species exhibits synchronous development amongst tubules, which made the sections studied for each individual representative of the individual as a whole (and thus appropriate for the current study). This dataset was compiled as part of a research project focusing on reproductive synchrony in deep-water echinoderms (Baillon et al. 2011). Briefly, samples were collected from depths of 798-1337 m off the south-west Grand Banks in October 2005. Standard histological and examination techniques were used on all specimens (similar to the present study) and four maturity stages were defined, analogous to those used for *O. aculeata*. The density and diameters of the first 100 oocytes were measured using the imaging software Simple PCI (v. 6.0). Data were used to calculate a MSI with all formulas detailed

above, although size of individual in this case was the body length between mouth and anus. Each set of MSI was compared to assess which formula would provide the best results as compared to results obtained with *O. aculeata*. The efficiency of each formula was evaluated in the same way as in *O. aculeata*, by determining whether each formula can differentiate between the various stages of the gametogenic cycle.

2.3.6 Statistical analyses

One-way ANOVAs (henceforth abbreviated as OWA) or their non-parametric counterparts (Kruskal-Wallis ANOVA on Ranks, or KWA) were used to investigate how well the respective indices obtained would discriminate among the gametogenic stages. Conventional methods (GI, oocyte diameter, oocyte density) were tested in the same manner. Post-hoc analyses were used to compare specific groups: Student-Newman-Keuls method (SNK) was used for one-way ANOVAs, and Dunn's method for Kruskal-Wallis ANOVAs on Ranks. Pearson and Spearman correlations were used to investigate the relationships between the different indices. Statistical analyses were conducted with the software package Sigmaplot/SigmaStat (version 11.0; Systat, Inc.)

2.4 Results

Each individual in the dataset was given a MSI calculated with each of the formulas (with all MSI values determined in triplicate from multiple gonad subsamples). It was also attributed one of the four gametogenic stages, a GI, an average oocyte diameter and an average oocyte density. Statistical analysis was

used first to determine how well MSI values obtained with the different formulas would delineate the corresponding qualitative stages. The MSI calculated with the most accurate method was subsequently compared to other indices (GI, oocyte diameter, oocyte density) in its ability to quantitatively define gametogenic development. Finally, the most accurate definition and applicability of the MSI was evaluated in another species.

2.4.1 Comparison of MSI obtained with different formulas

All MSI formulas tested were successful in showing overall differences among gametogenic stages in *O. aculeata*, but not all of them could distinguish clearly between successive stages with the same precision (Figure 2-2).

The MSI calculated with Formula 1 established significant differences among stages (KWA, $H = 94.7$, $df = 3$, $p < 0.001$), but MSI values between growth and mature, and between mature and early growth stages were not distinct (Dunn's, $p > 0.05$). Results for the MSI obtained with Formula 2 were similar, with differences evidenced among stages (KWA, $H = 124.1$, $df = 3$, $p < 0.001$), and post-hoc tests showing that MSI failed to differentiate growth and mature, as well as spent and early growth stages (Dunn's, $p > 0.05$). While clear distinctions among stages were obtained using Formula 3 (KWA, $H = 84.3$, $df = 3$, $p < 0.001$), the post-hoc analysis could only find differences between the MSI of the spent stage and all others (Dunn's, $p < 0.05$). Formula 4 was the only method that provided clear MSI boundaries among stages (KWA, $H = 157.8$, $df = 3$, $p < 0.001$), with post-hoc tests showing distinctions between all stages in

pairwise comparisons (Dunn's, $p < 0.05$). Formula 5 also worked well to provide distinct MSI values among stages (KWA, $H = 107.3$, $df = 3$, $p < 0.001$), although it could not differentiate clearly between the spent and early growth stages (Dunn's, $p > 0.05$). Furthermore, while results of MSI comparisons made using Formula 4 were significant when all data points collected during the experiment were used, Formula 5 only provided significant pairwise differences when outliers were removed.

Formula 4 was therefore chosen for further comparison of MSI with conventional methods of characterizing gametogenic maturity and used for the description of the reproductive cycle in the Chapter 3. Overall, the MSI corresponded to the different stages of development with the following values: early growth = MSI of 100-200, growth = MSI of 200-400, mature = MSI of 400-800, and spent = MSI of 0-100. Thus the quantitative MSI mirrored the qualitative staging patterns in monthly samples (Figure 2-3). MSI is small for early growth due to the dominance of very small oocytes. It is larger in the growth stage due to the proliferation of larger oocytes, though density remains relatively low. The mature stage has large MSI values due to the high density of uniformly large oocytes. Most individuals in this stage had MSIs from 400-500, though a few had much larger values, having higher densities and larger oocytes. The maximum MSI found was approximately 800, representing the highest measurements of oocyte size and density. The spent stage has low MSI due to the relative lack of oocytes, with only residual mature oocytes in the process of being lysed.

2.4.2 Comparison between MSI and other methods

For *O. aculeata*, the four gametogenic stages established visually from ovary sections were well distinguished by the MSI but were not as clearly defined by oocyte density, oocyte diameter or GI alone (Figure 2-4). While there was a significant difference among stages using oocyte density (KWA, $H = 92.5$, $df = 3$, $p < 0.001$), the early growth and growth stages, and the growth and mature stages did not differ significantly (Dunn's, $p > 0.05$). The same test with oocyte diameter found a significant difference among stages ($H = 97.3$, $df = 3$, $p < 0.001$), except between the growth and spent stages (Dunn's, $p > 0.05$). Analysis of the GI also found significant differences among stages (OWA, $F = 29.4$, $df = 3$, $p < 0.001$), except between growth and mature stages (SNK, $p = 0.132$) and between spent and early growth stages (SNK, $p = 0.236$). Nevertheless, there were positive correlations between MSI and GI ($r = 0.505$, $df = 139$, $p < 0.001$), MSI and oocyte density ($r = 0.402$, $df = 221$, $p < 0.001$), and MSI and oocyte diameter ($r = 0.677$, $df = 203$, $p < 0.001$). While the GI showed a positive correlation with disc diameter of individuals ($r = 0.386$, $df = 80$, $p = 0.0004$), the MSI did not ($r = 0.119$, $df = 145$, $p = 0.155$), demonstrating that the latter was independent of body size (Figure 2-5).

2.4.3 Determination of MSI in another species

All MSI values calculated with the holothurian *M. lactea* exhibited a downward trend as the gametogenic cycle progressed from early growth to spent (as opposed to increasing MSI in *O. aculeata*), due to the nature of oocyte

growth and proliferation. Nevertheless, clear distinctions among gametogenic stages were obtained when using MSI values calculated with the various formulas. Again, formulas were not equally successful in delineating adjacent stages. Values of MSI established with Formulas 1 and 2 were the most accurate in this particular case, despite not taking size of animal into consideration (Figure 2-6). Overall significant differences among stages were shown for both Formula 1 (OWA, $F = 37.9$, $df = 2$, $p < 0.001$) and Formula 2 ($F = 10.0$, $df = 2$, $p = 0.002$). Post-hoc tests confirmed that all stages were significantly different in pairwise comparisons (SNK, $p < 0.05$). For Formula 3, significant difference among stages occurred (OWA, $F = 10.2$, $df = 2$, $p = 0.002$), and post-hoc tests revealed differences between all stages (SNK, $p < 0.05$) except mature and spent (SNK, $p = 0.230$). Formula 4 resulted in a significant difference among stages (OWA, $F = 4.602$, $df = 2$, $p = 0.028$), but a post-hoc test could not find differences between any stages (SNK, $p > 0.05$). Formula 5 did not result in a significant difference among stages in this species (OWA, $F = 3.344$, $df = 2$, $p = 0.065$).

2.5 Discussion

The maturity stage index (MSI) was able to distinguish between gametogenic stages, quantitatively separating them based on measured variables. It was more precise than gonad index (GI), oocyte density or oocyte diameter alone, as none of these could distinguish between all stages in pairwise comparisons. The MSI was easily calculated, using the same data as classic techniques (disc diameter, oocyte diameters), thus not requiring any increase in

workload or development of new methods. In contrast to the visual assessment of the gonad and gametes to which it was compared, the MSI is a quantitative measurement that can be easily reproduced. Hence, it provides a reliable alternative (quantitative) methodology for studying gametogenic cycles, and possibly a more sensitive method of studying finer variations in the gametogenic cycle, including small-scale spatial distribution and inter-individual synchrony.

As shown in the current study, many of the traditional methods of studying gametogenesis have value when at least two of them are used together, though each can have some issues. Gonado-somatic indices can be affected by intrinsic variability in the chosen denominators. For instance, the variable water content in organs and tissues or the presence/absence of food in the digestive tract make it hard to standardize the GI especially in those invertebrates which use their gonads as storage organs. Gonad indices can also be influenced by the size of the organism, especially since the relationship between gonad size to body size can change throughout ontogeny (Devlaming et al. 1982; Hughes et al. 2006; Mercier and Hamel 2009; Ebert et al. 2010). The GI also tends to be more precise in large species possessing large gonads (J.-F. Hamel, personal communication). Gonad indices are best used for comparisons and correlations with other methods, such as histology. However, histological studies using gametogenic stages are qualitative assessments, which make it difficult to quantitatively study reproductive cycles and to make fine comparisons between samples. The maturity index (MI) as defined by Yoshida (1952) is a quasi-quantitative analysis, using the gametogenic stages to calculate an average

maturity value. It is useful when the gametogenic stages are the main focus of a study, as it can be used to assess the overall gametogenic state of a group. However, it is not a true quantitative assessment, and skews the data away from the first and last stages, being based on the average value. Tyler (1977) used the MI in conjunction with oocyte counts and the percent of individuals in each stage in a given month to increase the reliability of the analyses.

Studies which truly quantify gametogenic development in some way are rare; many were performed on species that are important for fisheries and aquaculture. The gamete volume fraction (GVF) method used in bivalve research is a reliable method for comparing genders or locations (Bayne et al. 1978; Lowe et al. 1982; Newell et al. 1982; MacDonald and Thompson 1986). However, it may not be as useful for fine-scale statistical comparisons, as the values obtained are fractions, and would be more difficult to compare statistically than values such as the MSI. While the GVF appears to have potential for use outside of bivalve research, it requires extensive additional analysis using imaging software, and thus was not measured in the present study. Urrutia et al. (1999) identified two problems with the GVF method. First, inter-individual asynchrony in gamete development and release could lead to underestimation of reproductive output, and second, partial spawning could lead to an overestimation of reproductive output. Singh et al. (2001) studied the commercial sea cucumber *Cucumaria frondosa* and quantified its reproductive condition using similar measurements of gonad volume fraction, as well as gonad dry weight, measurements of cross-sectional tubule area and tubule wall area, and levels of

haemal fluid in tubules. Some of these measures are partly qualitative in nature and many of them are very specific to holothuroid anatomy. In another quantitative study, Osada et al. (2007) used the incorporation of 5-bromo-2'-deoxyuridine (BrdU) in order to detect mitotically active gonial cells as a proxy of gametogenesis in a commercial scallop. Furthermore, Enríquez-Díaz et al. (2009) studied reproductive effort and investment in a commercial oyster by measuring gonadal biomass, soft tissue production and spawning intensity. While these methods both provide useful quantitative measurements, the former is based on incorporation of a chemical compound by live animals, and is therefore only applicable to laboratory studies. The latter is well suited to bivalves although it still relies significantly on the measure of weight and size of whole gonads, which does not accurately reflect reproductive status in all marine invertebrate species (similar issues as with the GI).

A long established method of quantifying changes throughout gametogenesis is oocyte size frequency distribution, which has been used for a variety of species. Patent (1969) found it necessary to use oocyte diameter to determine a precise gametogenic stage, as the definitive physical characteristics of a given stage were not always clear enough in histological sections. Oocyte size frequency distributions are generally accurate indicators of gametogenic activity and spawning, however it has been shown here that they are less accurate than the MSI, particularly in partial-spawning species such as *O. aculeata*. As most species of marine invertebrates do not completely shed all gametes upon spawning, the lingering presence of large mature oocytes in the

spent stage is a generalized concern with the use of oocyte size as a sole parameter. Oocyte size frequency also does not allow for fine-scale comparisons between individuals from different treatments, such as different habitats or laboratory experiments.

In the present study, Formula 4 (using oocyte density, size of individual and oocyte surface area) was determined to be the best proxy of gametogenic development in *O. aculeata* when compared to other formulas and to classical methods, and thus was chosen as the definition of MSI. One important advantage of this formula is that it takes the size of the individuals being studied into consideration. As in many species, this was desirable in *O. aculeata* because sexually mature individuals exhibited a range of sizes, with disc diameters varying between 8.5 and 20 mm (even though most were 10-14 mm). The GI was shown to be strongly correlated with disc diameter and was therefore size-biased. Using the same dataset, appropriate use of the GI would have required decreasing the number of samples in order to narrow the size range as indicated by Hughes et al. (2006) and Mercier and Hamel (2009). The MSI was not affected by the disc diameter in mature individuals; therefore it made fuller use of the samples. This can be particularly useful when sample sizes are low. More importantly, the wide range of disc diameters in our study represents the natural variation found in communities of *O. aculeata*, so that limiting the size range could have biased the study toward a select group, instead of being representative of the entire population.

One parameter that was integrated into several formulas but was found not to be a significant determinant of gametogenic development for this species was the coefficient of variation (CV) on the mean oocyte diameter. The oocytes measured throughout this study were small (10-120 µm), as is typical of planktotrophic species. Measures of oocyte size frequencies had a relatively narrow range (mean oocyte size was 46.0 ± 20.8 µm). Consequently, the CV values measured using oocyte diameter for this species had a relatively small range (mean CV was 32.5 ± 10.7%) that did not make a major contribution to the MSI relative to other parameters. Hendler (1991) summarized characteristics of different developmental patterns in ophiuroids and made the following generalizations: (1) for planktotrophic species, the maximum range of oocyte diameter was 70-170 µm and the maximum number of oocytes per individual was between 240,000 and 2.6 x 10^6; (2) for lecithotrophic species, the maximum range of oocyte diameter was 130-250 µm and the maximum number of oocytes per individual was 4,000-30,000. In other words, lecithotrophic species have fewer larger oocytes. Therefore, unless the oocytes are all nearly identical in development (which generally only occurs at full gametogenic maturity), it is likely that there will be greater variations in oocyte size, leading to a wider range of CVs, presumably making this parameter a more valuable one for species with lecithotrophic development (see below).

It is likely that the MSI formulated in this study will provide an accurate summary of reproductive activity in other species with similar types of gonads and oocyte size frequencies. Furthermore, the MSI can easily be adapted to

species with different reproductive strategies, as demonstrated here with *M. lactea* based on the data from Baillon et al. (2011). The assumption that certain formulas would work better for species exhibiting larger oocyte sizes was correct, as Formulas 1 and 2 worked for this lecithotrophic species without modifications to the data. The formulas that worked best for this species all contained a measure of CV, supporting the theory of CV working best for species with larger oocytes. Even more importantly, the data set was from a relatively poorly known species, for which other determinants of gamete development were not well established, and so the MSI can be a useful tool for assessing gametogenesis. The fact that Formulas 1, 2 and 3 worked best for this species despite not taking body size variation into account is likely because the size range of sampled individuals was relatively small (9-13 cm in length).

This is by no means a definitive study: the precise MSI formulas developed for *O. aculeata* and *M. lactea* may not be directly applicable to all other marine invertebrates. Further analysis using a variety of species with small and large oocytes will be required to fully assess the value of this novel analytical method. However, the principles presented here provide a useful framework toward the development of similar methodologies for other species. The current study lacked the time and resources that would have been necessary to develop a similar index for males, but future studies could develop variants of MSI for male specimens by measuring features that vary throughout gametogenic development, such as the thickness of the germinal epithelium and different layers of gametes (spermatogonia, spermatocytes and spermatozoa), and the

presence/absence of nutritive phagocytes. The onus is on future researchers to conduct complete studies (histology, biometrics) and develop their own variant of the MSI.

There are many potential benefits that could come from developing such a system, including and not limited to: ease and expedience of processing samples with standard methods, more easily replicated methodologies, and being able to quantitatively measure and compare gametogenic cycles with minimum skew or bias (contrary to maturity indices based on gametogenic stages). The results of the present study already show that this quantitative index can fairly easily be adjusted for different organisms. Any such undertaking must consider the fundamentals of the species in question, such as the reproductive strategy (brooding, broadcast spawning), the oocytes sizes, and the presumed periodicity of the reproductive cycle (annual, seasonal, aperiodic). Comparisons with other indices (GI, maturity indices, somatic indices) previously recorded for the species may be useful. For example, the present study found positive correlations between MSI and GI, oocyte diameter and oocyte density for *O. aculeata,* indicating that these are likely reliable indicators of gametogenic development and are valuable when used in conjunction with the MSI. While the easiest and most significant variables to measure in *O. aculeata* were oocyte density and surface area, this may not be the case for all species, and a wide variety of other factors that change over the reproductive cycle could potentially be used to contribute to future MSI formulas. The discarded variables from different formulations in this study (CV, oocyte diameter) may also prove valuable. Finally,

because the differing functions of gonads may affect the effectiveness of some measures of reproductive activity (such as gonad weight and presence/ dominance of nutritive phagocytes); special care must be exerted in adapting the MSI in species known to use gonads for both reproduction and energy storage, such as echinoids (Gonor 1973; Mercier and Hamel 2009). Our current work in this area is focusing on the development of similar techniques for use with male specimens, using variables that can be quantifiably measured throughout the gametogenic cycle.

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2.7 References

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2.8 Tables

Table 2-1. Research methods used in various studies on ophiuroid reproduction, where 'GI' represents gonad indices, 'staging' represents the use of visual gametogenic stages, 'MI' represents a qualitative maturity index, and 'OSFD' represents oocyte size frequency distribution. This list provides only some of the most recent studies, and is not exhaustive.

Study	GI	Staging	MI	OSFD
Patent 1969 *Gorgonocephalus caryi*		X	X	
Fenaux 1970 *Amphiura chiajei* Forbes		X		
Tyler 1977 *Ophiura* spp.		X	X	X
Bowmer 1982 *Amphiura filiformis*		X	X	X
Hendler and Tyler 1986 *Ophioderma brevispinum*	X			X
McClintock et al. 1993 *Asteroporpa annulata*		X		X
Lefebvre et al. 1999 *Ophiothrix fragilis*	X			
Sumida et al. 2000 *Ophiocten gracilis*		X	X	
Morgan and Jangoux 2002 *Ophiothrix fragilis*	X	X	X	X
Falkner and Byrne 2003 *Ophiactis resiliens*		X		
Grange et al. 2004 *Ophionotus victoriae*	X	X		X

2.9 Figures

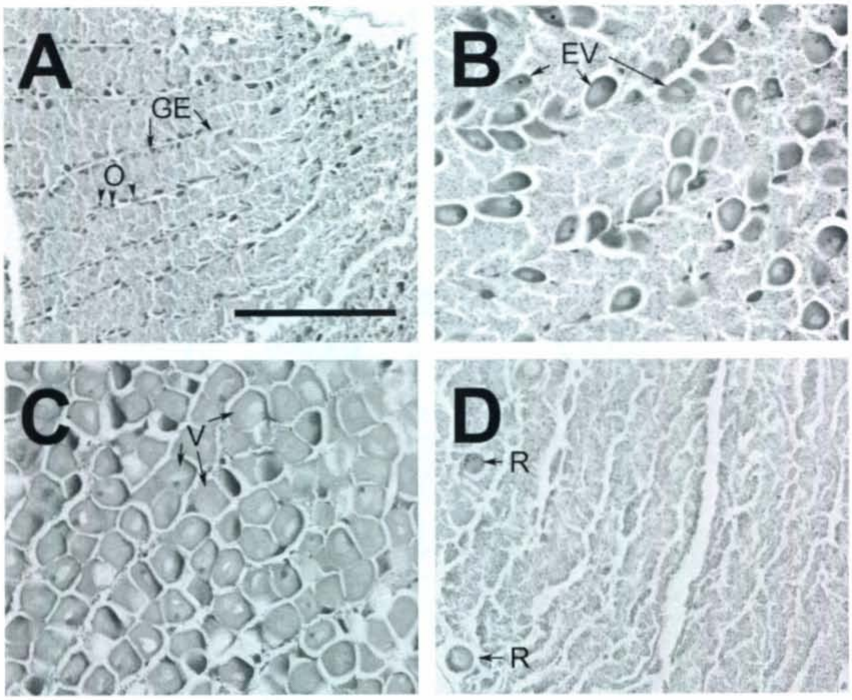

Figure 2-1. Oogenic stages in female *Ophiopholis aculeata*, where A = early growth (with oogonia [O] growing from the germinal epithelium [GE]), B = growth (with early vitellogenic [EV] oocytes), C = mature (with vitellogenic [V] oocytes, and D= spent (with residual [R] oocytes). Scale bar represents 400 µm.

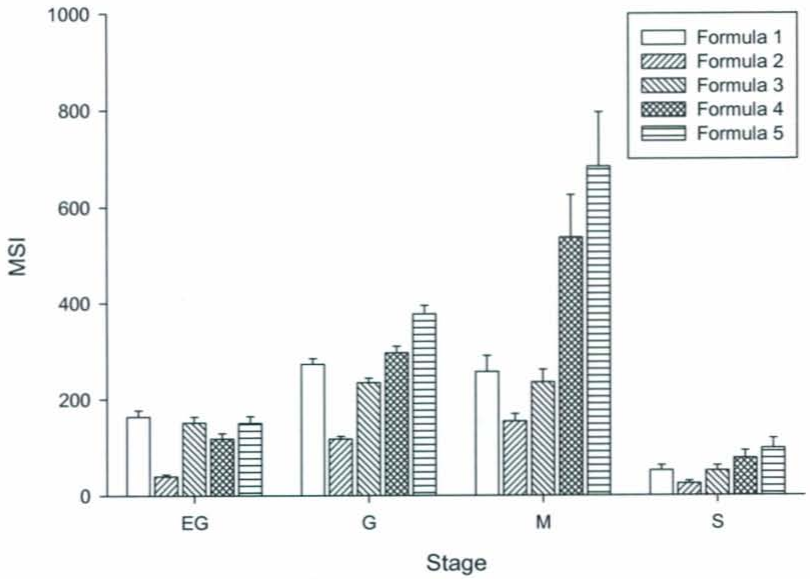

Figure 2-2. Maturation stage indices (MSI) obtained with the different formulas with corresponding oogenic stages of *Ophiopholis aculeata*, where EG = earth growth, G = growth, M = mature, and S = spent. Data presented as mean +standard error, n = 5-53.

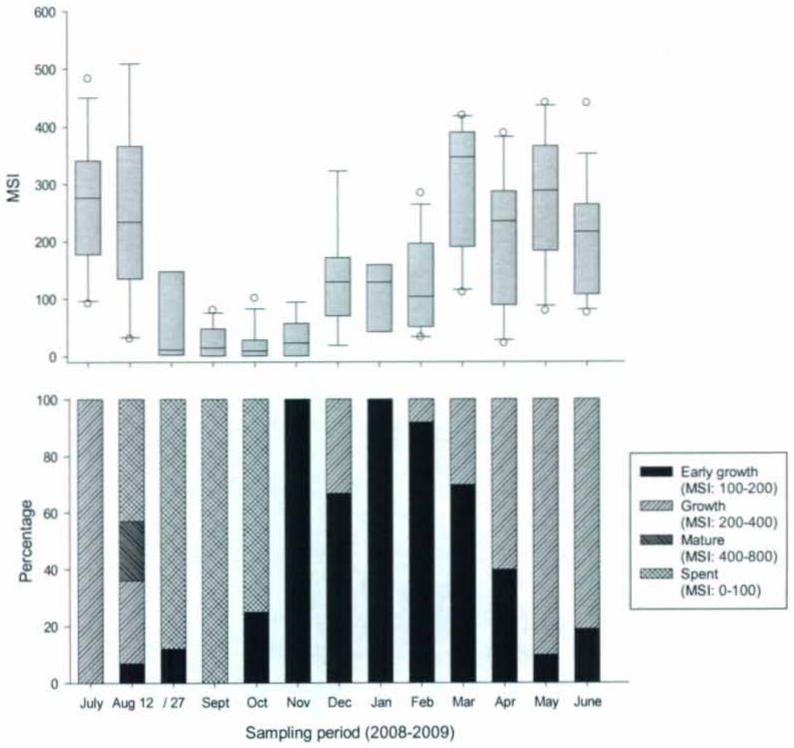

Figure 2-3. Mean MSI of female *Ophiopholis aculeata* (± standard error; n = 8-16) with corresponding proportion of individuals in each oogenic stage (early growth, growth, mature and spent; n = 5-53) throughout the sampling period.

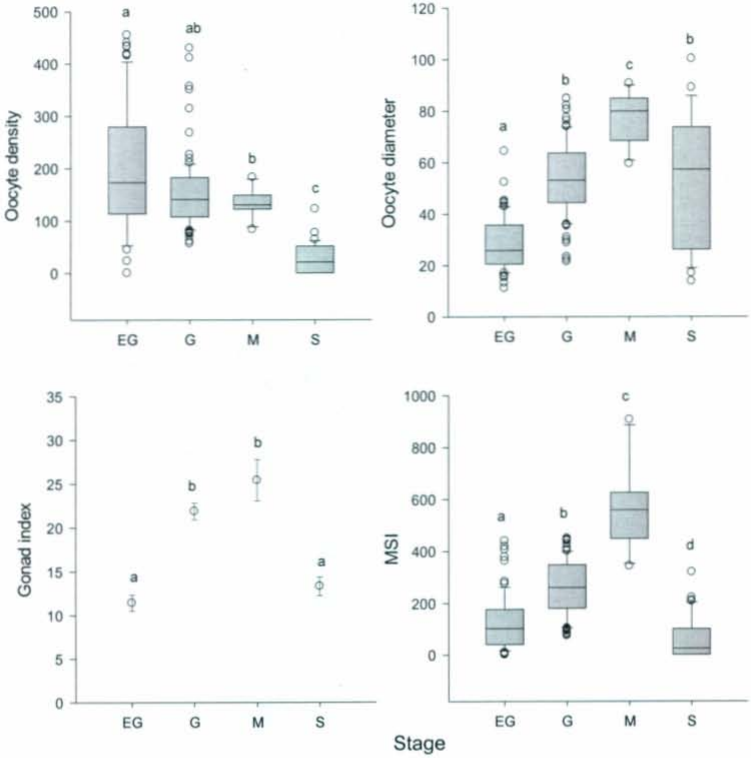

Figure 2-4. Plots of oocyte density (oocytes · mm^{-2}), oocyte diameter (µm), gonad index (% wet weight) and maturity stage index (MSI) across oogenic stages for *Ophiopholis aculeata*, where EG = early growth, G = growth, M = mature, and S = spent. In the box plots, the horizontal line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles and outliers appear as open circles. The scatter plot shows mean and standard error, n = 5-53. Means with different letters (a,b,c,d) are significantly different.

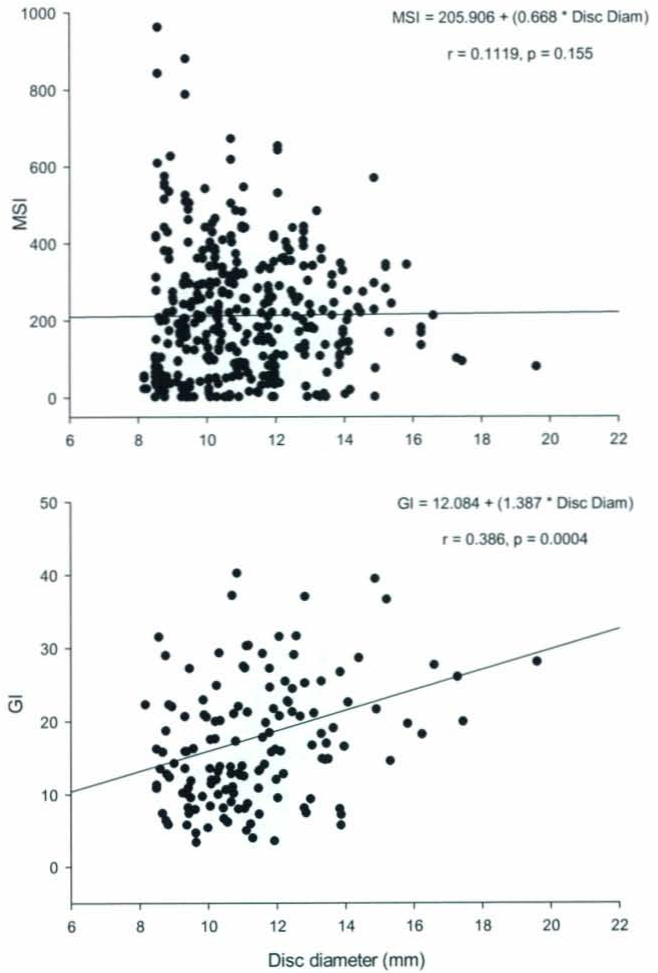

Figure 2-5. Scatter plots of relationships between disc diameter (mm) and maturity stage index (MSI; upper panel) and gonad index (% wet weight; lower panel) in *Ophiopholis aculeata*, where the lines represent linear regression and n = 147. For MSI, r = 0.119 and p = 0.155. For GI, r = 0.386 and p = 0.0004.

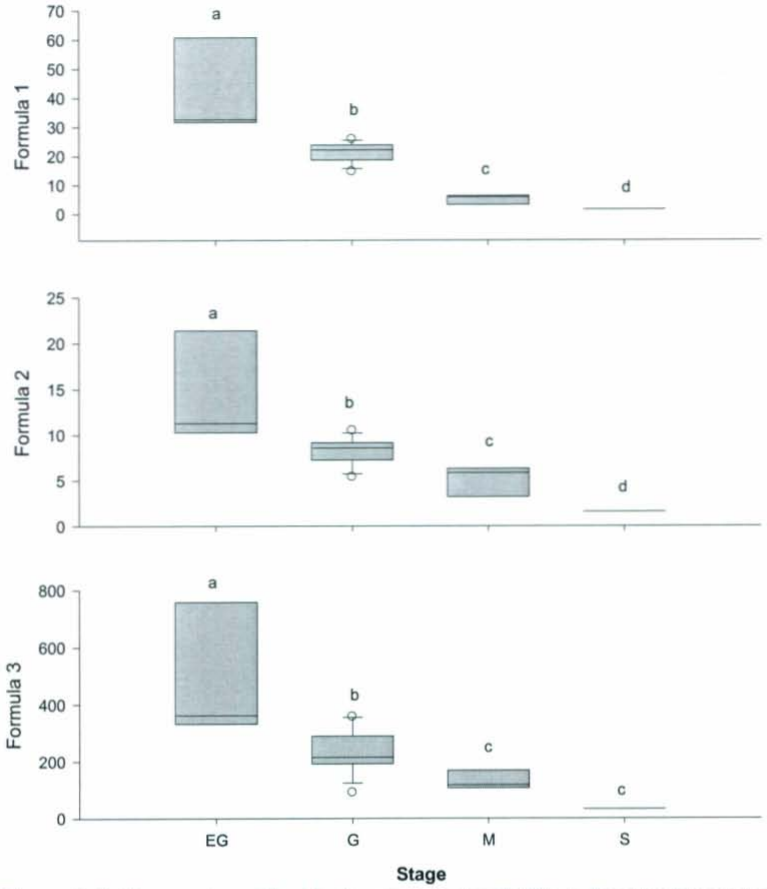

Figure 2-6. Comparison of maturity stage index (MSI) defined by Formulas 1, 2 and 3 across gametogenic stages of *Mesothuria lactea*, where EG = early growth, G = growth, M = mature, and S = spent. The horizontal line shows the median, the lower and upper limits of the boxes represent the 25^{th} and 75^{th} percentiles, whiskers represent the 10^{th} and 90^{th} percentiles and outliers appear as open circles. Different letters indicate significantly different MSI.

Chapter 3 - Influence of spatial distribution and environmental factors on the reproduction of the brittle star *Ophiopholis aculeata* (Echinodermata: Ophiuroidea)

3.1 Abstract

The brittle star *Ophiopholis aculeata* was used to examine variations in gametogenic activity on various scales: (1) in a given habitat over the annual cycle, (2) between two adjoining habitats, (3) among individuals in each of these habitats, and (4) within and among natural clusters of individuals. Complementary laboratory trials were conducted to further examine the influence of temperature and spatial distribution on gametogenesis. Statistical comparisons were made using a newly developed quantitative method called the maturity stage index (MSI). Results showed that *O. aculeata* exhibited an annual gametogenic cycle with a main spawning event in late summer. Multiple decreases in MSI throughout the year present the possibility of partial spawning events. MSI revealed significant differences in trends between the two habitats and among individuals from the same habitat in certain months. Gametogenic cohesion was generally greater just before spawning than at other times, and in nearby/grouped individuals than in distant/isolated individuals. The onset of rapid gametogenic development in March was correlated with an increase in seawater temperature and the early phase of a phytoplankton bloom; spawning was

correlated with a secondary phytoplankton bloom in late summer and the highest seawater temperatures of the cycle. However, laboratory experiments indicate that temperature does not act alone to drive maturation and spawning. The present study emphasizes the likely interplay of exogenous cues and inter-individual exchanges at different stages of gamete maturation in helping to coordinate the reproductive cycle of *O. aculeata*.

3.2 Introduction

Reproductive processes are among the most important factors that shape benthic invertebrate communities. Given the high species diversity of the benthos, reproductive strategies are varied and many (Thorson 1950; Giese and Kanatani 1987). Synchronized gamete maturation and release becomes crucial in order to maximize fertilization success and subsequent recruitment of offspring, especially in species with external fertilization which dominate in many benthic taxa, including echinoderms (Giese et al. 1991; Mercier and Hamel 2009). Fertilization rates and overall reproductive success in broadcast spawners can also be affected by the density and proximity of congeners within and between populations (Pennington 1985; Hamel and Mercier 1996; Kelly 2000; Mercier and Hamel 2009).

The generally positive relationship that exists between population density, overall numbers of conspecifics and per capita population growth rate is widely studied (e.g. demographic Allee effects) (Allee 1938; Courchamp et al. 1999; Stephens and Sutherland 1999; Gascoigne and Lipcius 2004). In contrast to how

spatial factors (e.g. density, proximity) may affect fertilization rates in broadcast spawners at the time of gamete release, the role of these factors on the synchronous development of gametes prior to spawning are poorly understood for most marine invertebrates, including echinoderms. Furthermore, studies of reproductive cycles relative to spatial distribution can be viewed from two different perspectives. Most have focused on species for which reproduction does not entail any major change in spatial distribution and fewer have considered species that aggregate prior to or during spawning, in order to synchronize reproduction or increase reproductive success (reviewed for echinoderms by Mercier and Hamel 2009). Therefore fine-scale spatial factors (e.g. intra-habitat proximity) have largely been studied in species that aggregate transiently (Selvakumaraswamy and Byrne 2000; Mercier and Hamel 2008) whereas large-scale factors (e.g. inter-site synchrony) were mostly investigated in species that do not display this behaviour (Lessios 1981; Bourgoin and Guillou 1990; King et al. 1994; Lefebvre et al. 1999; Guettaf et al. 2000; Kelly 2000; Lamare et al. 2002). The importance of spatial organization in species that are not known to consistently aggregate for reproduction remains poorly understood.

The effects of small- and large-scale spatial distribution on reproductive cycles within natural communities and populations have been widely studied in echinoids from boreal, temperate and tropical habitats (Mercier and Hamel 2009). While the reproductive biology of echinoids is somewhat unique, the shear amount of knowledge on echinoid reproduction makes it interesting as a basis of comparison. Many studies on echinoids have found that food availability and

accumulation of nutrients are integral to gametogenic regulation, with natural variation in food levels often leading to discrepancies in reproductive status among sites, depths and habitats, and some studies implicating a possible second factor such as temperature (Pearse 1970; Gonor 1973; Ernest and Blake 1981; Byrne 1990; King et al. 1994; Byrne et al. 1998; Meidel and Scheibling 1998; Seward 2002; Kelly 2000; Muthiga and Jaccarini 2005). Lessios (1981) found that echinoids in seasonally variable locations had more synchronous, well-defined reproductive cycles, while species from annually stable sites showed less defined reproductive periodicity, and concluded that biotic factors (i.e. density) affected populations as much as physical factors. Others evidenced differences among populations of the same species that were presumably resulting from varying levels of energy characterizing each habitat (Guettaf et al. 2000). Most inter-site studies have compared them on the basis of broad properties (i.e. Byrne 1990; Byrne et al. 1998; Meidel and Scheibling 1998; Seward 2002; Kelly 2000). Very few investigations compare reproductive variation within habitats across multiple sites, as most compare habitats at a single site, so it is difficult to accurately attribute changes in indices, such as gonad index, to habitat (Kelly 2000).

In holothuroids, small-scale spatial distribution has been shown experimentally to affect reproductive synchrony, in that isolated individuals exhibited less synchronous gametogenic development than those maintained in groups (Hamel and Mercier 1996). This supports the assumption that density and arrangement of individuals can play a role in the gamete maturation phase

leading to spawning. Exchange of waterborne chemicals presumably mediates fine-tuning of gametogenesis, and ensures synchronous spawning (Hamel and Mercier 1999). Similarly, reproductive aggregations and spawning chemicals have been shown or suspected to drive harmonious gamete development and spawning in certain species of asteroids (reviewed by Mercier and Hamel 2009). Studies of this nature are comparatively rare in crinoids, although McClintock and Pearse (1987) conducted a histological analysis of the Antarctic crinoid *Promachocrinus kerguelensis*, noting synchronous development between individuals and suggesting that spawning was coordinated within the sub-population they sampled.

Despite the common dominance of ophiuroids in benthic communities ocean-wide, relatively little is known about the effect of spatial distribution on their reproductive biology. Bowmer (1982) reviewed the ophiuroid *Amphiura filiformis* and noted that studies conducted at different sites reported a wide range of lifespan and reproductive periodicities, but offered no clear explanation for such variations. In reviewing the reproduction of *Ophioderma brevispinum*, Hendler and Tyler (1986) found reports suggesting that growth and spawning periods were shorter in more northern locations, and longer in more southern populations. They attributed this to a general trend of extended growth and breeding in warmer water. Lefebvre et al. (1999) studied the boreal species *Ophiothrix fragilis* and found high spatial variability between populations in the English Channel. Offshore populations had lower gonad indices, attributed to lower temperature and food availability.

It is becoming increasingly evident that spatial distribution has a major influence on reproductive strategies in many species of marine invertebrates and that this factor should be considered at various scales, i.e. based on (1) how distribution and density of conspecifics at a given site may influence inter-individual signalling, and (2) how some exogenous factors mediating reproduction may vary among sites.

Homospecific and heterospecifc communication for the purpose of reproduction has been documented in tropical, temperate and boreal echinoderms at various levels (reviewed by Mercier and Hamel 2009). Studies have suggested that hormones help synchronize gametogenesis (Hamel and Mercier 1996, 1999; Lamare et al. 2002) or spawning (Iliffe and Pearse 1982; Soong et al. 2005), with or without the additional influence of exogenous controls. Inter-individual chemical communication (or lack thereof) has been documented as having some effect on gametogenic development on various spatial scales, with some species showing inter-individual coordination in development of gametes based on spatial separation (Hamel and Mercier 1996, 1999; Lamare et al. 2002). Specific hormones and steroidal products have been documented in echinoderms and are thought to function in inter-individual chemical signalling, allowing individuals to synch their reproductive cycles (Soong et al. 2005; Georgiades et al. 2006; Wasson and Watts 2007; reviewed by Mercier and Hamel 2009).

Gametogenesis and spawning in echinoderms and other marine invertebrates have been shown to be influenced by numerous environmental

factors (see reviews by Giese and Kanatani 1987; Himmelman 1999; Mercier & Hamel 2009; and references therein). Photoperiod has been demonstrated experimentally to influence the pace of gametogenesis and hormonal levels in many echinoderms, though manipulation of this factor could not stop the progression of the cycle or the eventual spawning event after the process was engaged (Pearse and Eernisse 1982; Pearse et al. 1986; Xu and Barker 1990; Pearse and Bosch 2002; Dumont et al. 2006). The role of temperature on reproduction in echinoderms has also been well studied. Many studies considering the relationship between temperature and reproduction concede that temperature is a possible exogenous control of gametogenesis and/or spawning, but conclude that a) it was not studied finely enough to determine exactly what role it plays, and/or b) other linked factors (such as photoperiod or phytoplankton) likely play an important role as well (Pearse 1970; Gonor 1973; Himmelman 1980; Iliffe and Pearse 1982; Hendler and Tyler 1986; King et al. 1994; Kelly 2001; Morgan and Jangoux 2002; Grange et al. 2004; Himmelman et al. 2008).

It is generally thought that phytoplankton would be an advantageous spawning cue because it requires integration of multiple parameters (temperature, photoperiod) that lead to favourable larval conditions (Starr et al. 1990). Some studies have concluded that temperature and/or photoperiod were not the main causes in gonad growth and/or spawning, and that food levels were potentially more influential (Gonor 1973; Himmelman 1980; Grange et al. 2004). Himmelman et al. (2008) came to a similar conclusion during their study of multi-species mass spawnings, noting a correlation between spawning and sea

temperature spikes caused by downwelling, but acknowledged that other factors affected by downwelling could have induced spawning as well, particularly phytoplankton. In their study of multi-species mass spawnings, Mercier and Hamel (2010) concluded that reproduction in numerous boreal invertebrates was influenced by phytoplankton levels, but also found that most species used lunar cycles, time of day and inter-individual communication to finely coordinate gamete release. While spawning was successfully induced in echinoderms and other invertebrates using phytoplankton extracts (Starr et al. 1990; Watson et al. 2003), less definitive responses were also reported (Reuter and Levitan 2010), further suggesting an interplay among seasonal factors, lunar cycles and inter-individual signalling.

While ophiuroids are less often the focus of reproductive studies than asteroids, holothuroids or echinoids, some studies have examined the effect of environmental conditions on their reproductive cycle. The relationships these studies have produced are generally correlative, sometimes lacking strength due to only considering a single environmental factor at a time (Mercier and Hamel 2009). One of the few common threads in the literature is the suggestion that many boreal ophiuroids show a correlation between onset/increase in gametogenesis and rising water temperatures (Hendler and Tyler 1986; Hendler 1991; Stewart and Mladenov 1995; Lefebvre et al. 1999; Morgan and Jangoux 2002). Some studies have focused on food supply, concluding that a limited food supply can impair gonad growth, induce a resting period, or even cause mortality (Patent 1969; Tyler 1977), while higher food levels can trigger gametogenesis or

increase gonad growth (Bowmer 1982; Sköld and Gunnarsson 1996; Lefebvre et al. 1999; Pomory and Lawrence 1999). Hendler (1991) concluded that food availability often acts as an advantageous or disadvantageous factor in ophiuroid reproduction, but does not directly entrain reproductive cycles.

In the only dedicated study of the reproductive cycle of the brittle star *Ophiopholis aculeata* Blake (1978) used temperature as a major correlative factor, stating that populations in Maine (USA) and Newfoundland (Canada) spawned after a drop in water temperature. Other studies of *O. aculeata* also cite temperature as a spawning trigger (Mileikovsky 1960; Himmelman et al. 2008). However, these studies generally either did not study other factors (Blake 1978) or concluded that other factors could play some role as well (Himmelman et al. 2008). Mercier and Hamel (2010) implicated phytoplankton as a spawning trigger for *O. aculeata* maintained in a mesocosm, and further suggested that spawning had a lunar periodicity, and was related to time of day (or the slight change of water temperature that occurs throughout the day due to sunlight).

Although many studies have been conducted on reproduction in benthic communities, there still remains a large gap in our knowledge of how most benthic invertebrates proceed through the gametogenic cycle toward synchronous spawning. For many common species such as *O. aculeata*, the bulk of our knowledge comes from studies of gamete release, not gamete development, and large variations among investigations prevent clear conclusions from emerging (i.e. Hendler 1991). Part of this lack of knowledge comes from the fact that these largely cryptic animals are rarely the main focus of

a study. It may also come from their lack of economic value or their poorly understood ecological function, despite their dominance in several habitats.

The present work was partly aimed at closing gaps in our knowledge of reproduction in *O. aculeata* and ophiuroids in general. Furthermore, because of its abundance and prevalence in two common habitats in the Northwest Atlantic, *O. aculeata* provided an opportunity for a thorough investigation of reproductive processes at various scales. The main objectives of the study were therefore to: (1) determine the reproductive periodicity of *O. aculeata* in two habitats; (2) investigate how small-scale spatial distribution could affect the synchrony of gametogenic development over time (i.e. among natural clusters, among individuals); (3) establish correlations with environmental factors and (4) use experimental trials to further test the influence of temperature and inter-individual communication on the level of synchrony in gametogenic development.

It was hypothesized that *O. aculeata* would exhibit an annual reproductive cycle, peaking with spawning in summer. Based on research conducted on other ophiuroids and holothurians, it was thought that individuals in closer proximity to one another (within natural clusters) would be more synchronous in their reproductive cycle than those further away from one another (in separate clusters and other habitats). It was hypothesized that environmental factors would play an important role in initiating and regulating gametogenesis and spawning, particularly phytoplankton and seawater temperature. It was therefore believed that concurrent laboratory experiments controlling proximity and water temperature would further elucidate results from the field study.

3.3 Materials and methods

3.3.1 Sample site

All main collections of *O. aculeata* were made in St. Philips, Newfoundland and Labrador, Canada (N 47° 35.5, W 52° 53.5). The study compared two adjacent benthic habitats: a rocky bottom, and a rhodolith bed. These are the two most common habitats in which ophiuroids are found around Newfoundland, and both habitats are distributed around the island and mainland portions of the province. At the study site, these two habitats occurred in several distinct patches on a low-grade slope at 10-15 m depth. Both the rock and rhodolith habitats extended approximately 100-200 m across the field site, with an approximately 50 m wide boundary area between the two.

3.3.2 Reproductive cycle in rock and rhodolith habitats

Samples were collected monthly during dives around the 15th of each month, with an additional dive around the 30th during the presumed spawning month of August. Specimens were collected over a period of thirteen months, from June 2008 to June 2009. Each dive included collections from the rocky bottom and the rhodolith bed. Bags were used to separate *O. aculeata* collected from each rock (generally from underneath) and to collect and isolate each rhodolith (with its entire content, including brittle stars). For the rock samples, between twenty and thirty individuals (depending on dive conditions) were collected from a total of five to nine rocks, while a minimum of six rhodoliths

(maximum of ten) were collected. All specimens were preserved in the laboratory within 1 h of collection.

As part of the field study, environmental parameters were measured in St. Philip's. Two temperature/light logger HOBO® Pendants (UA-002-64) were cemented onto rocks between the two habitats at a depth of 10 m. The loggers were set to record temperature and light information every three hours. Water samples (20 L) were collected from the site to establish the abundance of phytoplankton. These collections were bi-weekly during the suspected spawning period (August-October), and monthly for the rest of the cycle. Plankton levels were determined by filtering 1 L subsamples through Nitex® mesh (5 µm). The filtered plankton was rinsed into a 50-ml Falcon® tube and homogenized using a VWR Mini Vortexer MV1®. Four 10-µl samples were removed from each tube and placed on a Neubauer® brightline haemocytometer for cell counts. Plankton data were compared to the 2008-2009 values collected by Fisheries and Ocean Canada at nearby Station 27 through the Atlantic Zone Monitoring Program. The 2008-2009 data on photoperiod were obtained from Environment Canada (Environment Canada 2010).

Comparison with samples from other sites

Specimens required for the field study and laboratory experiments were always collected from the same area in St. Philip's. However, samples were also concurrently collected in Harbour Main and Admiral's Cove for comparative gonad index comparisons, within two days of the initial St. Philip's samples (July

10 and 12, 2008, respectively). Samples were again collected in Admiral's Cove in December 2008 for full histological and quantitative comparison. Harbour Main (N 47°26.243, W 53°09.311) is a relatively sheltered inlet located approximately 55 km southwest of St. Philip's, at the base of Conception Bay. Admiral's Cove (N 47° 05.320, W 52°54.364) is a much more exposed and dynamic environment located on the opposite side of the northeast Avalon Peninsula. Samples were collected from underneath rocks in Admiral's Cove and from rhodoliths in Harbour Main.

3.3.3 Laboratory experiments

The laboratory component of this study consisted of three main experiments, where individuals of *O. aculeata* were either maintained 1) grouped in tanks submitted to naturally fluctuating environmental conditions and food supply; 2) isolated individually in tanks under naturally fluctuating environmental conditions and food supply, or 3) isolated in tanks under fixed temperature (with all other parameters fluctuating naturally). See below for the detailed experimental designs. Individuals for these experiments were collected in July 2008 from underneath rocks at 12 m depth in St. Philips. Rocks from the field habitat were also brought back and placed in all of the experimental tanks to provide shelter and mimic natural conditions. Only specimens with a disc diameter ≥ 8.5 mm were used, to ensure they had reached sexual maturity. Individuals were initially placed into holding tanks supplied with running ambient seawater for two weeks, to get acclimated and be measured, sexed, and

separated among experimental designs. Each experiment (see below) ran for twelve months, starting on August 1 2008 and ending on July 23 2009.

Each experiment was initially designed with the assumption that the individuals used were accurately sexed, but sex was only confirmed at the end of the trials. Sexing live specimens was based on the visual appearance of the gonad through the body wall (pale white or yellow in males, bright pink or orange in females). Preliminary investigations on mature specimens indicated fairly accurate sex determination, as confirmed by preserving sexed individuals and removing gonads for microscopic investigation.

(1) Grouped under naturally fluctuating conditions

This experiment involved a 20-L tank containing a mixed group of approximately twenty individuals of both sexes (bottom density of 154 individuals $\cdot$ m^{-2}) and a larger 200-L tank harbouring 100 individuals of both sexes (bottom density of 53 individuals $\cdot$ m^{-2}). These tanks were maintained under naturally fluctuating environmental conditions, based on average conditions of temperature and light intensity recorded at approximately 10 m depth in St. Philip's. They were exposed to natural light through large windows and supplemented by timer-controlled fluorescent lights, with global light intensity fluctuating between 0 and 200 lux daily. Both tanks had approximately 10 proportionately large rocks in them, to stimulate natural conditions. Unfiltered running seawater (with natural planktonic food) was provided at a rate of 5.5-6 L min^{-1}, and water temperature was adjusted in summer months using an inline

chilling unit when necessary. Temperature and light levels were continuously recorded by a Temperature/Light data logger HOBO® pendant (UA-002-64) placed in one of the tanks. Thirty individuals (fifteen males and fifteen females) freshly collected from the sampling site were processed at the onset of the experiment, to determine initial gametogenic status. A group of thirty individuals from the large tank were subsequently processed for biometrics, gonad index and histology in February 2009 (6-month sample) while another group of thirty individuals from the large tank along with all individuals from the smaller tank were processed in July 2009 (12-month sample).

(2) Isolated under naturally fluctuating conditions

This experiment was conducted to explore the role of inter-individual exchange (physical or chemical requirements) in synchronizing gametogenic development and to be compared with individuals kept in groups. The experimental design consisted of twenty 20-L tanks exposed to naturally fluctuating environmental conditions under flow-through conditions, as described above. Each of the tanks had one rock inside, to supply natural shelter. The Individuals in this experiment were collected at the same time as those in the experiment above; the same group of thirty freshly collected individuals was used to account for initial gametogenic status. Brittle stars were then placed individually in the tanks: ten contained a single male and ten others a single female. Of the total, five males and five females were preserved for biometrics, gonad index and histological analysis after 6 months and after 12 months.

(3) Isolated under fixed temperature

This experiment was conducted to test for the effect of environmental cues on ophiuroid reproduction in the absence of the linked temperature fluctuations. A multi-tank semi-open coldwater unit (Aquabiotech®) with electronic control of temperature and light was used. Photoperiod and light levels were set to automatically follow the natural regime at 10 m depth in St. Philip's throughout the year, as in the previously described experiments, while temperature was maintained at 6.5 ± 0.5°C, which is approximately mid-way between the minimum and maximum annual temperatures experienced by *O. aculeata* in the field. Each of the 9-L tanks had one rock inside, to supply natural shelter. The same unfiltered running seawater as used for the other experiments was provided at a rate of 5.5-6 L min^{-1}. Again, individuals were collected in St. Philips and 30 of them processed at the beginning of the experiment to establish gametogenic status. The remaining specimens were distributed in the experimental unit as follows: 8 tanks held a single male and 9 held a single female. Approximately half of these specimens were preserved for biometrics, gonad index and histological analysis in February 2009 (6-month sample), while the remaining specimens were preserved for processing after 12 months.

3.3.4 Biometrical analysis

The first step in processing samples from the field and laboratory experiments was a biometrical analysis, which was conducted on live specimens. For each individual, two perpendicular measurements of disc diameter were

recorded using the imaging software Simple PCI© (v. 6.0) from photographs taken under a Nikon SMZ1500® stereomicroscope attached to a Nikon DXM1200F® digital camera. After the initial analysis, all arms were removed using a scalpel, and the central disc was placed into 4% formaldehyde for a minimum of seven days.

3.3.5 Gonad index

The gonad index (GI) was measured in a minimum of twenty individuals for the field study, and in as many individuals as possible for laboratory experiments (4-20 individuals for the various experiments). The GI was calculated using the wet weight of all gonads divided by the wet weight of the entire central disc and multiplying by 100.

3.3.6 Histology

A total of 5-20 individuals per field sampling, and 4-20 individuals per laboratory trial (depending on the experiment) were examined. For each individual, standard histology procedures were used on two gonadal lobes. Gonad samples were dehydrated in a Leica TP1020 Semi-enclosed Benchtop Tissue Processor® using a graded series of ethanol baths (70% ethanol, Flex 80-95 and 100% ethanol), followed by clearing in two baths of Neo-Clear Clarifier®, and paraffin infiltration using two vacuum-baths of Paraplast® embedding medium. Both lobes for each individual were placed into the same paraffin block. For each sample, three 12-14 µm histological sections were made (2 lobes x 3 histological sections each = 6 sections per individual) and mounted

on each of three slides and stained using the periodic acid-Schiffs Reagent (PAS) method.

Gonadal sections were examined and photographed under a light microscope (Nikon Eclipse 80i®) attached to a Nikon DXM1200F® digital camera to visually assess the development of the gametes. Characteristics defined by Patent (1969), Fenaux (1970) and Falkner and Byrne (2003) were used; here, the 'post-spawning' and 'recovery' stages are merged into a single stage, thus creating a system of four gametogenic stages: early growth, growth, mature and spent. A detailed description of each stage is provided in Chapter 2 for females and in Appendix A for males.

3.3.7 Quantitative analysis of oogenesis

Micrographs of the ovarian sections were analysed using the software Simple PCI© (v. 6.0). For each photograph, this software was used to measure the maximum diameter of every oocyte present within the photograph. The total number of oocytes was then used along with the size of the photograph to determine oocyte density. As a quantitative measurement of gametogenic development, a formula coined the 'maturity stage index' (MSI) was developed which incorporates easily quantifiable variables that change throughout the gametogenic cycle (see Chapter 2 for details):

$$MSI = \frac{\text{oocyte density}}{\text{size of individual}} \cdot \text{oocyte surface area} \cdot 0.01$$

Where oocyte density is the number of oocytes present per mm^2, size of individual is based on the disc diameter in mm, oocyte surface area = $¼ \cdot \pi \cdot$ oocyte diameter^2, and oocyte diameter is the mean diameter of all oocytes present. The MSI of each individual was calculated separately for the three gonad sections. The MSI calculation provides a range of values from 0 to a maximum of approximately 800, where the lowest values were seen post-spawning (in spent ovaries), with values increasing incrementally until maturity.

The coefficient of variation (CV) on the MSI was calculated for individuals or groups of individuals using:

$$CV = \frac{\text{standard deviation of MSI}}{\text{mean MSI value}} \cdot 100$$

3.3.8 Statistical analyses

Chi-square tests were used to test for any deviation of the sex ratio from equality (1:1 ratio). One-way analyses of variance (abbreviated OWA) were used to determine differences among sampling dates, between habitats, among individuals and between trials for different variables (GI, disc diameter, CV, MSI). When assumptions of normality and equal variance were not met, the non-parametric equivalent (Kruskal-Wallis ANOVA on Ranks, abbreviated KWA) was used. Two-way ANOVAs were used to test for inter-substrate, inter-individual, and intra-substrate differences in MSI. Post-hoc pairwise tests (Holm-Sidak method for one-way ANOVAs, Dunn's method for Kruskal-Wallis ANOVAs on Ranks) were conducted to compare specific groups. Spearman correlations were

used to test for correlation between MSI and GI. Statistical analyses were conducted with the software package Sigmaplot/SigmaStat (version 11.0; Systat, Inc.)

3.4 Results

3.4.1 Sex ratio

Although more males than females were collected in both habitats, the overall sex ratio of all *O. aculeata* collected from St. Philip's did not significantly differ from equality in rocks ($\chi^2 = 1.36$, $p = 0.244$, $n = 355$), in rhodoliths ($\chi^2 = 1.76$, $p = 0.185$, $n = 252$), or when both habitats were pooled ($\chi^2 = 3.18$, $p = 0.075$, $n = 607$). Some months sampled during the field study showed an apparently uneven distribution of sexes among and between habitats and months, i.e. March through May samples from rocks, where the male to female ratio ranged between 1.7:1 and 2.1:1. However, distribution never significantly differed from a 1:1 ratio.

3.4.2 Maturity Stage Index (MSI)

MSI generally corresponded to the different stages of development with the following values: early growth = MSI of 100-200, growth = MSI of 200-400, mature = MSI of 400-800, and spent = MSI of 0-100 (Chapter 2).

3.4.3 Field study – rock and rhodolith habitats

Ophiopholis aculeata displayed an annual reproductive cycle, based on samples collected from both rocks and rhodoliths (Figure 3-1). Changes in GI

were generally similar in males and females, except where mentioned below. The GI (measured in both sexes) and MSI (measured in females) indicated that there was relatively little gametogenic activity in the 1-2 months following spawning in August, other than the histological evidence of phagocytosis of relict oocytes, and minimal GI (~10%) and MSI (0-100). Some individuals first showed signs of early gametogenic growth in October 2008, and virtually all individuals were in early growth by November, though the oocytes present at this point were very small and infrequent and caused minimal change to the GI and MSI. Oocyte proliferation and growth increased in December, reflected in the GI and MSI rising from minimal values (GI of 10-15%, MSI of 100-200). Gametogenesis slowly progressed throughout winter and spring months (GI increasing to 20%, MSI increasing to 200-400), culminating with individuals reaching reproductive maturity in summer (GI of 20-35%, MSI of 400-800). In 2008, most individuals were in late growth or reproductive maturity by mid-August, and spawning occurred by late August. In 2009, GI and MSI indicate that at least some individuals were mature earlier and underwent at least a partial spawning in June. The GI decreased between December 2008 and January 2009, more noticeably in males than females (male ophiuroids from rocks decreased from 10 to 5%, those from rhodoliths decreased from 18 to 9%), though the MSI showed little variation (Figure 3-1). A similar decrease in GI of males from both habitats (with minimal change in females from rhodoliths) was seen between February and March 2009. The MSI decreased in rock samples between March and April, and again between May and June, but did not show similar decreases in

rhodoliths. Gonad index and MSI decreased in female ophiuroids from rhodoliths between July and August, but increased again before the major spawning event at the end of August. Males from rhodoliths showed a similar decrease, but beginning in June, and decreasing consistently from June to August without showing an increase prior to the major spawning even at the end of August. Both GI and MSI indicate that *O. aculeata* mostly undergoes partial spawning, whereas totally empty gonads were rare and only found toward the end of the spawning period. Many spent females collected post-spawning harboured large vitellogenic oocytes in the process of being lysed by phagocytes. Although males were not analysed using the MSI, the GI was generally similar between sexes for both rock and rhodolith habitats, with the exception of some delay in GI drop in female samples from the rock habitat in August 2008. Males from rhodoliths also showed an increase in GI in November and December 2008, with a decrease after December, followed by another increase in February and decrease in May.

Nested ANOVAs were used on ophiuroids from rocks and rhodoliths to test for differences in MSI intra-individually (using three samples from the same individual). For each month throughout the field study, nested ANOVAs were unable to find differences among gonad replicates for either habitat ($p > 0.05$), suggesting that the multiple samples produced for each individual were not significantly different, and that gonad condition was similar throughout the gonads of an individual. Therefore, the replicates for each individual were pooled to obtain an average MSI value for each individual to use in broad inter-site and inter-month comparisons.

Kruskal-Wallis ANOVAs on Ranks were used to test for differences in GI, MSI and disc diameter between individuals from rocks and individuals from rhodoliths throughout the field study. Analyses showed that disc diameter was significantly smaller in individuals from rhodoliths ($H = 18.73$, $df = 1$, $p < 0.001$). When months were tested individually, most were found to have a significant difference between individuals from rocks and rhodoliths, excepting November and December 2008 and April 2009. GI and MSI were both significantly different between the two habitats for some, yet different months, over less than half of the sampling period, with no discernable pattern or relationship to one another. When all data throughout the year was considered as a whole, no significant difference in GI between rock and rhodolith samples was found ($H = 1.58$, $df = 1$, $p = 0.209$). However, a significant difference occurred between the two habitats when comparing MSI ($H = 11.88$, $df = 1$, $p < 0.001$). This was expected from a comparative study that showed the greater sensitivity of the MSI (Chapter 2). Hence, the fine analysis will focus on the female MSI, and results from other methods will only be used to substantiate some of the comparisons.

Rocky habitat

As expected in an annual cycle, there was a significant difference among MSI of samples from different sampling months (KWA, $H = 83.00$, $df = 12$, $p < 0.001$). Post-hoc tests found that MSI in post-spawning months (September, October, November) were significantly lower than in months when individuals were in gametogenic growth and just prior to spawning (March to August 12)

(Dunn's, $p < 0.05$; Figure 3-2). More differences were revealed when the MSI was analyzed with the addition of a second factor, such as individuals or subgroups (individuals collected under the same rock). A two-way ANOVA of MSI (using month x individual) also showed that the mean MSI values were significantly different across months ($F = 10.72$, $df = 12$, $p < 0.001$), but not among individuals in a month ($F = 0.11$, $df = 5$, $p = 0.990$). Along with the same differences between months seen in the previous test, two additional months (May and July) were determined to be significantly different from August 27, thus including the latter date in the post-spawning period (Holm-Sidak, $p < 0.001$). This relationship was further strengthened in another two-way ANOVA for MSI (month x subgroup), which showed that sampling months were significantly different ($F = 10.90$, $df = 12$, $p < 0.001$), but subgroups were not ($F = 1.55$, $df = 6$, $p = 0.168$). The same differences were seen as in the previous test, with the addition of August 27 being significantly different from August 12. Furthermore, January and February were significantly different from March and other pre-spawning months (Holm-Sidak, $p < 0.001$).

Finer analyses within each month generally showed that individuals collected at the same date were significantly different from one another when MSI values were compared regardless of which rock each individual was collected from (KWA, $p < 0.05$). Maturity stage index values were also compared after taking provenance into account (i.e. subgroups of individuals coming from the same rock) using two-way ANOVAs (rock x individual). For this, months were selected for which there were at least four rocks harbouring a minimum of two

individuals each. Globally, analyses showed that pre-spawning months (June and July) exhibited greater MSI homogeneity both among individuals and among subgroups (rocks) than spawning and early growth months (August and February; Figure 3-3). In June, the MSI was not significantly different among individuals ($F = 0.58$, $df = 1$, $p = 0.453$), but was different among subgroups of individuals from rocks ($F = 4.63$, $df = 5$, $p = 0.005$), and there was an interaction between the factors ($F = 4.49$, $df = 5$, $p = 0.005$). Only two of the six pairs of individuals collected under the various rocks had a significantly different MSI (Holm-Sidak, $p < 0.05$). In July, the MSI did not vary inter-individually ($F = 1.15$, $df = 1$, $p = 0.297$), but varied among subgroups from rocks ($F = 4.58$, $df = 4$, $p = 0.009$), and there was no interaction between the factors ($F = 2.23$, $df = 4$, $p = 0.102$). The MSI therefore did not differ between individuals in any of the five pairs examined (Holm-Sidak, $p > 0.05$). On August 12, the MSI differed both among individuals ($F = 49.79$, $df = 1$, $p < 0.001$) and among subgroups from rocks ($F = 14.02$, $df = 3$, $p < 0.001$), and there was a significant interaction between the factors ($F = 9.35$, $df = 3$, $p < 0.001$). Individuals in three of the four pairs showed a significantly different MSI (Holm-Sidak, $p < 0.05$). In February, the MSI was again different among individuals ($F = 11.51$, $df = 1$, $p = 0.003$) and subgroups from rocks ($F = 8.19$, $df = 4$, $p < 0.001$) with a significant interaction ($F = 8.23$, $df = 4$, $p < 0.001$). The MSI was significantly different between individuals in three of the five pairs sampled (Holm-Sidak, $p < 0.05$).

The variance (CV) of the MSI was also examined across the sampling period. Within each month, the CV generally differed among subgroups. Again,

this was particularly pronounced in spawning and post-spawning months (August 27 through October), which showed larger CVs than most other months (Figure 3-4). In contrast, periods of pre-spawning (June, July, August 12) showed smaller inter-month and inter-subgroup variances (Figure 3-4). Statistical analysis of CV on subgroup means confirmed that CV was significantly different among months (OWA, $F = 2.42$, $df = 12$, $p = 0.026$; Figure 3-5), though a post-hoc test was unable to identify months that were significantly different from one another (Holm-Sidak, $p > 0.05$). The CV on mean MSI was greatest in the three months following spawning (Figure 3-5).

There was a positive correlation between MSI and GI ($r = 0.368$, $df = 80$, $p < 0.001$).

Rhodolith habitat

The structural complexity of the rhodoliths (due to branching and growth around/on rocks and shells) led to individuals in rhodoliths being more difficult to sample than those living under rocks. This was compounded by the dominance of sexually immature individuals (2-6 mm in disc diameter) in the rhodoliths, providing few mature adults in most samples.

A KWA indicated that the MSI was significantly different among months ($H = 37.01$, $df = 11$, $p < 0.001$). Post-hoc comparisons showed that the MSI of samples in July was significantly different from that of samples collected between September and December (Dunn's test, $p < 0.001$; Figure 3-6). The relationship between MSI and month was further studied using two-way ANOVAs (i.e. month

x individual and month x subgroup). This analysis found a significant difference among months ($F = 3.83$, $df = 11$, $p < 0.001$) but not among individuals ($F = 0.47$, $df = 14$, $p = 0.937$). Post-hoc tests indicated that along with July being different from September-December (as determined previously), April was also significantly different from July (Holm-Sidak, $p < 0.001$). Like in the rock samples, this relationship was refined when comparing MSI of subgroups (individuals collected from the same rhodolith) within each month. There was a significant difference among months ($F = 4.32$, $df = 11$, $p < 0.001$), but not subgroups ($F = 0.74$, $df = 4$, $p = 0.571$). The original difference was confirmed (July different from September-December), with the addition of July as also being significantly different from all months between February and May (Holm-Sidak, $p < 0.001$).

Many rhodoliths harboured only one mature individual, which did not allow for any robust intra-rhodolith analysis. Furthermore, within a given month, this meant that the variation of individuals among different rhodoliths was often equal to the inter-individual variation outlined above. In months with at least 2 samples from a minimum of 2 different rhodoliths (March, April, May, June), individuals within a given month were significantly different from one another in MSI value when considered regardless of which rhodolith they were collected from (KWA, $p < 0.05$). When the individuals were compared based on which rhodolith they came from, subgroups were found not to be significantly different from one another, except in June 2009 (Two-way ANOVA, $F = 11.276$, $df = 4$, $p < 0.001$). None of the other months tested using two-way ANOVAs (rhodolith x individual)

showed differences among subgroups of individuals from rhodoliths or between paired individuals ($p > 0.05$).

There was no significant difference in the variance of MSI among months (KWA, $H = 13.37$, $df = 10$, $p = 0.204$). Because there were few rhodoliths with multiple mature females, it was difficult to accurately compare CVs among them, with the exception of the April-June 2009 interval. Of note was the prevalence of large CV values (> 50%) in post-spawning and recovery months (August-February), and smaller values (< 50%) in growth and pre-spawning months, suggesting a higher degree of synchrony in MSI in the latter period.

There was positive correlation between MSI and GI for rhodolith samples as a whole ($r = 0.646$, $p < 0.001$, $df = 59$).

Other field locations – Harbour Main and Admiral's Cove

Preliminary samples collected in Harbour Main and Admiral's Cove in July 2008 were compared with samples from both rocks and rhodoliths collected at the same time in St. Philip's. There was no significant difference in GI among the samples from the 3 locations at that time (KWA, $H = 5.93$, $df = 4$, $p = 0.204$). Similarly, for December 2008, no significant difference was shown between samples from St. Philip's and Admiral's Cove based on GI (OWA, $F = 3.10$, $df = 1$, $p = 0.092$). The MSI of Admiral's Cove samples could only be calculated for December 2008 because the earlier samples were ruined during the initial histology process (as were those from Harbour Main). The MSI values of St. Philip's samples were significantly higher than those of samples from Admiral's

Cove at that time: the median MSI for the latter was 43.4, while that for the former was 130.8 (KWA, $H = 14.84$, $df = 1$, $p < 0.001$; Figure 3-7). The St. Philip's samples were further along in development than those from Admiral's Cove, as many were in the process of early growth, while most of those from Admiral's Cove were still in the spent stage and had not begun to generate new oocytes.

3.4.4 Laboratory study

Individuals collected at "Time 0" (collected in July 2008 for experiment beginning August 1 2008) were significantly different from one another in terms of MSI (KWA, $H = 37.84$, $df = 16$, $p = 0.002$; Figure 3-8). The mean MSI at time 0 was 331.6 ± 23.1, which is indicative of individuals being in the growth stage. The CV for the group was determined to be 32%.

Grouped under naturally fluctuating conditions

After 6 months (February 2009), the mean MSI was 143.68, indicative of individuals in early stages of gametogenic growth. A significant difference in MSI was seen among individuals maintained within a group under natural conditions (KWA, $H = 30.65$, $df = 11$, $p = 0.001$). Post-hoc tests showed that a single individual was significantly different from two others (Dunn's, $p < 0.05$), while all other individuals were similar ($p > 0.05$). When all data were considered, the CV was 86%, over double the initial variance. When the individual that differed from others was removed, the CV was 66%, still twice the initial variance.

After 12 months (July 2009), the mean MSI in the two tanks was 44.84 and 28.55, respectively, suggesting that individuals had recently spawned. No significant difference between samples in the two grouped tanks was observed at the time (KWA, $H = 0.37$, $df = 1$, $p = 0.544$). There was a significant difference in MSI among individuals when considered regardless of which of the two tanks they were from ($H = 37.27$, $df = 16$, $p = 0.002$; Figure 3-9). Graphical analysis shows that grouped individuals are split into two clusters based on MSI, most exhibiting low MSI (0-70) and three showing higher MSI (90-190). Only one individual bridged between the two clusters. The CV on mean MSI for the two groups was 116% and 123%.

When comparing MSI across the sampling period, a steady decrease was detected: the mean MSI was 332 at time 0 (August 2008), 144 after 6 months (February 2009), and around 45 after 12 months (July 2009), with a global statistically significant difference among the values (KWA, $H = 88.29$, $df = 2$, $p < 0.001$). Post-hoc tests indicated that the significant difference was between the initial samples and all others (Dunn's, $p < 0.05$; Figure 3-9).

Isolated under naturally fluctuating conditions

At the 6-month sampling, the mean MSI was 66.29, suggesting that individuals were spent, with one showing signs of early growth (MSI of 100-200). A statistically significant difference was detected among individuals (KWA, $H = 10.42$, $df = 3$, $p = 0.015$). Post-hoc analysis showed that two individuals were different from one another (Dunn's, $p < 0.05$; Figure 3-10). The CV was found to

be 86%, over twice that measured at the onset (32%), but identical to that measured in grouped individuals after the same interval (86%).

After 12 months, the median MSI was 172.08, suggesting that individuals were in early growth and growth stages. Individuals were significantly different from one another (OWA, $F = 5.15$, $df = 4$, $p = 0.016$). Post-hoc tests showed that one individual differed from several others (Holm-Sidak, $p < 0.05$; Figure 3-10).

A statistically significant difference among MSI at 0, 6 and 12 months was found (KWA, $H = 46.99$, $df = 2$, $p < 0.001$). Post-hoc tests showed significant differences between 0 and 6 months and between 0 and 12 months (Dunn's, $p < 0.05$; Figure 3-11). After 6 months, the variance on MSI of isolated individuals was more than twice that measured initially (86% vs. 32%), whereas after 12 months the CV was lower (26%).

Comparison between laboratory and field studies

Samples collected at the 6-month interval (early February 2009) from the field and laboratory studies were contrasted based on GI, MSI (Figure 3-12) and CV. There was no significant difference between the grouped samples in the lab and individuals from the rock and rhodolith habitats in the field based on GI (OWA, $F = 0.055$, $df = 2$, $p = 0.947$) or MSI (KWA, $H = 2.17$, $df = 2$, $p = 0.338$), though there was a difference based on CV (KWA, $H = 30.00$, $df = 2$, $p < 0.001$). Within the field, the rock and rhodolith samples had similar relatively low CVs (51% and 53%, respectively), while the grouped individuals under natural conditions in the laboratory had a CV of 86%.

Also at the 6-month interval, there was no significant difference among the grouped individuals under natural conditions, isolated individuals under natural conditions, and isolated individuals under controlled temperature based on GI (OWA, $F = 0.73$, $df = 1$, $p = 0.412$), or MSI (KWA, $H = 1.70$, $df = 2$, $p = 0.429$), though again, there was a difference based on CV (KWA, $H = 18.00$, $df = 2$, $p < 0.001$). Both the grouped and isolated individuals under natural conditions had a CV of 86%, while the individuals from the controlled temperature experiment exhibited a much higher CV than the others (116%), though this was due to a single individual with a much higher MSI than the others.

Comparisons were also made for samples collected at the 12-month interval (July 2009) in the various studies based on GI, MSI and CV (Figure 3-13). There was a significant difference between the grouped samples in the lab and individuals from the rock and rhodolith habitats based on GI (OWA, $F = 16.67$, $df = 2$, $p < 0.001$), as all three treatments were different from one another (SNK, $p < 0.05$), with individuals from the grouped laboratory experiment having significantly lower GI (mean = 10%) than individuals from rocks (mean = 22%), which were also significantly lower than GI of individuals from rhodoliths (mean = 29%). There was also a difference based on MSI (KWA, $H = 27.08$, $df = 2$, $p < 0.001$), with the grouped individuals in the laboratory having significantly lower MSI (mean = 41) than both field experiments (individuals from rocks = 197, individuals from rhodoliths = 331) (Dunn's, $p < 0.05$; Figure 3-13). Finally, there was a difference based on CV (KWA, $H = 43.00$, $df = 2$, $p < 0.001$), as the rock

and rhodolith samples had CVs of 29 and 34%, respectively, while the grouped individuals under natural conditions in the laboratory had a CV of ~120%.

Again at the 12-month interval, there was no significant difference between the grouped individuals under natural conditions, isolated individuals under natural conditions, and isolated individuals under controlled temperature based on GI (KWA, $H = 6.98$, $df = 3$, $p = 0.073$), though there was a difference based on MSI (KWA, $H = 9.131$, $df = 2$, $p = 0.010$; Figure 3-13), as the isolated individuals under natural conditions had a significantly higher MSI (mean = 188) than grouped individuals under natural conditions (mean = 41) (Dunn's, $p < 0.05$). There was a difference based on CV (KWA, $H = 23.287$, $df = 2$, $p < 0.001$), as the grouped individuals under natural conditions had significantly higher CV (~120%) than the isolated individuals under natural conditions (~25%) and controlled temperature (~104%) (Dunn's, $p < 0.05$).

Samples for all three laboratory experiments were collected within two days of those from the field at the zero and six month samplings (July 14-16 and February 15-17, respectively), but the final laboratory samples were collected approximately three weeks after those from the field experiment (July 23 as opposed to July 1; Figure 3-14). This must be considered when comparing individuals from the laboratory and field experiments, as the field samples collected on July 1 were in the later phase of the growth stage, with many individuals appearing more gametogenically advanced than conspecifics collected at the same time in the previous year. Many individuals from the

laboratory experiment had spawned when collected on July 23, with some in spent and early growth stages.

Additional study on temperature

In the study of individuals maintained isolated under stable temperature, the median MSI was 56.76 after 6 months, indicating that individuals were spent. There was no statistically significant difference in MSI among individuals at that time (KWA, $H = 2.76$, $df = 2$, $p = 0.296$), but there was one after 12 months (OWA, $F = 58.70$, $df = 6$, $p < 0.001$). This relationship is best seen graphically (Figure 3-15), with individuals being separated into two distinct clusters: those with a very high MSI (530-630) indicative of readiness to spawn and those with low MSI (1-105), which appeared to have recently spawned. A statistically significant difference in MSI among individuals sampled at 0, 6 and 12 months was found (KWA, $H = 9.12$, $df = 2$, $p = 0.010$). Post-hoc tests showed that only samples from 0 and 6 months were significantly different (Dunn's, $p < 0.05$; Figure 3-16). The variance on the mean MSI increased sharply in the first 6 months (from 32% to 116%) and showed little difference after 12 months (104%).

3.4.5 Environmental parameters

Temperature and phytoplankton levels were recorded throughout the study (Figure 3-17). Temperature in the field and laboratory reached a minimum value of -1°C in winter (January through March) and maximum values between 15-16°C in summer (July through September), with the field values recorded at 10-15 m depth and the laboratory temperatures measured independently in two

separate experimental tanks. Although temperature in the laboratory was variable (particularly during summer months), it remained generally close to that at the field site (Figure 3-17). Phytoplankton levels from water samples collected in St. Philips showed a small increase in August 2008 (from $0.5 \cdot 10^5$ to $1.0 \cdot 10^5$ cells $\cdot L^{-1}$) followed by a 5-fold increase in April and May 2009 (from $0.5 \cdot 10^5$ to $2.5 \cdot 10^5$ cells $\cdot L^{-1}$; Figure 3-17). Photoperiod ranged from a general minimum of 9 h daily in late December-early January to a maximum of 17 h in June.

3.5 Discussion

Ophiopholis aculeata has an annual reproductive cycle culminating with spawning in summer. Based on the field study, synthesis of new gametes begins as early as fall in some individuals with maximum activity recorded in the following spring in correlation with the onset of the main phytoplankton bloom and the first increase in temperature. The brief period during which visibly mature specimens occurred indicates that gametogenic development is slow and protracted until shortly before spawning. Several decreases in GI and MSI throughout the year present two possibilities: first, the usage of the gonad as a storage organ, and second, small partial spawning events spread throughout the year. The frequency of decreases suggests that both are likely. The main spawning period coincides with maximum water temperatures and the late summer phytoplankton bloom. The fact that individuals maintained under constant temperature in the laboratory were gametogenetically similar to those exposed to ambient temperatures at corresponding dates indicates that

temperature does not act as a sole or main driving cue. Fine analysis of the gametogenic cycle showed that it differed on several spatial scales. Ophiuroids sampled within 200 m but in distinct habitats (rocks or rhodoliths) were not entirely synchronous in oogenesis, as individuals from rhodoliths experienced an approximately two-week delay in spawning. Greater discrepancies were observed when comparing individuals from the same substrate sampled in distant sites in a given month (120 km along the coastline), as these were in different gametogenic stages. Furthermore, laboratory work showed that grouped individuals were synchronous with those from the field in gametogenic development and spawning, whereas individuals maintained in isolated tanks were gametogenetically heterogenous and out of phase. This highlights the importance of social interaction in the fine tuning of gametogenesis in *O. aculeata*, similar to what was demonstrated by Hamel and Mercier (1996; 1999) with the sea cucumber *Cucumaria frondosa*.

The annual reproductive cycle of *O. aculeata* is typical of boreal ophiuroids (Hendler 1991), and the evidence of spawning in the field in August 2008 supports Blake's (1978) preliminary findings for *O. aculeata* studied in southeast Newfoundland. Bowmer (1982) reviewed available literature on ophiuroid reproductive cycles, and found that 14 of the 25 species studied had short, discrete, annual maturations, and that nearly all species spawned in summer and/or autumn, regardless of the environmental regime to which they were exposed. This is in line with most temperate and boreal ophiuroids, in which the

spawning period is reported as relatively short (Bowmer 1982; Hendler and Tyler 1986; Bourgoin and Guillou 1990; Lefebvre et al. 1999).

The MSI used as a proxy of gonad maturation in the present study is a reliable tool to investigate fluctuations in oogenesis over time and among conspecifics (see Chapter 2). It showed that substantial gametogenic development occurred in June-July followed by spawning in August. Individuals collected in July 2008 were in the growth stage, and some of those collected in mid-August were spent, eliciting a large change in MSI between these samples. Such findings indicate that final maturation of gametes can occur within a relatively short period. While the decrease in MSI between August 12 and 27 was clear in individuals sampled from rocks, suggesting that spawning occurred in that interval, individuals from rhodoliths demonstrated a more prolonged spawning period, with a minor MSI decrease in July, and a later major one between August 27 and September 15. For individuals from both habitats, December to February corresponded to the recovery months where relict gametes were degraded by a growing number of phagocytes. There was minimal gamete synthesis activity at this point, with most individuals slowly progressing through early gametogenic growth. When MSI data were examined using month and subgroups of individuals from the different rocks sampled, December 2008 was identified as a boundary month between post-spawning and recovery months, while January and February acted as recovery months, and were significantly different from March and other pre-spawning months. The same relationship was seen in individuals from rhodolith samples, except January was

found to be the boundary month. In individuals from both habitats, March to May marked the shift between slow early growth of gametes and the onset of more rapid oogenic growth and vitellogenesis as shown by sharper increases in MSI.

Oogenesis was synchronous among individuals from rock and rhodolith habitats during periods of slow development, but at other times changes were seen in individuals from the one habitat but not the other. The clearest example of this was a delay of roughly two weeks in the major spawning event in individuals from the rhodolith habitat, followed by the same delay in the shift between spent and early growth stages. Are the reproductive cycles slightly out of phase because of the nature of the habitats, because of the size structure of the individuals in each habitat, or because of some other factor? Several studies have reported size differences of ophiuroids within specific substrates, with smaller individuals generally preferring more complex substrata, as it is the case here with smaller individuals found in rhodoliths. In a study conducted in Brazil, Boffi (1972) indicated that calcareous algae were a preferred habitat, with 80% of them harbouring a variety of brittle star species, compared to 50% for non-calcareous algae. Hendler and Littman (1986) found that the smallest ophiuroids living on the Belize Barrier Reef were also in calcareous substrates, while the largest were in coral rubble. The calcareous algae supported denser populations and more immature individuals than other habitats; they were proposed to act as a refuge substratum as well as a nursery habitat. Hendler and Littman (1986) were unable to conclude exactly how calcareous algae (i.e. rhodoliths) were dominated by smaller ophiuroids; likely factors include preference at larval

settlement, migration or differential survival, and possibly a diet-based discrimination. Hendler and Littman (1986) concluded that juvenile brittle stars occupy the refuge substratum temporarily, and move to different substrata over time, which is consistent with observations in Newfoundland from the present study and that of Goobie (2009). Similarly, McClintock et al. (1993) found size distribution to be skewed toward small individuals in *Asteroporpa annulata* from rhodoliths in Florida. It is also possible that individuals within rhodoliths become sexually mature at smaller sizes, as their growth patterns may be constrained in crevices. The fact that sexually mature individuals of *O. aculeata* living in rhodoliths exhibited erratic fluctuations in GI and MSI and a prolonged spawning relative to conspecifics in an adjacent rocky habitat could be a result of their smaller size (and lower fecundity) and/or low numbers (most individuals in rhodoliths were sexually immature).

Data analysis at the fine scale (intra-habitat) in females from the rock habitat showed that the mean MSI was relatively homogeneous (low CV) among individuals and groups just prior to and at the beginning of the spawning period, while months corresponding to ongoing and post spawning were relatively heterogeneous (high CV). While previous studies in ophiuroids were conducted at larger spatial scales, the trends are comparable. For instance, Lefebvre et al. (1999) found that individuals of *O. fragilis* in each of two populations in the English Channel were gametogenically synchronous prior to spawning and asynchronous following spawning. Furthermore the review by Hendler (1991) mentioned how, for some species (including *O. aculeata*), asynchrony occurred

during most of the gametogenic cycle, often followed by a more synchronous and discrete spawning period, though several studies making this conclusion do not consider spatial distribution. The present study went further by distinguishing inter-individual patterns at the various spatial scales.

For most months over the sampling period, individuals within a given month were overall different from one another, but less so when compared within subgroups living under the same rock. The highest degree of synchrony within rocks (as defined by individuals not being significantly different from each other) was observed in June and July with 67% and 100% synchrony among ophiuroids from the same rock, respectively. Conversely, spawning and early growth months exhibited low synchrony (intra-rock synchrony being 25% on August 12 and 40% in February). The original hypothesis supposed that gametogenic synchrony would be maximal at close quarter and decrease with increasing inter-individual distance, i.e. between habitats at a given site and ultimately among distant locations. Clear hints of this were seen at the intra-habitat and inter-habitat levels at the same site, but only preliminary results were obtained for the inter-site study. Unfortunately, logistical and temporal constraints (especially the difficulty associated with diving in Newfoundland outside summer months) prevented more than a single full sampling event at an additional site (Admiral's Cove in December 2008). Nevertheless, the latter sample revealed a marked difference in MSI of ophiuroids between Admiral's Cove and St. Philip's at that time. Many individuals collected from Admiral's Cove were totally spent (mean MSI of 46.5); they had not begun a new gametogenic cycle, and very few individuals were in

the earliest stages of growth. In contrast, individuals from St. Philip's were all in gametogenic growth stages (mean MSI of 133.6). This asynchrony was interesting, as individuals from rocks and rhodoliths in St. Philip's were relatively synchronous in MSI during this time, i.e. there was marked inter-site variation but little intra-site (inter-habitat) variation. The most obvious differences between the sites relate to general oceanographic conditions: St. Philip's is protected by an island within a deep bay (Conception Bay), is less exposed to large waves and high winds, and is not exposed to pack ice during winter. In contrast, Admiral's Cove is open to the Atlantic Ocean, is exposed to colder water due to large upwelling events, is submitted to larger waves and stronger winds, and can be exposed to icebergs and pack ice during winter and spring months. This is reflective of a study on the echinoid *Paracentrotus lividus* by Guettaf et al. (2000), in which gonad index and development were affected by the hydrodynamism of a site, but not by depth or substrate/algal community. The gonad index was highest at the most sheltered site, and lowest at the harshest site. Similarly, Lefebvre et al. (1999) studied an ophiuroid in the English Channel and found that gonad index was lower at sites with lower water temperature and food levels. Thus, the delayed gonad development of ophiuroids in Admiral's Cove may reflect the generally harsher environmental conditions.

The relationship between spatial distribution and reproduction was further elucidated with the laboratory experiments. At the six month mark, the MSI was similar among all experiments, and comparable to that of concurrent field samples. It was slightly lower in the isolated than in the grouped individuals,

suggesting that isolation had a small but not significant effect on gonad development. At the twelve month mark, the grouped individuals were similar in development within groups, and the isolated individuals were similar to one another, but the grouped and isolated individuals (as a whole) were quite different from each other. The isolated individuals were synchronous (in the growth stage) with a small CV of 25%, while the grouped individuals were less synchronous (in spent or early growth stages) with a CV of 116-123%. The numbers here can be deceptive and warrant an in-depth investigation. The grouped individuals had high CV because most were spent but a few were in early growth, leading to a wide range in MSI. Furthermore, the last field samples were collected on July 1 2009, approximately three weeks before the laboratory experiments were completed (July 23). Field samples from June 2009 were notably more gametogenically advanced than those from June 2008 (mostly in advanced growth or mature growth), and appeared to have begun spawning. Given that the individuals collected from rocks on July 1 2009 showed signs of an early spawning event, it is likely that individuals in the field were spent by late July in 2009. Therefore, individuals kept in groups under natural conditions at this time spawned relatively concurrently with individuals from the field. This would explain their small gonad indices, large CV and small MSI values in late July 2009. The isolated individuals were synchronous with one another, but were less developed and not all had spawned, suggesting some degree of spawning asynchrony with conspecifics from grouped lab conditions and from the field.

Together these results suggest that short-lived asynchrony during gametogenic development does not impede reproductive success. As gamete synthesis progresses, cohesion among conspecifics starts to appear and spreads from natural clusters to relatively restricted areas of a few hundred meters, to reach a maximum before spawning. A synchronous spawning period at the local scale is an evolutionary advantage, as it could allow for higher probability of fertilization.

While few studies have considered the importance of spatial organization on reproduction in ophiuroids, Hendler (1991) noted the obvious differential between reported spawning periodicity for *O. aculeata* across various areas of its range. Blake (1978) reported spawning from August-October in the northwest Atlantic, while a variety of references cited by Hendler (1991) gave different spawning periods including February-May (in reports from the UK, USA, Russia, Norway and the North Sea), and May-September (in Russia). More recently, Himmelman et al. (2008) reported that a majority of visible *O. aculeata* participated in a multispecies spawning in the western Gulf of St. Lawrence in June-July, and a small number of individuals did the same in April during a mesocosm study in Newfoundland (Mercier and Hamel 2010). Such variations suggest that disparities in regional exogenous stimuli can lead to out-of-phase spawning. However they might also illustrate the capacity of *O. aculeata* to undergo numerous partial breeding episodes.

The direct observations of gamete release by *O. aculeata* in June-July or April reported by Himmelman et al. (2008) and Mercier and Hamel (2010) are

contradictory to the indirect determination of later spawning by Blake (1978) and the current study. The disparities suggest the possibility of *O. aculeata* undergoing multiple annual spawning events, based on different triggers, particularly since individuals collected in the same location have been reported as spawning at different times (Mercier and Hamel 2010; present study). Dips in the MSI were noted, such as between March and April 2009, which might be interpreted as secondary spawning events and evoke the concept of partial spawning. In the current study, individuals collected with only part of their gonad emptied of mature gametes were presumed to have partially spawned, supporting the idea of protracted or successive gamete release(s). Himmelman et al. (2008) recorded changes in gonad index during spawning of *O. aculeata* in the Gulf of St. Lawrence, and found that individuals there underwent large decreases in GI over ~4 weeks (from ~20-13%) in June and July 2003, followed by slow decrease in July and August 2003 to a GI of ~10%. These changes in GI were similar to those seen in the current study. Himmelman et al. (2008) felt that the initial decrease in GI was due to a mass spawning event, and that the latter slower drop was due to resorption of gonadal tissue, rather than further spawning. This was similar to Olsen (1942), who reported that *O. aculeata* spawned all viable oocytes in a single episode. Holland (1979), however, found that individuals of *O. aculeata* only released anywhere from hundreds to several thousand oocytes at a time, making it likely that there is more than one spawning event per spawning season. The present study clearly demonstrated that a major fairly restricted spawning episode occurred during the annual cycle, but that

participation of a small number of individuals to less generalized events remains likely, especially in the context of entrained heterospecific mass spawnings, such as the ones discussed by Mercier and Hamel (2010).

When considering the rhythmicity of oogenesis in *O. aculeata*, it is interesting to examine the environmental parameters occurring throughout the cycle. Temperature and phytoplankton have been cited as mediators of gametogenic activity and spawning in several species of ophiuroids, though most studies are preoccupied with factors that control spawning, and few focus on controls of earlier stages of oogenesis. Temperature in particular has been studied as a factor that can induce spawning or affect the pace of gametogenesis in ophiuroids (i.e. Fenaux 1970; Bowmer 1982; Tyler 1977; Yamashita and Iwata 1983; Hendler and Tyler 1986; Stancyk 1974; Hendler 1991; Stewart and Mladenov 1995; Lefebvre et al. 1999; Morgan and Jangoux 2002; Grange et al. 2004). For many echinoderms, specific temperature triggers have been discovered to induce pre-spawning and/or spawning behaviour (Hamel and Mercier 1995; Mercier and Hamel 2008; Mercier and Hamel 2009). Since Mileikovsky (1960) suggested that *O. aculeata* in the White Sea required a dip to temperatures of 5-5.5°C to spawn, it was not expected that the species would receive such a cue, if it existed, when maintained in a laboratory experiment between 6-7°C. However, the apparent spawning noted in individuals maintained under constant temperature in the current study indicates that they did not require some minimum temperature throughout the early stages of gametogenesis to trigger maturation, although there was some inter-individual

asynchrony in the early gametogenic process. As in the grouped individuals under natural conditions, the high variance in MSI at the end of the experiment is most likely explained by the fact that some individuals were mature, ready to spawn, while others were already spent. As it is likely that individuals from the field were spent by late July 2009, the presence of mature and spent individuals in the constant temperature experiment leads to the conclusion that stable temperature did not inhibit or significantly delay oogenesis, but rather that it generated some asynchrony (relative to individuals kept under natural conditions).

In the current study, gametogenic development was largely initiated during the period of increasing temperature and the main phytoplankton bloom in the spring, whereas spawning occurred during the period of highest water temperature during a small fall phytoplankton bloom. The rapid final maturation of gametes likely occurs in response to an exogenous cue such as increased phytoplankton levels that marks a relatively short period of time with ideal conditions for feeding and nutrient transfer to the growing gametes. Such timing with food availability has been suggested by previous research (e.g. Himmelman et al. 1980; Starr et al. 1990; Lefebvre et al. 1999). Inter-annual difference in development in the field makes it unlikely that photoperiod affects spawning in this species, although it could play some role in inducing the onset of oogenesis. Individuals maintained under constant water temperature in the laboratory were mostly synchronous in development with conspecifics under ambient

temperature, both in February and June, suggesting that phytoplankton levels are most likely affecting both gonad development and gamete release.

Many previous studies have cited water temperature as a spawning trigger for *O. aculeata* (Mileikovksy 1960; Blake 1978; Himmelman et al. 2008). However, Blake (1978) only recorded temperature and did not investigate other closely related factors. Himmelman et al. (2008) suggested that spawning in the field was related to upwelling causing a warming of seawater temperatures, but like Mercier and Hamel (2010) and the current study, also noted that *O. aculeata* spawned following large increases in phytoplankton level. Ultimately, reports of reproductive periodicity in *O. aculeata* varying between sites in the same year (Blake 1978) and between times of year in various studies (Hendler 1991; Mercier and Hamel 2010) strongly suggest that some combination of exogenous factors and inter-individual chemical communication act to control reproductive periodicity.

In summary, the field study of *O. aculeata* revealed an annual reproductive cycle with one major spawning event in late summer that may shift slightly from one year to the next according to environmental conditions. Data also suggested that the rapid onset of gametogenic development in spring led to a smaller partial spawning in March-April, especially since the previous summer spawning did not empty the gonad of all individuals. Gametogenic cohesion was generally greater just before spawning than at other times, and in nearby/grouped individuals than in distant/isolated individuals. Significant heterogeneity was observed between distant populations. Laboratory experiments showed that grouped individuals

were more similar in development with those from the field than isolated ones and that individuals exposed to constant temperature were not prevented from developing gametes and spawning at approximately the same time as conspecifics under natural conditions. Therefore, phytoplankton is more likely to be the main proximate cue both for the onset of gamete synthesis and for spawning. If, and how, specific temperature thresholds act on early gametogenesis in *O. aculeata* will require further examination.

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3.8 Figures

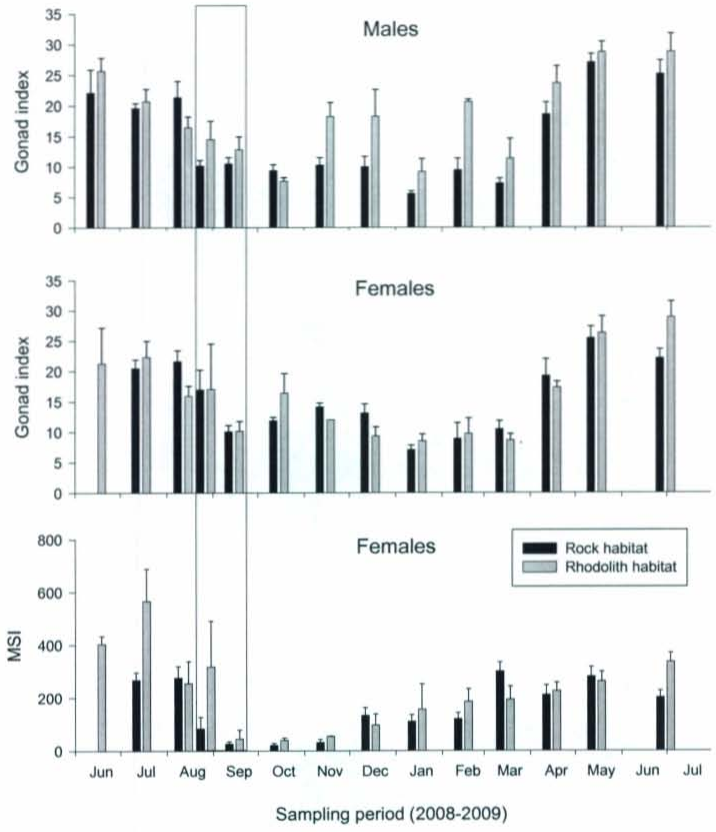

Figure 3-1. Gonad index (%) of males and females and maturity stage index (MSI) of females in *Ophiopholis aculeata* collected from rocks and rhodoliths between June 2008 and June 2009. Estimated major spawning period is indicated by a box. The error bars represent standard error and n = 3-16.

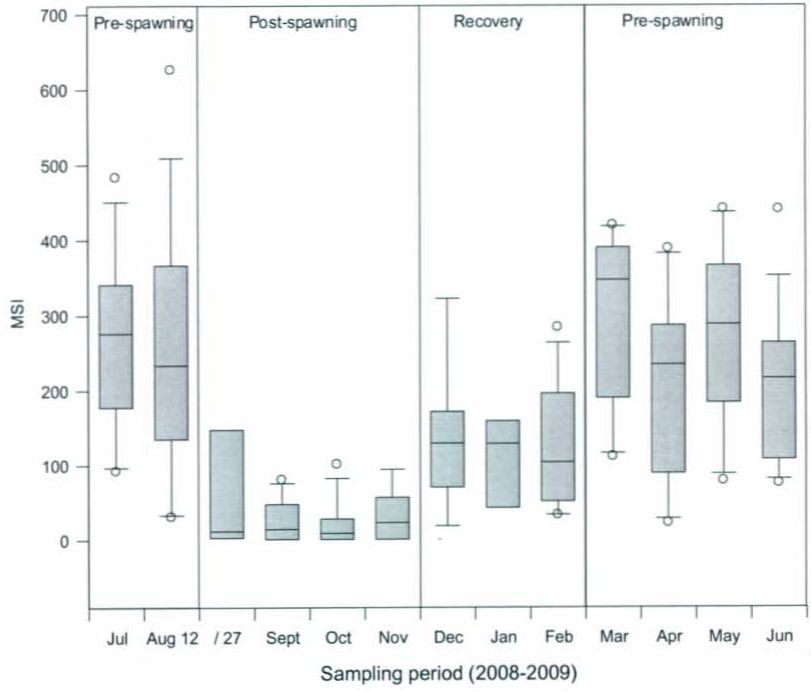

Figure 3-2. Box plots of maturity stage index (MSI) in *Ophiopholis aculeata* collected from rock habitat between July 2008 and June 2009. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles, and n = 8-16. Maturity was reached and individuals spawned in a short period of time (less than 2 weeks), thus the progression from pre- to post-spawning between August 12 and 27 2008.

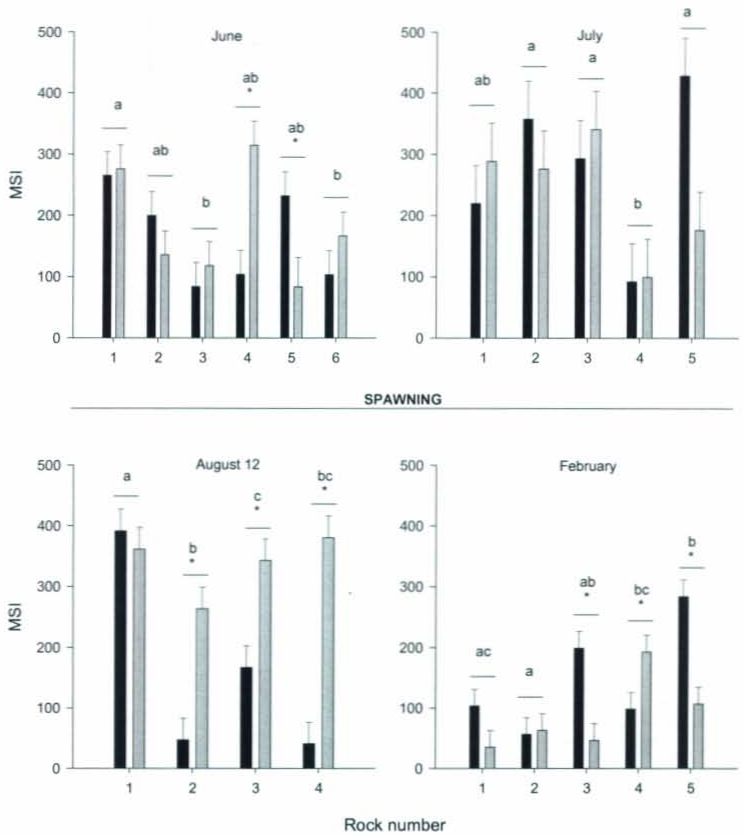

Figure 3-3. Variations in maturity stage index (MSI) among groups of two individuals (with each individual represented by a black or grey bar) of *Ophiopholis aculeata* collected from 4-6 rocks on pre-spawning dates (June-July) and post-spawning dates (August 12, February). Statistical results are illustrated where groups that share the same letters do not have significantly different mean MSI values. Individuals paired under the same rock that exhibit significantly different MSI are identified by asterisks. Error bars represent standard error.

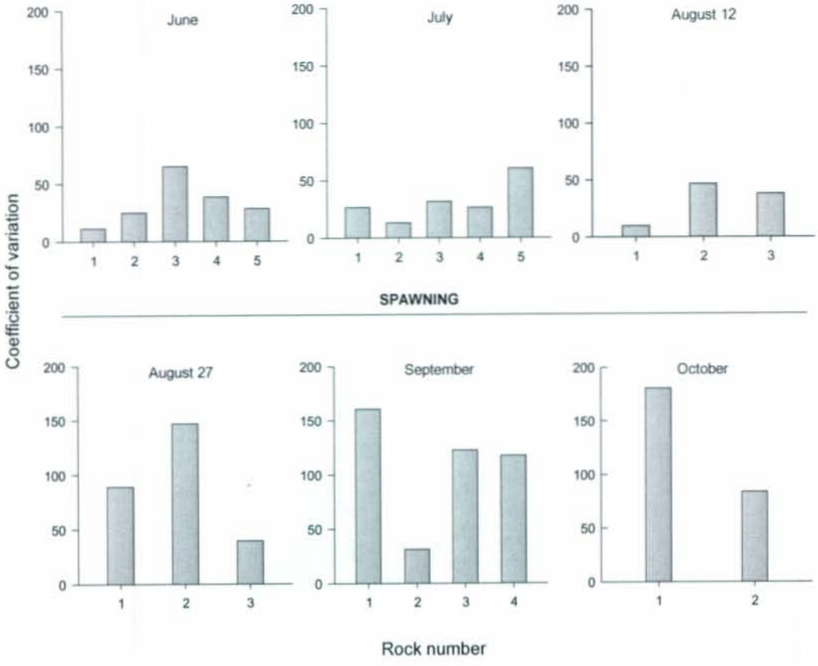

Figure 3-4. Coefficient of variation (%) on mean maturity stage index (MSI) for subgroups of *Ophiopholis aculeata* collected from 2-5 rocks on pre-spawning dates (June-Aug 12) and post-spawning dates (Aug 27-Oct). n = 2-5.

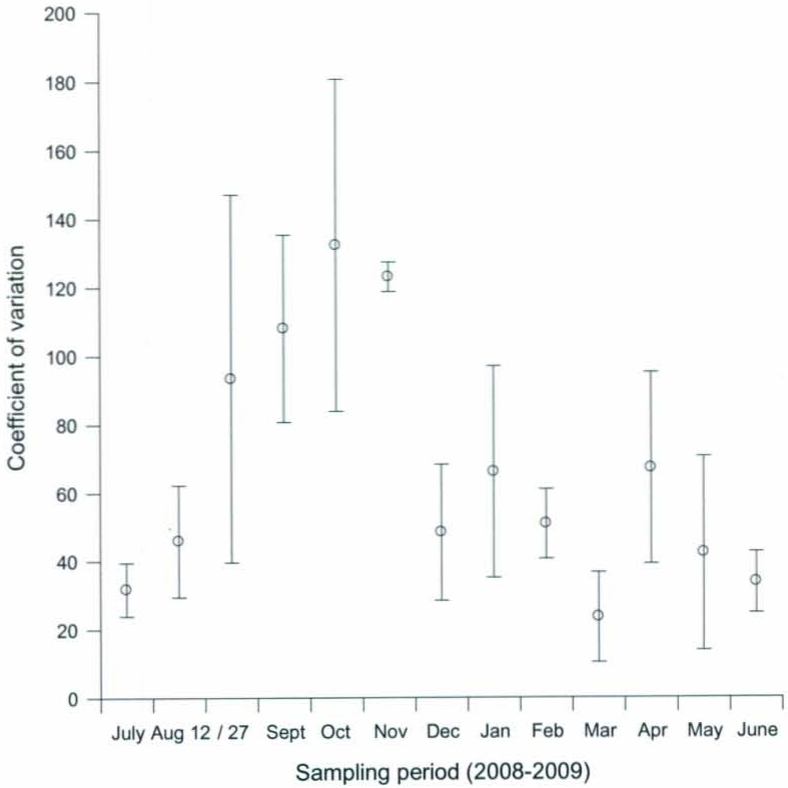

Figure 3-5. Range of coefficient of variation (%) on mean maturity stage index (MSI) for subgroups of *Ophiopholis aculeata* collected from rocks between July 2008 and June 2009. n = 2-5 subgroups of individuals per month. Error bars represent standard error.

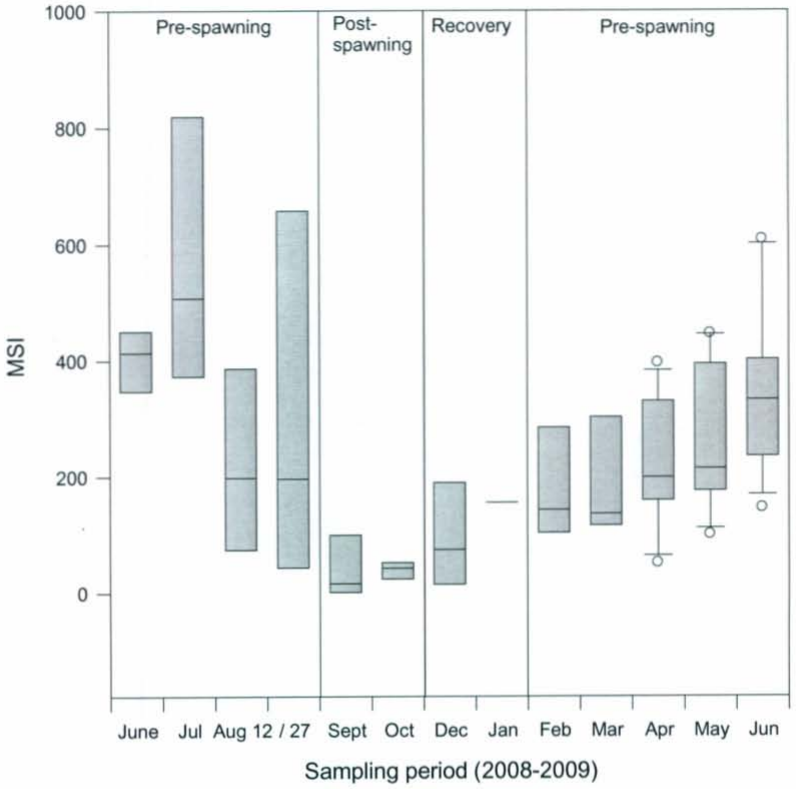

Figure 3-6. Box plots of maturity stage index (MSI) in *Ophiopholis aculeata* collected from rhodoliths between June 2008 and June 2009. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles, n = 3-14.

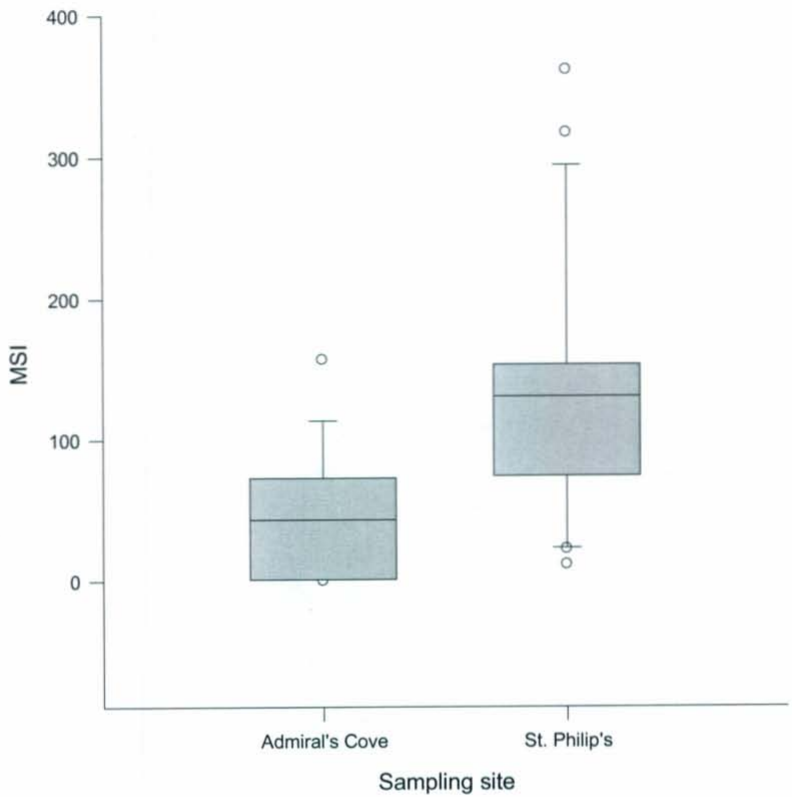

Figure 3-7. Box plots of maturity stage index (MSI) in *Ophiopholis aculeata* collected from rocks in two different locations, St. Philip's and Admiral's Cove, in December 2008. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles and n = 9-12.

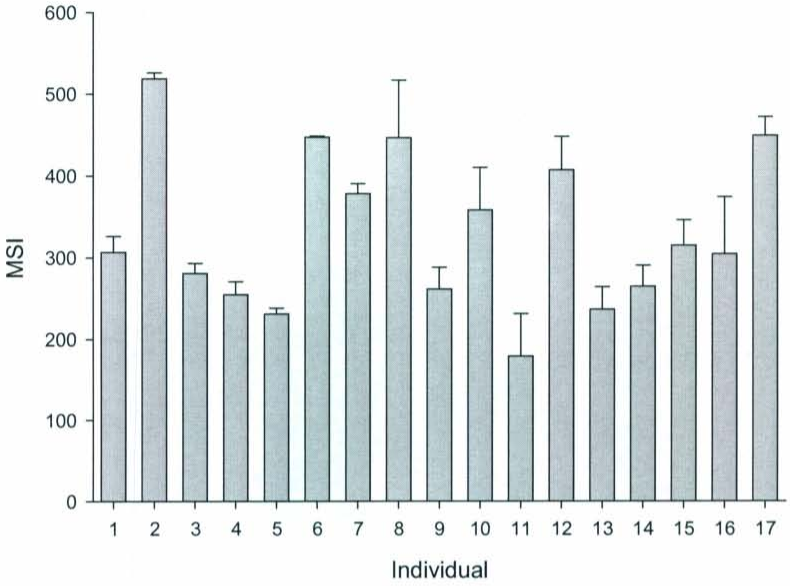

Figure 3-8. Maturity stage index (MSI) in *Ophiopholis aculeata* collected from rocks in July 2008 for use as 'Time Zero' samples in the laboratory experiments. The mean MSI value for each individual was determined using three separate gonad samples, and error bars represent standard error.

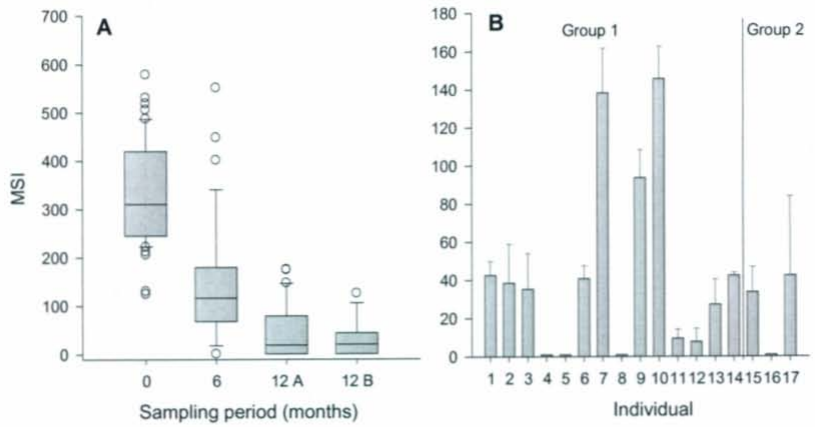

Figure 3-9. Box plots of maturity stage index (MSI) in *Ophiopholis aculeata* maintained grouped in the laboratory under natural conditions. (A) MSI at the various sampling dates (0, 6 and 12 months), where 12A = Group 1 and 12B = Group 2. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles and n = 12-15. (B) MSI of individuals in the two tanks (Groups 1 and 2) at the 12-month sampling date. The mean MSI for each individual was determined using three separate gonad samples, and error bars represent standard error.

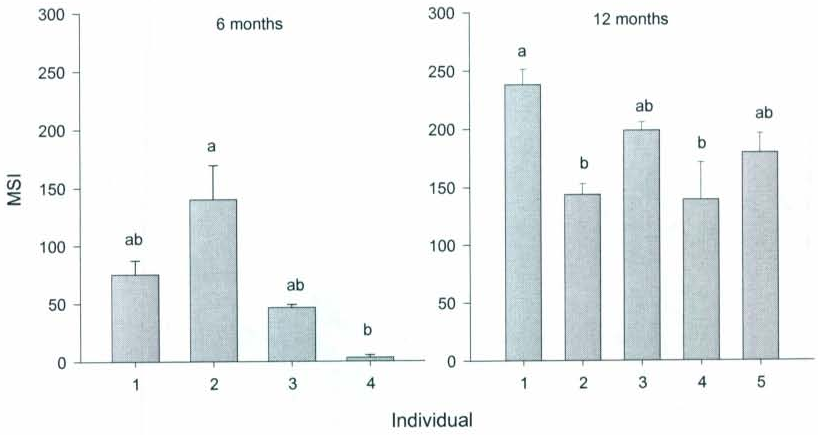

Figure 3-10. Maturity stage index (MSI) in *Ophiopholis aculeata* kept isolated under natural conditions in the laboratory. Results are shown for the 6 and 12-month sampling dates. The mean MSI for each individual was determined using three separate gonad samples, and error bars represent standard error. Individuals with different letters exhibit significantly different MSI.

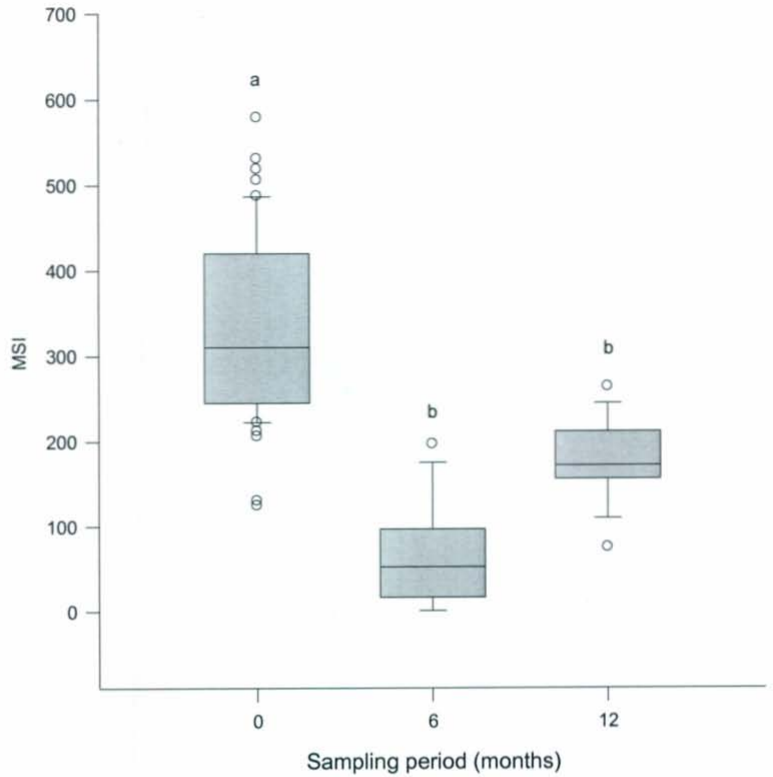

Figure 3-11. Box plots of maturity stage index (MSI) in *Ophiopholis aculeata* sampled after 0, 6 and 12 months during a laboratory experiment where individuals were kept isolated under natural conditions from August 2008 to July 2009. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles and n = 4-15. Different letters indicate significantly different MSI.

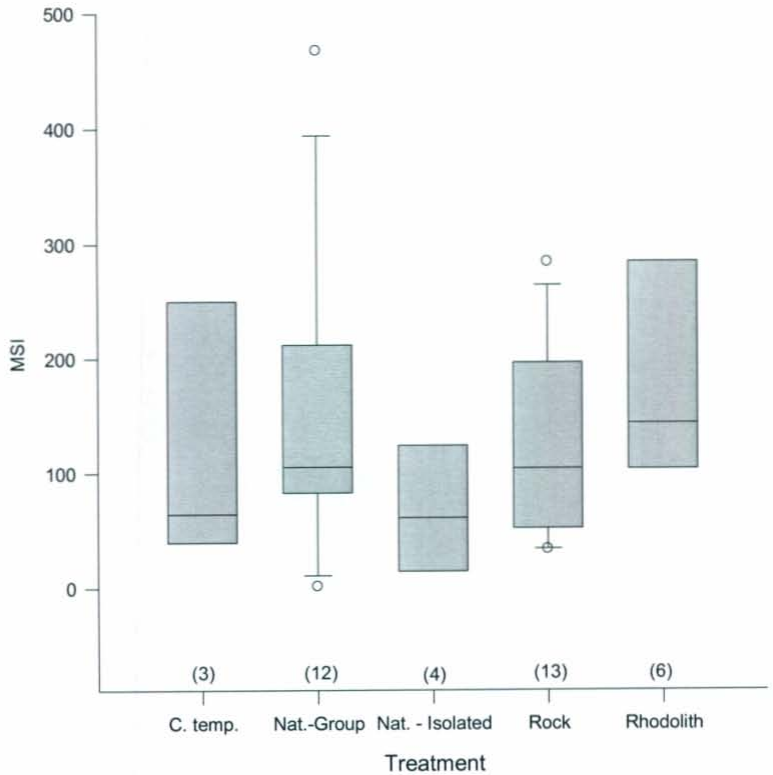

Figure 3-12. Box plots of maturity stage index (MSI) of *Ophiopholis aculeata* in laboratory experiments and in the field at the 6-month sampling date (February 2009). C. temp. = constant temperature, Nat. = natural conditions. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, and outliers are shown as hollow circles. Sample size are represented as (n) under each box.

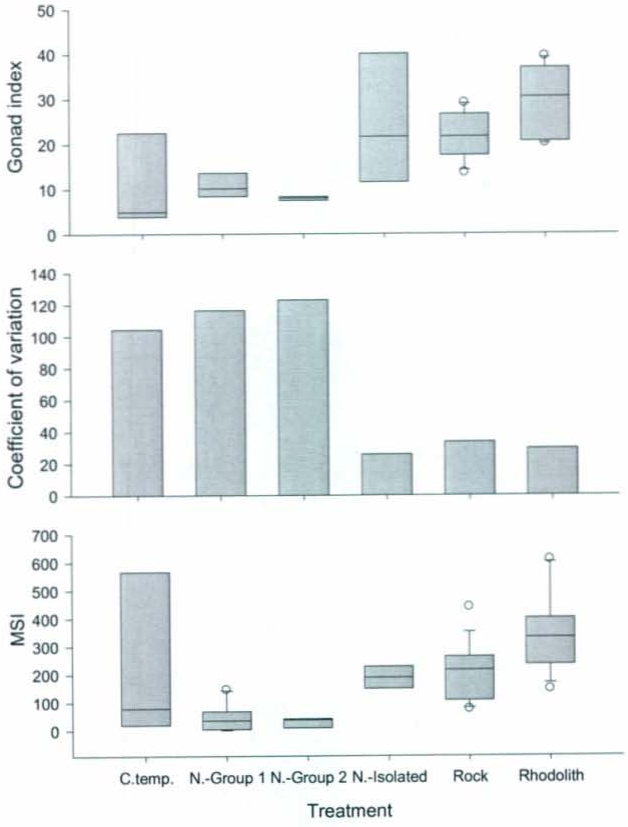

Figure 3-13. Gonad index (% wet weight), maturity stage index (MSI) and coefficient of variation on mean MSI (%) in *Ophiopholis aculeata* from laboratory experiments and field samples at the 12-month sampling date (July 2009). C. temp. = constant temperature, N. = natural conditions. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles and n = 3-16.

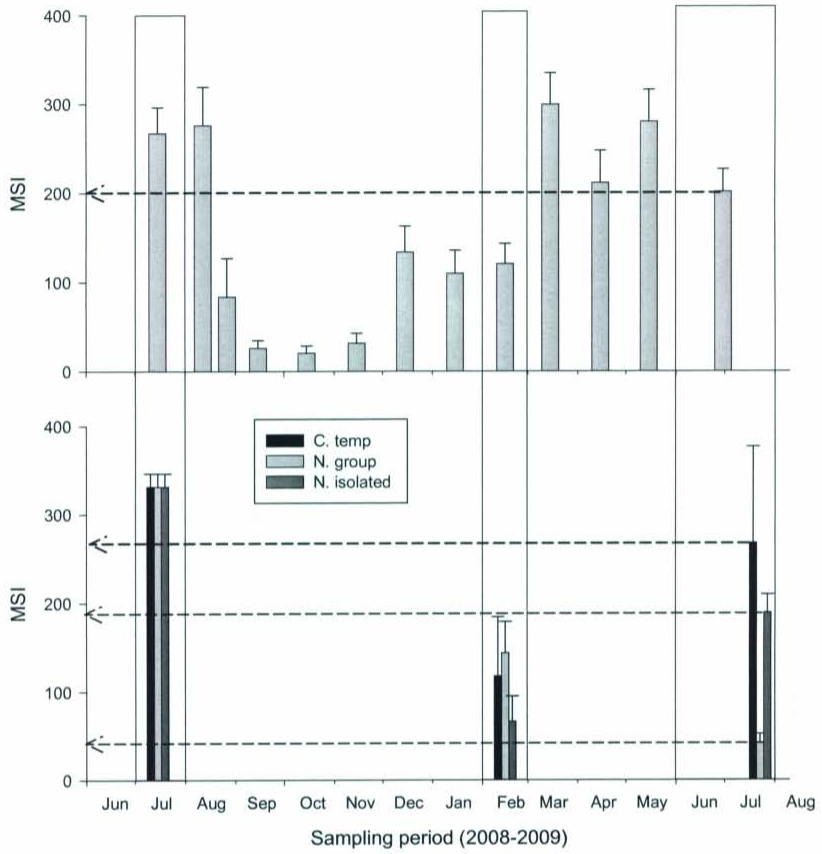

Figure 3-14. Maturity stage index (MSI) in *Ophiopholis aculeata* from the rocky habitat (upper panel) and laboratory experiments (lower panel) from August 2008 to July 2009. C. temp. = constant temperature, N. = natural conditions (with either grouped or isolated individuals). Concurrent laboratory and field samples are enclosed in boxes, and dashed lines indicate mean MSI in the various treatments at the final sampling in July 2009. Error bars represent standard error.

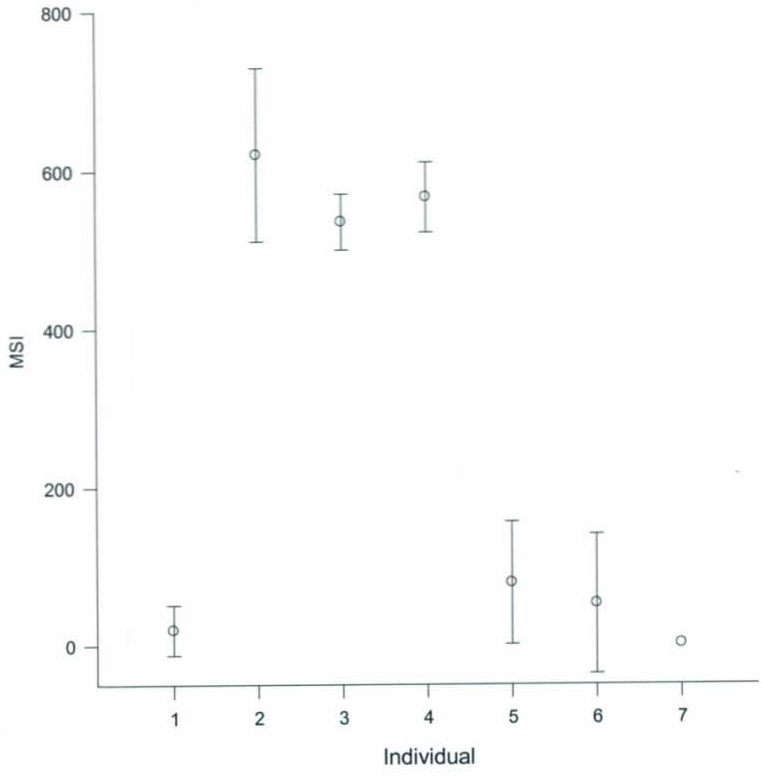

Figure 3-15. Maturity stage index (MSI) in *Ophiopholis aculeata* collected at the 12-month sampling date (July 2009) of a laboratory experiment, where individuals were kept isolated under constant water temperature (6.5 ± 0.5°C). The mean MSI for each individual was determined using three separate gonad samples, and error bars represent standard error.

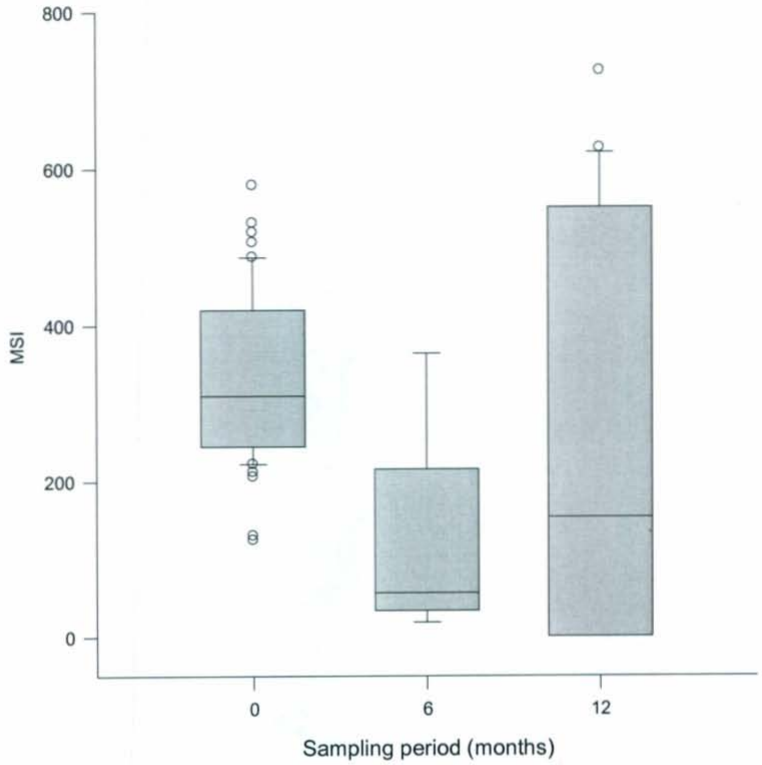

Figure 3-16. Box plots of maturity stage index (MSI) in *Ophiopholis aculeata* sampled at 0, 6 and 12 months during a laboratory experiment, where individuals were kept isolated under constant water temperature (6.5 ± 0.5°C) from August 2008 to July 2009. The center line shows the median, the lower and upper limits of the boxes represent the 25th and 75th percentiles, whiskers represent the 10th and 90th percentiles, outliers are shown as hollow circles, and n = 3-15.

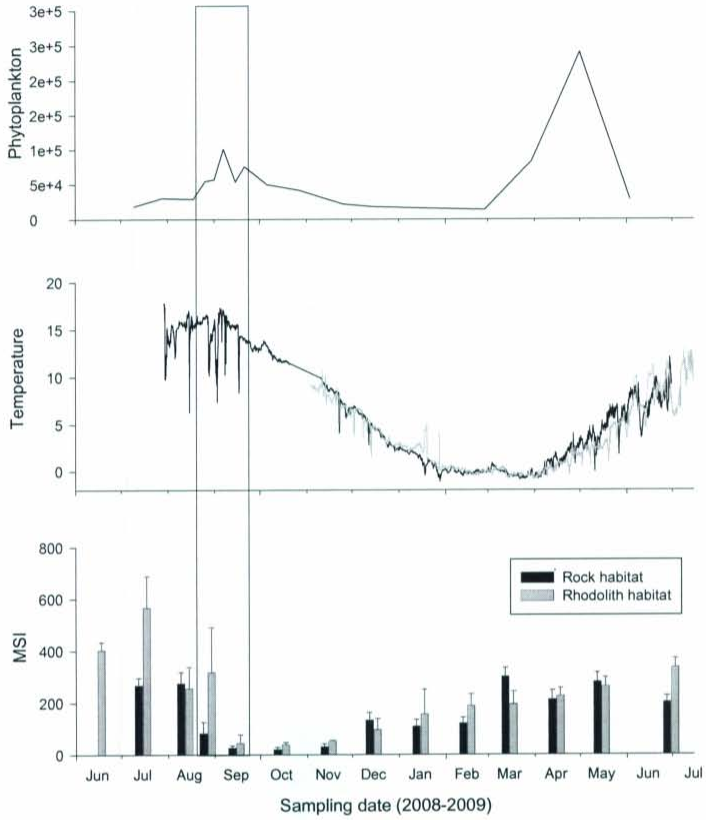

Figure 3-17. Environmental parameters from July 2008 to June 2009, as compared to variations in the maturity stage index (MSI) of *Ophiopholis aculeata* in the study site. The top panel shows estimated abundance of phytoplankton (cells·L^{-1}); the center panel shows the temperature (°C), where black = St. Philip's (12-15 m), grey = laboratory, and the bottom panel shows the MSI. Error bars represent standard error. Estimated major spawning period is indicated by a box.

Chapter 4 - Conclusions and perspectives

4.1 Major findings

This project had several major findings and outcomes. First of all was the development of a quantitative maturity stage index (MSI) that is sensitive enough to allow for in-depth analysis of the gametogenic cycle. The MSI proved to be a reliable tool for measuring gametogenic development in the two echinoderm species tested. It provides a quantitative measure of oogenic activity at a higher resolution than would have been possible using conventional methods, such as gonad index, oocyte size frequencies or gametogenic stages. Using a formulation of measurements of oocyte density, oocyte surface area and size of the individual allowed variations in the gametogenic cycle to be determined with minimum bias, compensating for the wide size range of individuals used in the study. Overall, the MSI is a straightforward method that uses simple histological measurements similar to other methods with the advantage of being fully quantitative.

The MSI was subsequently used to investigate monthly and inter-individual variations in gametogenic development of *Ophiopholis aculeata* with an aim to define and understand the influence of spatial distribution and environmental factors on reproductive patterns. The species was found to exhibit an annual reproductive cycle culminating with a broadcast spawning in mid- to late summer. It was also determined to be a partial spawner, releasing only

varying proportions of gametes in multiple spawning events. Notable decreases in MSI at various times throughout the year in the current study, along with other published reports (including some conducted in the same laboratory) listing spawning periods for *O. aculeata* as ranging throughout spring and summer, further suggest that *O. aculeata* can spawn multiple times within the same year in response to different cues. The gametogenic cycle of *O. aculeata* differed on several spatial scales. Individuals collected from rock and rhodolith habitats were periodically synchronous and asynchronous throughout the reproductive cycle, with those from rhodoliths delayed in maturation and spawning by about two weeks. Since reproductive synchrony in this species tends to be highest just prior to the spawning period, this result suggests differences in individuals living within rhodoliths (as compared to those from rocks and other loose substrata). The most likely explanation is the dominance of small, sexually immature individuals within the confined available space in this type of rhodolith, low numbers of mature individuals, and consequent reduced exposure to mature conspecifics.

Apart from the inter-habitat differences, variations in the MSI also revealed gametogenic asynchrony among brittle stars living in similar rocky habitats but in two distant locations. At the finer scale, a study of individual ophiuroids within and between rocks showed inter-rock variation in all months, though there was more variation in ongoing- and post-spawning months (August 12 and February). The same relationship was seen when studying intra-rock variation, as individuals within rocks were shown to be more gametogenically variable in spawning and post-spawning months.

Grouped individuals maintained under natural conditions in a laboratory setting were mostly synchronous in development with those from the field, while isolated individuals under the same conditions experienced some delay in maturation and spawning. Isolated individuals kept under fixed temperature settings in the laboratory were less synchronous than grouped conspecifics and those from the field, but underwent maturation and spawning at approximately the same time, suggesting that temperature does not act as a proximate reproductive cue, particularly for maturation and spawning.

This study, along with the two most recent studies on *O. aculeata*, all indicate that phytoplankton levels are likely the dominant environmental factor mediating the annual reproductive cycle, particularly the onset of maturation and spawning. Since there is so much variation in spawning periodicity throughout spring and summer months, photoperiod is likely playing a lesser role, though it possibly plays a more important role in the onset of gametogenesis, as the rapid gametogenic proliferation in spring is parallel with the annual increase in day length. Lunar cycles were implicated in having some role in spawning periodicity by a recent study, but could not be studied within the sampling framework of the present project.

In summary, the development of the MSI allowed a fine-scale study of gametogenesis in *O. aculeata* that confirmed several hypotheses concerning the effect of small-scale spatial distribution and environmental factors on gametogenesis. The resolution of this study at the spatial level is probably unique; it brings strong evidence of the importance of this factor in gametogenic

development. Results of environmental mediation are consistent with those of Himmelman et al. (2008) and Mercier and Hamel (2010), who similarly implicated phytoplankton levels as possible spawning cues, although they did not study any other stages in gametogenesis.

4.2 Main issues and possible solutions

There were a number of problems encountered during this study that were overcome but need to be acknowledged to assist future research. The most obvious issue was the difficulty in distinguishing between male and female samples during recovery stages of gametogenesis. Many such individuals had no or poorly identifiable gametes, and sometimes no relict oocytes, whereas the germinal inner epithelium left no clear traces of whether the organism was male or female based on basic histology and microscopy methods. Even with careful examination and assistance of more experienced researchers, some samples could not be sexed and thus could not be included in the data sets. This possibly skewed the MSI away from zero, as these individuals would have had MSIs of zero. Fortunately, there were very few such samples that could not be sexed (< 10 of ~900 individuals), so this problem had little impact on the overall quantitative analysis. Other researchers studying ophiuroids have reported similar drawbacks. Falkner and Byrne (2003) found it hard to distinguish between male and female specimens during the recovery stage because of the similarities between spermatogonia and oogonia, and Borges et al. (2009) were unable to

sex a good proportion of their samples (11 of 99) either due to missing gonads or because they could not be conclusively categorized.

A similar difficulty in the study of gametogenic cycles is the often unclear distinction between certain gametogenic stages. This is intrinsic to the qualitative staging system. The most common stages that cannot be distinguished are recovery and early growth, when the gonad exhibits relatively little activity. In her study of the tropical holothurian *Actinopyga echinites*, Conand (1982) noted that: "During (resting and immature) stages, sex cannot be determined microscopically. It is impossible or very difficult to distinguish resting gonads from immature gonads." The use of the wholly quantitative MSI proved useful to circumvent this problem.

An unexpected drawback of the present study was the uneven availability of sexually mature specimens (≥ 8.5 mm in disc diameter). During summer months and those with bursts in gametogenic activity (March and April 2009), it was easy to collect sufficient numbers of large individuals, and field collections from both rocks and rhodoliths were completed without difficulty. During post-spawning months and winter months, it was difficult to collect enough large sexually mature individuals to conclusively quantify gametogenesis at the finer scales (e.g. among subgroups coming from the same rock or rhodolith). The winter months were especially problematic in the rhodolith habitat, as upwards of 15 rhodoliths were collected and less than 15 individuals greater than 8.5 mm in disc diameter were found, which unexpectedly reduced our sampling design to 0-2 individual(s) per subgroup. This finding is similar to that of Hendler and Littman

(1986), who studied various substrata and found that calcareous algae had the densest population of ophiuroids, but had the smallest individual ophiuroids and more immature individuals than other habitats, acting as a refuge substratum (and not just as nursery habitat), likely reducing predation and environmental stress. Other studies have found that rhodolith substrata supporting ophiuroids tend to be skewed towards small individuals (McClintock et al. 1993) and are dominated by smaller specimens in fall and winter (Borges et al. 2009). It is likely that a similar effect was seen during this study, and that the rhodolith habitat is mostly supporting communities of small, sexually immature individuals. Countering this during the sampling process would be difficult, as many rhodoliths would need to be collected during winter months to obtain enough individuals for statistical analysis. Otherwise, it is difficult to compare individuals from rhodoliths to those from rocks. However, if the very nature of the rhodolith habitat is different from that of the rock habitat, it is difficult to accurately compare them statistically, since they have a different demographic of individuals present. Future work in this area should consider the effect of demographics and population distribution among both habitats. Part of this relationship could consider movement of individuals between habitats as they age, and whether juveniles only occupy refuge substratum temporarily, perhaps moving to different substrata over time (i.e. Hendler and Littman 1986).

Another issue that arose during the laboratory experimentation is the presence of small conspecific brittle stars living throughout the pipes in the facility in which this study was conducted. The pipes carrying seawater have extensive

fouling communities within them, with a variety of invertebrate and algal species settling in the header tanks through which water is distributed. Brittle stars living in the pipes are morphologically different from those collected in the field (discs small and squat, arms thin and underdeveloped), but analysis using dissecting scopes indicated that they are the same species. However, their presence in the pipes and header tanks which deliver water to the experimental individuals may have some effect on individuals that were meant to be isolated. Although it is just hypothetical at this stage, this potential issue would need to be further investigated. For the present, it was assumed that physical contact among congeners was essential for induction of homogeneous gametogenesis.

Although it is a shortcoming common to most investigations of the reproductive cycle, it is of note that this study was only conducted over a single year. There is value in following reproductive cycles over multiple years, particularly for species living in temperate and boreal climates where environmental cues (particularly temperature and phytoplankton) can vary greatly from one year to the next. Those few studies that were able to follow reproduction over multiple years have found significant annual differences. Byrne (1990) studied a commercial sea urchin off the coast of Ireland and found that differences in sea temperature led to changes in the periodicity of spawning from year to year. Because the photoperiod cycle did not change annually, she concluded that temperature was a major mediator of gamete release. In a study of the Antarctic ophiuroid *Ophionotus victoriae*, Grange et al. (2004) found inter-annual variation in reproductive effort, associated with nutritional condition. Even

Blake (1978) noted that the two spawning seasons he studied for *O. aculeata* were different in timing. However, the focus of the present study was to assess the influence of spatial distribution with only a secondary interest in identifying the annual spawning period and its mediator(s).

4.3 Future research

There were many important relationships touched upon in this study that could not be studied in their own right and that warrant further research to elucidate some of the mysteries associated with ophiuroid reproduction. One such concept would be the various spatial scales at which studies are conducted. For example, it is widely reported that large-scale spatial distribution often leads to differences in reproduction and spawning (i.e. Hendler 1979; Hendler 1991), so it would be interesting to conduct more reproductive studies at a variety of spatial scales to determine at which point synchrony becomes more/less apparent. One example of this is the relationship between site and MSI variation, particularly if sites could be standardized for environmental conditions. In the current study, individuals collected from Admiral's Cove in December 2008 had progressed less through the gametogenic cycle than conspecifics from St. Philip's at the same time. Most individuals from Admiral's Cove were totally spent, and had not begun a new gametogenic cycle, while those from St. Philip's (from both rock and rhodolith habitats) were all in growth stages. This asynchrony was especially interesting, considering that individuals from rocks and rhodoliths in St. Philip's were relatively synchronous in MSI at this point.

Another topic for future research should be further uncoupling of the roles of certain environmental factors. Inter-annual variation in the gametogenic cycle of *O. aculeata* (Hendler 1991; present study) makes it unlikely that photoperiod acted as the dominant control for gametogenesis or spawning for this species in this location. Studies focusing on photoperiod manipulation could further elucidate any relationship between photoperiod and gametogenesis (particularly the onset and early development, as this could be partially controlled by photoperiod). However, it was outside the scope of this particular study. It would also be fascinating to examine the role of the lunar cycle on the control of gametogenesis and spawning, as Mercier and Hamel (2010) noted that spawning in *O. aculeata* followed lunar periodicity. However, further study of this would require more frequent samplings than were possible for the present investigation.

One topic that was not considered at all in this study was the relationship between arm regeneration and fluxes in energy output to reproduction. It is often difficult to find large specimens of *O. aculeata* that do not have at least one arm in the process of regeneration (R. Hooper, personal communication). Pomory and Lawrence (1999) found that the ophiuroid *Ophiocoma echinata* stored less organic material in the form of gonad production when they were regenerating arms, and concluded that these individuals may have less energy available for reproduction. This relationship was not considered when selecting individuals for use in the current study, although care was taken not to use individuals missing more than two arms. However, most individuals collected were missing at least part of one arm, mostly the very tips. Future research could focus on the effect of

arm regeneration on reproductive activity in this particular species, as many individuals appear to be in the process of limb restoration.

Finally, an important factor that could not be fully studied and expanded upon in the current study was the male gametogenic cycle. Although histology was performed on all individuals, the time required to develop and test the various formulas of the MSI essentially prevented the development of an equivalent formulation for males. Several variables have been considered for measurement in male specimens, and a male version of the MSI should be developed in the near future.

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Appendix A - Male gametogenic stages in *Ophiopholis aculeata*

Stage 1 – Early Growth (Figure 5-1A): The testes are very small. There is a thin layer of spermatogonia visible along the germinal epithelium. The lumen is mostly empty.

Stage 2 – Growth (Figure 5-1B): Early in this stage, there are several layers of sparse and unevenly distributed spermatogonia along the germinal epithelium. Spermatocytes have formed a layer either along the germinal epithelium or overlaying the spermatogonia, and columns of them begin to grow into the lumen. Later in this stage, the layer of spermatocytes has become thicker, and the distinctive layers of developing spermatogonia, the columns of spermatocytes and the presence of spermatozoa become prominent. Spermatozoa are accumulating in the lumen but do not form a separate mass.

Male Stage 3 – Mature (Figure 5-1C): The testes are swollen. Spermatogonia and spermatocytes are barely visible along the germinal epithelium and the accumulating spermatozoa in the lumen reach maximum density; they are visually separate from the remaining precursor cells.

Male Stage 4 – Spent (Figure 5-1D): In the early part of this stage, some testes are only partially spawned, in which case spermatozoa in the lumen are still present but sparse (sometimes forming pockets separated by empty sections). In the later part of this stage, some spermatozoa and spermatogonia

may be present along the germinal layer, but most have been resorbed by the growing number of phagocytes.

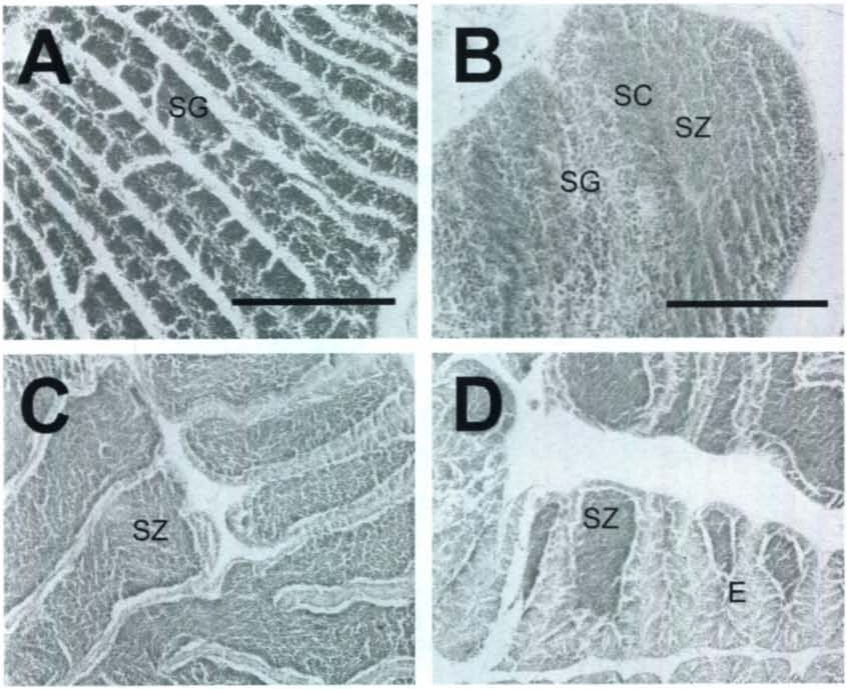

Figure 5-1. Gametogenic stages in male *Ophiopholis aculeata*, where A = early growth (with spermatogonia [SG] lining the germinal epithelium]), B = growth (with spermatogonia [SG] lining the epithelium, spermatocytes [SC] extending in columns into the lumen, and spermatozoa [SZ] accumulating in the lumen), C = mature (with separate mass of spermatozoa [SZ] formed in the lumen), and D= spent (with pockets of residual spermatozoa [SZ] and some emptied sections of lumen [E]). Scale bar in A represents 200 µm; scale bar in B (also applied to C and D) represents 400 µm.