Characterization of lecithotrophic propagules in echinoderms

by

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A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the degree of
Master of Science in Marine Biology

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September 2021

Abstract

Reproduction often involves use of propagules (e.g. spores, seeds and fruit, fertilized eggs, embryos, and larvae), which are frequently dispersed in a medium such as air or water. These propagules possess characteristics (e.g. size, shape, viscosity, biochemical composition) that may influence their viability. Lecithotrophic (maternally-provisioned) eggs of echinoderms display a diversity of colours and buoyancies worldwide, yet these characteristics are rarely investigated in depth. Much more is known on the biochemical composition of propagules in relation to the ecology of lecithotrophic echinoderms. Thus, the objectives of this thesis were to quantify and find trends in the externally observable biophysical properties (colour and buoyancy) and connect them to trends found in the internal biochemical composition (lipids and pigments) across 1) the oocytes of different species of echinoderms with lecithotrophic development (sea stars: *Leptasterias polaris*, *Henricia sanguinolenta*, *H. perforata*, *Crossaster papposus*, *Solaster endeca*, sea cucumbers: *Cucumaria frondosa* and *Psolus fabricii*) and 2) across stages of oogenesis and development in the sea cucumber *C. frondosa*. Using an analytical chemistry technique, thin-layer chromatography with ionization detection, chromaticity, and spectrophotometry, I found 1) a trend in most metrics (density, chromaticity colour coordinates, percentages of glyceryl ethers and triacylglycerols, and pigment concentration) of mature oocytes associated with the phylogeny of the seven focal species, and 2) a bell curve trend in all metrics associated with the eco-physiological constraints of each life stage through the ontogeny (early vitellogenic oocytes to early juveniles) of *C. frondosa*. An additional contribution of this study was the exploration and development of methods to assess the density and colour of the propagules of seven focal species by reappropriating techniques from the fields of analytical chemistry and colour science (chromaticity x,y coordinates). Future research should extend to other invertebrates or externally fertilizing species, and explore the driving effects of visual predators, ultraviolet radiation, and dispersal on propagule characteristics.

Acknowledgements

First of all, I would like to thank my supervisor Annie Mercier, and Jean-Francois Hamel, for the extensive time and effort that they have put into my project, and for giving me the opportunity to participate in my first scientific research cruise and to mentor undergraduate students. As well, I would like to thank my committee members, Rachel Sipler and Craig Purchase for their thoughtful feedback on our work as well as Chris Parrish for his life advice throughout my graduate school experience.

Thank you to our former post-doc Emy Montgomery for her guidance, friendship, and our inquisitive conversations about biophysics in the first year of my program. Thank you to Derya Akkaynak, for putting so much focused effort into customizing and troubleshooting the Matlab code for my colour quantification and for being so supportive and fun to work with. Thank you to Chris Parrish for the advice on lipid and pigment techniques as well as Jeanette Wells for patiently teaching me, for tolerating all of my questions, and for helping me double-check my results during my lipid analysis. Thank you to our former post-doc Guillaume Caulier for his protocol design for extracting the pigments. Thank you to my lab members for being there throughout and for our shared experiences in the Mercier Lab.

Thank you to the science team and crew of the *CCGS Amundsen* ISECOLD cruise 2019 for your positivity, ability to push through, and for being a part of my first research cruise experience.

Thank you to all the people who have been there throughout my masters that supported me either directly or indirectly, including broader OSC (Ocean Sciences Centre) community—we were always there for each other, whether to give feedback on presentations, or just to be there when others were having a hard time; you all always greeted me smiling and I am very grateful for that. Thank you, MacGregor Parent and Samantha Crowley for teaching me the value of friendship, small adventures, and to appreciate the little things. Thank you to Kate, my fitness instructor, and fitness class members for your

support in keeping up a healthy lifestyle in the last year before COVID-19. Thank you to all others who were there at times in the process who were not already mentioned.

Most of all, I would like to thank my mom, who has supported me both emotionally and financially at times throughout my journey into academia. I would not be here without her. Thank you for giving me the values of education and truth and for always encouraging me to pursue what was most meaningful to me.

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Lp= Leptasterias polaris
Hs= Henricia sanguinolenta
Hp= Henricia perforata
Cp= Crossaster papposus
Se= Solaster endeca
Cf= Cucumaria frondosa
Pf= Psolus fabricii

CIE=Commission Internationale de l'Eclairage (International Commission on Illumination)

HC=hydrocarbons
GE= glyceryl ethers
EKET=ethyl ketones
TAG= triacylglycerols
FFA=free fatty acids
ALC=alcohols
ST=sterols
AMPL=acetone mobile polar lipids
PL=phospholipids

PV=previtellogenic oocytes
EV= early vitellogenic oocytes combined
EV1=early vitellogenic 1 oocytes
EV2=early vitellogenic 2 oocytes
M=mid-vitellogenic oocytes
L=late vitellogenic oocytes
S=spawned
B=blastula
G=gastrula

V=vitellaria
P1=pentactula 1
P2= pentactula 2
P3= pentactula 3
J=juvenile

M1=Mother 1
M2=Mother 2
M3=Mother 3
M4=Mother 4
M5=Mother 5
M6=Mother 6
M7=Mother 7
M8=Mother 8
M9=Mother 9
M10=Mother 10
M11=Mother 11
M12=Mother 12
M13=Mother 13
M14=Mother 14
M15=Mother 15

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Chapter 1: General Introduction

Reproduction often involves propagules, which are commonly defined as any material used for the purpose of propagating an organism to the next stage in their life cycle via dispersal. They are either fertilized (2n; zygote or embryo) or are able to form a new individual without fertilization (e.g. 1n spores, plant cuttings, parthenogenic eggs). Propagules are widespread amongst biological kingdoms and can be found in many different forms, from the powder-like spores of mushrooms to the fertilized seeds of the berries of the crackerberry plant and the sporophytes of ferns, to the fertilized eggs of beetles, the embryos of marine polychaete worms, tadpoles of frogs, and the larvae of fishes. Because of their potential for dispersal, propagules have long been of interest in the fields of ecology (e.g. interspecific interactions such as symbioses through dispersal of fruits via animal ingestion, competition via recruitment of invasive mussels, and parasitism in the form of fungi on insects) and population genetics.

Invertebrates (including, for example, phyla Porifora, Cnidaria, Annelida, Arthropoda, Echinodermata, and Tunicata) have a diversity of modes of reproduction. Many benthic invertebrates rely on dual pelago-benthic ('complex') life cycles in which the propagules transiently occupy a different (pelagic) habitat in a manner that is considered to favour dispersal. Studying the characteristics of benthic marine invertebrate oocytes (unfertilized germ cells) and eggs (fertilized oocytes, zygotes), embryos (early stages of development), and larvae (intermediate stages between embryo and adult) can help us understand how they successfully employ these dual life cycles.

Characteristics of propagules influence their behaviour and overall response to environmental conditions. The main propagule characteristics include but are not limited to: size (Moran et al., 2009); external shape, morphology and ultrastructure (e.g. Mazzini et al., 1984; Kihm et al. 2020); physical properties (Thomas et al. 1999); composition such as proteins, lipids and carbohydrates (McAlister et al., 2012); chemical defenses such as antioxidants (Li et al., 2017), antimicrobials (Benkendorff et al., 2001) and toxins (Okabe et al., 2019); the ability to undergo diapause or survive under extreme external

conditions (Strader et al., 2016); appropriately timed appearance of feeding structures (McAlister et al., 2018); and hairs, spines to prevent predation (Morgan et al., 1989).

1.1 Historical Perspective on Marine Invertebrate Propagules

Descriptions of the stages of oogenesis of benthic marine invertebrates have largely focused on cellular attributes. The classification of the stages of embryogenesis began with Hippocrates, who provided the first written record of embryology followed by elaboration on the subject by Aristotle, Galen, Vesalius, and Harvey (Needham, 1932). From there on, a series of contributions were made including the first mention of the 'blastodermic vesicle' by Foster et al. (1883) and gastrulation by Heisler (1907).

Between 1972-1992, a handful of disparate scenarios had been proposed, but no generally accepted comprehensive account existed on the origin of larval forms (Strathmann, 1993). Then, in 1993, Strathmann introduced a framework for testing, refuting, and generating new hypotheses on the origin of larval forms, which focused on i) morphological traits, ii) larval capabilities, and iii) the metamorphosis between two pre-reproductive stages. Two years later, Havenhand (1995) discussed the evolutionary origins of larvae, the ecological importance of larval type, dispersal, and gene flow. In 2001, Williamson challenged a widely held assumption (influenced by Darwin's concept of "descent with modification') that larvae and corresponding adults have always evolved together by introducing a concept of 'larval transfer', in which genes specifying larval forms have been transferred between taxa at infrequent intervals during the course of animal evolution. A year later, Young et al. (2002) put together an atlas of marine invertebrate larvae, including reproductions of the earliest drawings of larvae and details of the development and morphology of larval types from each of the major taxa and some minor taxa. In 2006, Raff et al. provided a review on the evolution of larval forms of living echinoderms, detailing origins of larvae, living diversity, molecular clocks, and developmental-genetic approaches that can be taken to study echinoderm larval evolution. In 2007, Williamson et al. proposed that mismatches between the forms of adult animals and their larvae may reflect fused genomes, expressed in sequence in complex life

histories. More recently, Carrier et al. (2017) have provided a 20-year update to the original overview put together by McEdward (1995) on the reproductive, biological, and oceanographic aspects of larval ecology, as well as reviewed several newly-developed branches of larval ecology and evolution, including anthropogenic stressors and current technological advances.

1.2 Life History and Developmental Modes

The classification of life histories and developmental modes of marine invertebrates has been debated since the early 20th century. The first large-scale classification of modes of reproduction and larval types was proposed by Thorson (1950) who provided an overview of benthic marine taxa in Scandinavian seas, in which he covered spawning and fertilization, types of larval development, causes of mortality in larvae ('sources of waste'), and modes of reproduction. Mileikovsky (1971) pointed out that Thorson overlooked variability in larval types in certain molluscs and demersal taxa. Focusing on the evolutionary change in development of asteroids (sea stars), McEdward et al. (1993) stated that while strides had been made in larval forms and developmental patterns between 1978 and 1993, classification of developmental patterns had been ambiguous because patterns had not been defined as unique sets of ecological and developmental character states. They proposed a multi-factor classification system based on habitat (pelagic or benthic), mode of nutrition (feeding or non-feeding), and type of morphological development (indirect or direct). Levin et al. (1995) took an ecological perspective on larval development and created a classification scheme comprising 4 categories: 1) mode of larval nutrition, 2) location or site of development, 3) dispersal potential associated with a particular developmental type, and 4) the morphogenesis involved in development, of which could be mutually or not mutually exclusive. Poulin et al. (2001) took an ecological perspective on the developmental patterns of marine benthic invertebrates stating that previous classification schemes did not effectively discriminate between developmental patterns. They proposed a new multifactor classification that discriminated between eight developmental patterns and encompassed any potential ecological pattern of development in benthic marine taxa and described it as a model with 8 combinations of 2-state characters (pelagic or benthic, free or protected,

and feeding or non-feeding), which were shown conceptually as a cube shape with each combination on a separate corner (e.g. pelagic, free, feeding; Figure 1.1). Many of the later attempts at classification include multi-factor models, each varying in perspective on which and how many factors are involved, as well as how factors relate to one another. The repeated use of multi-factor approaches has demonstrated the challenges and complexity of classifying complex life histories and developmental mode of marine invertebrates.

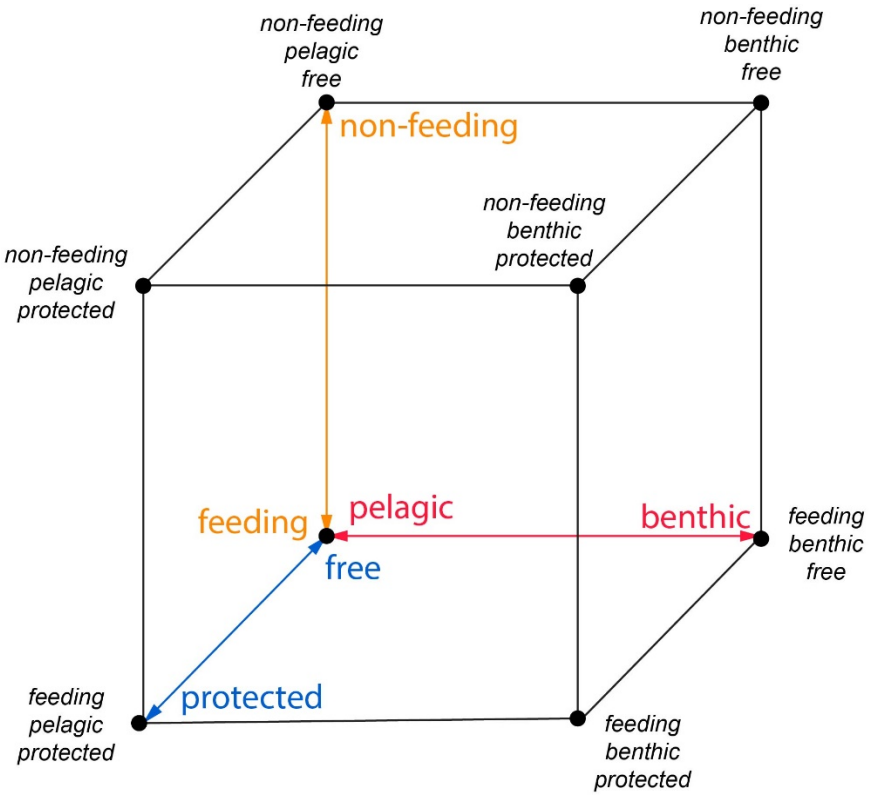

Figure 1.1 Multi-factor classification of developmental patterns in marine invertebrates. Adapted from Poulin et al. (2001).

1.3 Maternal Provisioning and Larval Nutritional Modes

Nutrition is supplied to the embryo by the mother and may either continue to sustain it until the juvenile stage or it may be depleted once the larval stage is reached. Maternally-derived nutrition can be obtained by 1) the transfer of nutrients directly from the mother during development (matrotrophy during

development within the mother's body), 2) lecithotrophy, which is supply of reserves (usually yolk) placed in the egg during oogenesis, or 3) adelphophagy, which is supplying eggs or siblings within the brood for a select portion of the brood to feed upon (Levin et al., 1995). Of these, lecithotrophy is most common (Levin et al., 1995). Lecithotrophic propagules are costly for the mother to produce because of the large energy and nutrient requirements for producing each egg, making production of only a comparatively small number of eggs possible on a fixed energy budget; however, they are at an advantage in that they do not dependent on the ocean's particulate food supply (Vance, 1973). An alternative nutritional mode is planktotrophy, whereby propagules switch to feeding on phytoplankton at the larval stage. This is the most common larval nutritional mode used by benthic marine taxa (Thorson, 1950). Planktotrophic larvae must feed because they have small amounts of yolk (Levin et al., 1995). Morphology of planktotrophic propagules allows a particular locomotory (Thorson, 1950) and feeding functionality, different in lecithotrophs (Montgomery et al., 2017b). It has also been shown that both lecithotrophs and planktotrophs are able to uptake dissolved organic matter from the water column (Manahan, 1990); however, the importance of this is unknown in most species (Havenhand, 1995). Because much of the literature in development has focused on planktotrophic species such as the sea urchin *Strongylocentrotus purpuratus*, there have been comparatively fewer studies on lecithotrophic propagules (Thorson, 1950; Byrne et al., 2019).

1.4 Developmental Site and Depth

The term 'site of development' (Levin et al., 1995) or 'developmental site' (Poulin et al., 2001) refers to the location of development relative to the mother and describes the tendency of benthic species to either brood or free-spawn their propagules. The spectrum between brooding and free spawning has also been described as being parental or aparental (Levin et al., 1995) in which benthic development may occur in direct association or independently of the parent in an attached or free mode (Levin et al., 1995). Parental association can occur by either internal brooding or external brooding, where embryos are either held and maintained on the inside or outside the female's body, respectively. For example, the sea star

Asterias groenlandica holds the developing embryos in pouches of the cardiac stomach (Lieberkind, 1920); similarly, two Australian species of the dwarf cushion star *Parvulastra* incubate their eggs within the gonads and juveniles are released via the gonopore (Byrne, 1996). In contrast, eggs are brooded outside the female's body, between the "ventral" side and the substrate in the sea cucumber *Cucumaria lubrica* (Engstrom, 1982); similarly, other species in the genera *Cucumaria*, *Psolus*, and *Thyonepslus* collect eggs with their tentacles and transport them to external wall pits or depressions (Hyman, 1955). Instead of remaining under constant care, development can occur in the absence of a parent, but with some level of parental care. The most common form of this is encapsulation, in which embryos are encased in gelatinous masses, capsules, or cocoons which are often attached to substrates, other plants or animals, or tubes and burrows (Levin et al., 1995). In contrast to brooding or encapsulation, many species free spawn (also referred to as broadcast spawning) leading to 'planktonic, or 'pelagic development' whereby oocytes are expelled into the water column as in the brittle star *Ophioderma brevispina* (Grave, 1916) and the crinoid *Metacrinus rotundus* (Nakano et al., 2003). A species' ability to disperse influences its access to resources and the genetics of its population. Species that brood, whether internally or externally, will receive more protection from the mother; however, they will risk offspring competing with the mother and siblings for resources and potential inbreeding. Species that free-spawn or broadcast, will travel further, which may allow them to exploit resources in new locations and incorporate more diversity into their genes (Todd et al., 1988); however, it is possible that they may travel to unsuitable habitats. Developmental site influences characteristics of propagules.

Threats to the survival of propagules can include mismatches between propagule physiological tolerances and environmental parameters (e.g. ultraviolet radiation, temperature, salinity, pH, oxygen levels), mechanical stresses such as strong currents, lack of resources such as food or proper substrate to settle upon, microbial infection (Dyrynda et al., 1995), and visual and non-visual predation (e.g. Järnegren et al., 2005). For example, there are various costs that may be associated with metamorphosing in response to specific chemical cues and postponing metamorphosis in the absence of those cues (Pechenik,

1999). Propagules that are brooded must remain at one depth for the entirety of their development. If they are brooded in shallow waters, more food sources are available for the mother, but there can be greater fluctuations in conditions than those on the deep sea. If propagules are brooded in the deep sea, they must be equipped to withstand or seek refuge from combinations of stressors unique to the deep sea, such as colder temperatures and crushing pressure. For example, the octopus *Graneledone boreopacifica* was found to have the longest known egg brooding period of any animal, which was proposed to be driven by the cold temperatures occurring in the deep sea (Robison et al., 2014). Embryos and larvae of sea urchin *Sterechinus neumayeri* were found to be able to tolerate higher pressures if the water is warmer (Tyler et al., 2000). Propagules that are free spawned and have pelagic development will experience more dynamic conditions than those that are brooded because they must ascend through a suite of conditions to reach the surface of the ocean. Propagules originating in the deep sea will encounter a greater range of depths (kilometers) than those originating from shallow waters (meters). Characteristics of propagules are likely tailored to address the threats in their respective environments.

1.5 Challenges of Working with Oocytes, Embryos, and Larvae

Certain taxa are difficult to isolate gametes from because modes of development need to be considered and there is no one protocol for all species (e.g. brittle stars; Ettensohn et al., 2004; Hodin et al., 2019). Chemical induced spawning of oocytes allows for oocytes to be gathered quickly; however, depending on the purpose of the study, the technique risks unnecessary or premature exposure to reproductive stimulants (e.g. 1-methyladenine in asteroids: Hart, 1995). Mechanical extraction is an alternative to this but can be tedious and may cause rupture of oocytes due to rough handling or too much time outside the mother's body. In studies of oocyte maturation and ovulation in tunicates, one of the greatest difficulties is the isolation and fractionation of follicles and oocytes at each developmental stage (Matsubara et al., 2019); however, a new technique to separate the various stages of oocytes has been recently developed using stainless steel sieves at varying particle sizes (Matsubara et al., 2019), which can potentially be applied to other organisms in the future. Unfortunately, after extraction and fertilization of

oocytes, many embryology labs studying echinoderms are not well-equipped to continue to rear post-embryonic stages (Hodin et al., 2019). It has been emphasized that when rearing echinoderm larvae, it is imperative that cultures are kept free of contamination, which is most often caused by molds, bacteria, and ciliate protozoans (Wray et al., 2004); otherwise, contaminated cultures will quickly "crash" or experience 100% mortality (Wray et al., 2004; preventative measures are detailed in Hodin et al., 2019). Although culturing embryos and larvae is a delicate process, it is well worth while and can help answer many important and interesting questions. Overall, technical difficulties may arise in studying propagules because of their delicate nature.

1.6 Methods for Studying Characteristics of Oocytes, Embryos, and Larvae

Egg characteristics are defined differently by field of biology and several techniques have been used to quantify them. Some of these include: egg size using an ocular micrometer (e.g. Lin et al., 2012) or calibrated ocular graticule on a compound microscope (Thomas et al., 1999); various metrics of size (length, volume, asymmetry, angle of curvature) measured off published images of insect eggs (Church et al., 2019); surface topography of fertilization membranes using light microscopy, and scanning and transmission electron microscopy in crinoids (Holland, 1977) and insects in the order Hemiptera (Rivas et al., 2016); viscosity of urchin eggs using a cone-and-plate viscometer (Thomas et al., 1999); colour of echinoderm eggs and lepidopteran larvae by obtaining red, green, and blue values off photos (Montgomery et al., 2017; Grant, 2007). Many biochemical analyses require large amounts of material (e.g. polychaete, Brachet et al., 1986), but efforts have been made to develop techniques for much smaller samples, such as those in Holland et al. (1971) which were used to analyze protein, carbohydrate, free reducing substances, total lipid, phospholipid, and RNA from only 5-10 mg of freeze dried oyster larvae. Some of these techniques are still used today (e.g. Whitehill et al., 2012). Pigments including those bound to proteins have been isolated by purification, homogenization, then extracted, separated by thin-layer chromatography, and identified via absorbance maximums in prawn eggs (e.g. Zagalsky et al., 1967). Developmental changes in morphology has been studied by scanning electron microscopy (e.g. McEuen

et al., 1985) and histology. Other properties have been measured on a cellular level: potential difference across cell membrane (Hiramoto, 1959), rheological properties during cell cleavage (Hiramoto, 1982), and tensions exerted by cleavage furrows (Rappaport, 1977). Although there are many, the techniques available for measuring characteristics of propagules may still limit which characteristics are possible to study.

1.7 Outline and Objectives of My Thesis

The above information raises the question 'Why does diversity in propagule characteristics exist in marine invertebrates?'. A striking diversity of colours and buoyancies has been reported anecdotally amongst oocytes in phylum Echinodermata (includes animals such as sea stars, sea cucumbers, urchins, and feather stars; summarized by Montgomery et al., 2017), particularly ones that develop into non-feeding larvae (termed 'lecithotrophic'). There have been many accounts of oocytes either sinking, remaining static in the water column, or floating, and appearing in an array of colours, including red, orange, yellow, green, brown, grey, or black (e.g. red and floating in the sea cucumber *Cucumaria frondosa*). Differing combinations have even been found within the same species (e.g. yellow and floating, as well as grey and sinking in the sea star *Henricia lisa*). While extensive observations exist, the colour and buoyancy of propagules is most often tangential to the study and is only described qualitatively. Because of the obvious external characteristics on the colour and buoyancy of lecithotrophic propagules and the lack in knowledge on the topic, echinoderms are an appropriate study organism to begin to answer the broader question 'Why does diversity in propagule characteristics exist in marine invertebrates?'.

In my thesis, I conducted a two-part exploratory study, which begins to establish associations between colour and buoyancy of lecithotrophic echinoderm propagules. In the present chapter (Chapter 1), I provide background information, as well as outline my scientific question and objectives. More knowledge exists on biochemical composition in relation to the ecology of lecithotrophic echinoderms (e.g. Hamel et al., 1996; Falkner et al., 2015; Prowse et al., 2017; Byrne et al., 2019). Thus, in my two

data chapters, I quantified and revealed patterns in the external biophysical properties (buoyancy and colour), then linked them to patterns found in the biochemical composition (lipids and pigments) across oocytes of different species of local lecithotrophic echinoderms: *Leptasterias polaris*, *Henricia sanguinolenta*, *Henricia perforata*, *Crossaster papposus, Solaster endeca*, *Cucumaria frondosa*, and *Psolus fabricii* (Chapter 2), and across stages of oogenesis and development of the locally abundant sea cucumber *C. frondosa* (Chapter 3). Another important contribution that came out of this study was in the methods, which I was able to reappropriate a set of techniques from other fields of science to measure characteristics of lecithotrophic propagules of echinoderms. In the final chapter (Chapter 4), I summarize the scientific question and objectives of my thesis, discuss the challenges of conducting the study (including re-appropriation of techniques), provide a summary of key findings, and provide suggestions for further research. As is often the nature of exploratory work, sampling of animals and propagules was highly opportunistic and so I have included diagrams in Appendix A that provide details for the reader to understand how the sampling influenced how the statistics were conducted and why particular ways of analyzing the data were chosen. Appendix B is a visual explanation of the concept used to measure oocyte density. A published article is included in Appendix C and includes work that was complementary to (I determined the sex of many *C. frondosa* during my thesis work and supplied photos of genital papillae for the paper) but is not an integral part of my thesis. I intend to publish Chapters 2 and 3, details of which are provided in the co-authorship statement. This work has explored new tools to investigate an exciting question in the field of reproductive ecology.

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Co-authorship Statement

The research described in this thesis was carried out by Janet M. Ferguson-Roberts, overseen by her supervisor, Annie Mercier, with input from Jean-Francois Hamel, and with guidance from her committee members, Craig Purchase and Rachel Sipler. This thesis is presented in thesis format, with no co-authors listed. Janet M. Ferguson-Roberts conceptualized the idea for the multi-metric aspect of this project and the interspecies comparison for Chapter 2 and Annie Mercier conceptualized the oogenesis and development idea for Chapter 3 (as a replacement for the initial idea of examining deep-sea oocytes). Janet M. Ferguson-Roberts was responsible for the data collection and analysis (with assistance from Annie Mercier and Jean-Francois Hamel) for Chapters 2 and 3. All chapters were written by Janet M. Ferguson-Roberts with extensive editing and writing by Annie Mercier and Jean-Francois Hamel. The grant for this research was provided to Annie Mercier.

Publication and submission status:

Chapter 2: (Ferguson-Roberts, Hamel, Mercier) in prep—Journal under consideration.

Chapter 3: (Ferguson-Roberts, Hamel, Mercier) in prep—Journal of Experimental Biology

Chapter 2: Interspecific variations in colour, buoyancy, and composition of oocytes in lecithotrophic echinoderms

2.1 Abstract

The propagules of benthic marine species face a number of challenges but possess characteristics that may aid in their survival. Globally, lecithotrophic echinoderms show a diversity in colour and buoyancy of oocytes, yet they have mostly been described qualitatively. The present study links biophysical attributes (buoyancy and colour) of oocytes to the composition (lipid and pigment) across different species of echinoderms with lecithotrophic development (sea stars: *Leptasterias polaris*, *Henricia sanguinolenta*, *H. perforata*, *Crossaster papposus*, *Solaster endeca*, and sea cucumbers: *Cucumaria frondosa* and *Psolus fabricii)*. This was done by analyzing four metrics in tandem: oocyte density, chromaticity colour coordinates, percent lipid class composition, and pigment concentration. Overall, I found a common trend across nearly all metrics that seems to be related to the phylogenetic relationships of the species (*L. polaris*, *H. sanguinolenta*, all other species) as well as hints at their roles in protecting propagules against visual predators and ultraviolet radiation. Unlike external colouration, pigment identity and concentration was not associated with phylogeny. Future research could make use of the techniques I adapted from other scientific fields for use on oocytes of lecithotrophic echinoderms to explore the capacity for colour and buoyancy to contribute to propagule survival and to broadening our knowledge to other invertebrates and externally fertilized species. Of particular interest is the role of propagule characteristics for evasion of visual predators, protections from ultraviolet radiation, and dispersal to appropriate habitats.

2.2 Introduction

The propagules (eggs, embryos, and larvae) of benthic marine species develop in various locations and conditions. In some species, propagules benefit from post-zygotic care (e.g. brooding), while in others, the eggs are released directly into the water column, where fertilization and development occur

(Poulin et al., 2001). Pelagic propagules can be carried by water currents towards favorable or unfavorable environments, and they may experience major changes in physico-chemical parameters, which can stress/damage them, or alter their development timing and survival. Propagules at various stages may also encounter predators (Iyengar et al., 2001; Lindquist, 2002; Mercier et al., 2013a) and face exposure to ultraviolet radiation near the surface (Cheesman et al., 1967; Lesser et al., 2003; Lamare et al., 2011). It is likely that the challenges faced by propagules during pelagic development are lessened by some of their attributes, which may help ensure survival until settlement (Mercier et al., 2013a). However, interspecific studies of physico-chemical metrics associated with oocytes remain limited.

The larval nutritional mode is a broad determinant of the physical and chemical characteristics of propagules, which are well exemplified in lecithotrophic propagules of echinoderms. The oocytes of echinoderm species with lecithotrophic development are typically larger (150–3400 µm diameter, Montgomery et al., 2017) than those of planktotrophs (~136 µm diameter) (Mercier et al., 2013b). The former are rich in yolk (a mix of protein, lipid, and carbohydrates) that supply the developing embryos and larvae with energy, circumventing the need for external nutrition (Levin et al., 1987). In addition, the yolk composition of lecithotrophic oocytes can lead to a range of buoyancies (e.g. negative, neutral, or positive), which may be influenced by the development site of the propagules (internally-brooded, externally-brooded, demersal free-living, or pelagic free-living; Poulin et al., 2001). The buoyancy of pelagic propagules further determines their positioning in the water column and their dispersal potential at various developmental phases (Petereit et al., 2014). The yolk of lecithotrophic oocytes may exhibit an array of rich colours ranging from brown, red, orange, and yellow to green, black or grey (Cheesman et al., 1967; McEuen, 1988; Montgomery et al., 2017), whereas planktotrophic oocytes are typically transparent or faintly coloured (Montgomery et al., 2017). Colour has been proposed to act as a means to confuse benthic and pelagic predators, as an indicator of toxicity (especially reddish pigmentation), or as protection against ultraviolet radiation at the ocean surface (Lamare et al., 2011) where broadcast lecithotrophic propagules are typically found.

Buoyancy of oocytes can be achieved in a number of ways. Because oocytes do not display autonomous locomotion, their buoyancy is fully determined by their morphology and chemical composition (Denton, 1974). For instance, the oocytes of the crinoid *Comanthus japonica* have ridges on the fertilization envelope whose main function has been suggested to reduce the sinking rate (Mortensen, 1920). Some studies have quantified buoyancy in lecithotrophic oocytes by calculating density (Young et al., 1987) and by measuring ascent velocity (Young et al., 1987; Hamel et al., 1993); others refer to buoyancy qualitatively (Montgomery et al., 2017). While there are several ways to quantify buoyancy of oocytes, some are more feasible or accurate than others. Overall, measuring ascent velocity as a proxy is just that—a proxy, which is appropriate for some studies where the intent is to obtain a general idea of the buoyancy for only propagules found within the properties of the seawater tested (pressure, temperature, density). Use of a density gradient column is the most accurate and direct way to measure propagule density but is also the most expensive option. Thus, if money is limited, sharing use of one or asking to use one from another discipline (e.g. chemical engineering) may be a way to gain access.

Proteins and lipids can make up most of the composition of echinoderm oocytes. The relative amounts of protein, lipids, and carbohydrates in the oocytes of the echinoid *Dendraster excentricus* are 63%, 31% and 6%, respectively (Turner et al., 1979). Propagules are primarily constituted of lipids in lecithotrophs, as opposed to proteins in planktotrophs (Villinski et al., 2002; Falkner et al., 2015). In light of recently proposed revisions to the lipid classes identified in studies of asteroids and echinoids (Villinski et al., 2002; Prowse et al., 2008), and ophiuroids (Falkner et al., 2006), lecithotrophic echinoderms are believed to provision their eggs primarily with a type of glyceryl ether (GE) called diacylglyceryl ether (DAGE) (Prowse et al., 2009), which is a type of glyceryl ether. Ross et al. (2013) examined how eurybathic echinoderm species adapt their life histories to variation in depth range (shallower than 20 m to deeper than 850 m). They used both classic reproductive metrics and lipid analysis to detect potential adaptations across three echinoderm species (*Cucumaria frondosa*, *Solaster endeca*, and *Henricia sanguinolenta*), as well as across the species' depth range. They found that *C.*

frondosa lacked oocytes at deeper depth, which suggested limited reproductive capabilities at the limit of their depth tolerance. *Solaster endeca* exhibited a higher gonad index at depth, suggesting greater investment into their oocytes without reducing the number produced (Ross et al. 2013). It is likely that GE were classified as wax esters (WE) in Ross et al. (2013), as proposed by Prowse et al. (2009). Regardless, WE are known to play an important role in zooplankton buoyancy control, similar to GE in echinoderms. Their ability to transition from solid to liquid phase, or inversely, is used across different marine taxa to change buoyancy during vertical migration (e.g. copepods: Pond et al., 2011; orange roughy: Phleger et al., 1990). A comparison of maternal provisioning in the oocytes of an ophiuroid (*Ophionereis schayeri*) supported this finding, and additionally found that the relative amounts of DAGE differed across species with lecithotrophic oocytes >250–300 µm diameter compared species with oocytes <250 µm (Falkner et al., 2015). Falkner et al. (2015) noted that WE and DAGE are more buoyant than triacylglycerol (TAG) due to a lower specific gravity and suggested that the smaller lecithotrophic oocytes of ophiuroids may be negatively-buoyant because of their small size and that differences in lipid contributions in oocytes may be due to differences in diet or basic physiology of the species.

Montgomery et al. (2017) recently conducted a global meta-analysis that examined the influence of development site, ocean basin, egg size, adult size, buoyancy, and taxonomic class on the colour of lecithotrophic oocytes across the phylum Echinodermata. They found that colour was not randomly distributed across development sites, ocean basins, and phylogenies, and that within species, colour was linked to the size of both the oocyte and the mother.

Most other research on pigments in oocytes has been conducted on crustaceans (Tessier et al., 1932; Stern et al., 1937; Goodwin, 1951; Zagalsky et al., 1967; Wallace et al., 1967), with more limited work on echinoderms (Young, 1958; Agatsuma et al., 2005) and terrestrial arthropods (Burgess et al., 1949). Work on crustaceans has focused on identifying the main pigment of eggs and describing their chemical properties. For example, carotenoids were identified as the source of the green colour in the eggs of water flea *Daphnia pulex* (Tessier et al., 1932). Then, ovoverdin, the water-soluble pigment

responsible for the green colour of the eggs of the lobster *Homarus americanus* was first named (Stern et al., 1937). From there, it was suggested that pigments in the eggs of a number of other crustaceans (the crab, *Cancer pagurus*; the scallop, *Pecten maximus*; and the shrimp, *Plesionika edwardsi*) were similar to ovoverdin and that astaxanthin, another pigment, was the sole carotenoid in *P. edwardsi* (Zagalsky et al., 1967). The principal pigmentary component in the ovaries of several decapod crustaceans (carotenoproteins) was characterized as lipovitellins, a name homologous to the major high-density lipoprotein found in animal eggs (Wallace et al., 1967). Eventually focus shifted toward metabolism of pigments; it was discovered that carotenoids were not metabolized in lobster embryos as had been found in a study conducted on locust embryos (Goodwin, 1951). Otherwise, study of the of pigments in invertebrates has been confined to developmental origin, as in the sea urchin *Lytechinus variegatus*, where echinochrome-forming cells begin to appear at the gastrula stage (Young, 1958). There has also been work done to determine the kelp pigments responsible for colouration of sea urchin gonads for application in aquaculture (e.g. Agatsuma et al, 2005). Thus, the work so far on pigment in marine invertebrate oocytes has focused largely on identifying and characterizing pigments, and to a lesser extent metabolism of pigments or examining when pigments appear in embryonic development.

The bulk of research on oocyte characteristics in echinoderms has been focused on size (diameter, volume) because of its importance in size-number trade-offs in fecundity (Emlet et al., 1987); however, few attempts have been made to quantify and explain other physical properties such as oocyte colour and buoyancy. The present study seeks to 1) find quantitative alternatives to the currently anecdotal, qualitative biophysical characteristics of colour and buoyancy across species of lecithotrophic echinoderms, and 2) tie them to shifts in chemical composition (lipids and pigments). Overall, this work proposes to tease out trends and develop a framework for the study of functional ecology through a broad, multi-metric, integrative approach, testing the assumption that colour and buoyancy vary across developmental locations in a way that mirrors known eco-physiological constraints of certain developmental modes.

2.3 Materials and Methods

2.3.1 Species Collection and Maintenance

Subtidal species of asteroids/sea stars (*Solaster endeca*, *Crossaster papposus*, *Leptasterias polaris*, *Henricia sanguinolenta,* and *H. perforata*) and holothuroids/sea cucumbers (*Cucumaria frondosa* and *Psolus fabricii*) exhibiting lecithotrophic development were used. All species were collected by divers through the Department of Ocean Sciences Field Services (Memorial University), off the eastern coast of the Avalon Peninsula, Newfoundland, Canada at depths of ~4.5–240 m between July 2017 and February 2018.

All individuals were held in 20-L tanks inside a flow-through system. Seawater parameters (e.g. temperature, pH, plankton) fluctuated naturally with local conditions (e.g. tides and seasons). Unfiltered seawater remained at temperatures of 1–3°C and was supplied at a rate of at 30 $L \cdot h^{-1}$. Tanks were located near large windows that provided natural lighting conditions to a maximum daily light intensity of ~300 lux over the study period (November 2017 to late April 2018). Sea cucumbers (*C. frondosa* and *P. fabricii*) fed upon natural phytoplankton present in the seawater (Hamel et al., 1998), *S. endeca* was fed sea cucumbers *C. frondosa* (So et al., 2010), *C. papposus* was fed sea urchins *Strongylocentrotus droebachiensis* (Gaymer et al., 2004), *L. polaris* was fed mussels *Mytilus edulis*; (Hamel et al., 1995; Gaymer et al., 2001), and both *H. perforata* and *H. sanguinolenta* were fed a variety of sponges (Sheild et al., 1993). *S. endeca*, *C. papposus*, and *L. polaris* were fed every week and *H. sanguinolenta* and *H. perforata* were supplied sponges to feed on *ad libitum*.

2.3.2 Spawning and Sampling of Oocytes

Oocytes were obtained during the natural reproductive seasons of all focal species (A2.1; Appendix A; Mercier et al., 2010): *L. polaris* and *C. papposus* were sampled in late January, *S. endeca* in late March, *H. sanguinolenta*, *H. perforata*, and *C. frondosa* in early April, and *P. fabricii* in mid-April (Figure 2.1; A2.1). For sea stars, mature oocytes were obtained by inducing final maturation in isolated

ovaries exposed to a solution of 1-methyladenine (1-MA) in seawater (0.6 µM) for 45 min. For sea cucumbers, mature oocytes were extracted surgically from the gonadal tubules by removing the gonad, and gently pushing the oocytes out of each tubule with a blunt probe. Oocyte sample collection corresponded to the parameters laid out in A2.1.

2.3.3 Density of Oocytes

The density of the oocytes was measured using a method reappropriated from analytical chemistry typically used in materials science (described in Kenkel, 2003). For this, the density of the oocytes was considered to be equal to the density of seawater in which oocytes reach hydrostatic equilibrium (i.e. neutral buoyancy; see Appendix B). Increasing densities of artificial seawater were mixed (Instant Ocean with distilled water; 1.000, 1.010, 1.015, 1.020, 1.025, 1.030, 1.040, 1.050, 1.060, 1.070, and 1.080 $g \cdot cm^{-3}$) and each placed in separate glass vials (11 mL) and maintained in a water bath (Boekel Scientific Inc.; heater: GD120L, chiller: IC-400) at the temperature at which spawning occurs (3°C; Mercier et al., 1996; Gianasi et al., 2018). Densities higher than 1.080 $g \cdot cm^{-3}$ could not be tested because of the potential risk of damage to oocytes via osmotic shock and the inability to keep the amount of salt dissolved at 3°C. Each vial was divided into 4 equal vertical strata by placing it in front of a visual guide during measurement.

Each measurement was performed using 20 oocytes (the number of replicates is provided in Table 2.1), which were released in the middle of each density-specific vial using a 1000-µl pipette (Eppendorf Research) with truncated tip then allowed 5 min to reach stasis. The density of the vial in which the oocytes reached hydrostatic equilibrium (i.e. evenly spread in suspension, not in contact with the surface of the water or bottom of the vial) was reported as the density of the oocytes. In cases where all oocytes sank in one density and floated in the next highest density, the intermediate density value was scored.

2.3.4 Colour of Oocytes

A single sample consisted of 30 oocytes for each species, which was photographed using an automated stereomicroscope (Leica M205 with DFC7000T camera) fitted with a ring light and diffuser dome (Leica LED5000 HDI) on a white background next to a small-scale colour standard (Pico ColorGauge, matte; Edmund Optics Inc., NJ, USA).

Colour values for each oocyte were obtained directly from digital images using a customized Matlab code (Akkaynak et al., 2014). To ensure that values remained camera-independent, XYZ values were used as opposed to RGB values (Choudhury, 2014). The area in the photograph occupied was averaged to yield one set of values per oocyte. XYZ values were then converted to 'x' and 'y' chromaticity coordinates as described by Mortimer et al. (2011), then plotted on the Commission Internationale de L'éclairage's (CIE) 1931 Chomaticity Diagram using the 'pavo' package in R. Reference values for the colour standard were obtained from the Image Science Associates (ISA) Products webpage in the Excel spreadsheet entitled 'Micro/Nano Target Colorimetry Data' (http://www.imagescienceassociates.com/mm5/merchant.mvc?Screen=PROD&Store_Code=ISA001&Product_Code=CGNT&Category_Code=TARGETS). The inter-mother variability in colour of oocytes was plotted on the 1931 CIE Chromaticity Diagram.

2.3.5 Total Lipids in Oocytes

Lipid analysis required at least 0.1 g of material and so 0.1-0.5 g of oocytes was collected, following the sampling in Table 2.1. Oocyte samples were then preserved in 100% chloroform under nitrogen gas and held at -80°C until use.

Total lipids were analyzed using a standard procedure (Parrish 1999). In brief, all total lipids (i.e. not only those containing both glycerol backbone and fatty acid chains, as opposed to only those with fatty acid chains) were extracted from oocytes samples; separated into their respective classes using thin-layer chromatography with flame ionization detection (TLC-FID); and scanned to obtain chromatograms

which were used to identify and quantify lipid classes by comparing their chromatograms to those of a prepared standard.

Total lipids were extracted using a 2:1 chloroform to methanol mixture. They were then separated by TLC using in a 3-step development process (Parrish, 1987): 1) most non-polar lipids, 2) somewhat non-polar lipids, and 3) most polar lipids. In the first step, the lipid mixture was spotted onto TLC Chromarods and focused twice using 100% acetone; then, the most non-polar lipid classes were separated by developing the lipid mixture twice in a hexane:diethyl ether:formic acid (98.95:1:0.05) mixture, the first development for 25 min and the second for 20 min. In the second step, the somewhat non-polar lipids were separated by developing the lipid mixture in a hexane:diethyl ether:formic acid (79.9:20:0.1) mixture for 40 min. The third step separated the most polar lipid classes by developing the Chromarods with the spotted lipid mixture twice in 100% acetone for 15 min each, then developing it twice in chloroform:methanol: chloroform-extracted water (5:4:1) for 10 min each.

Chromatograms were obtained by scanning TLC Chromarods using an Iatroscan MK6 analyzer (Iatron Laboratories, Tokyo, Japan) and then analyzing using PeakSimple Chromatography software (4.54, SRI Inc.). Total lipids were identified by comparing peaks with those of a prepared standard, which included 9 classes: hydrocarbons (HC), ethyl ketones (EKET), glyceryl ethers (GE), triacylglycerols (TAG), free fatty acids (FFA), alcohols (ALC), sterols (ST), acetone mobile polar lipids (AMPL), and phospholipids (PL). Percentages of each lipid class in a sample were calculated using chromatograms. The percentages of each lipid class within a sample were calculated as relative weight (g).

The difference between GE and TAG was confirmed by running select samples (that required confirmation) next to a standard for TAG only. The same three-step development system was used as above except in the second step, four times the amount of hexane:diethyl ether:formic acid was used and the lipid mixture was developed for 50 min instead of 40 min. If a peak ran before TAG, it was considered GE. If a peak ran with the saturated TAG standard, it was considered saturated TAG; if a peak ran after the saturated TAG standard, it was considered unsaturated TAG.

2.3.6 Pigments in Oocytes

Pigments in oocytes were identified by comparing the absorbance spectra of the pigments in the oocyte lipid extracts (same as used above in the lipid analysis) to those of known pigments in the literature (ketocarotenoids, astaxanthin; e.g. Mochimaru et al., 2008; Sandmann et al., 2006; Buckwald et al., 1968).

Absorbance spectra were obtained using a Cary 6000i UV-VIS spectrophotometer. The lipid extract was diluted to a 1 in 6 concentration using chloroform as a solvent and placed into a 3-ml quartz cuvette. Acetone is typically used as the solvent for pigments, though the effects of using chloroform in place of acetone can be considered negligible (Wellburn, 1973; confirmed by personal communication with C. Parrish). Spectra were scanned in both the ultraviolet (230-400 nm) and visible (400-700 nm), and infrared range (>700nm). Note that after lipid extraction, no colouration had been observed in the remaining oocyte material.

Pigment concentration of each oocyte sample was calculated using the following formula from Abouzaytoun (2017) and expressed in $\mu g \cdot g^{-1}$, adjusted from Takeungwongtrakul et al. (2012):

$$\text{Concentration of pigment in lipid} = \left(\frac{A_{468}}{0.20}\right)\left(\frac{\text{volume of extract * dilution factor}}{\text{weight of lipid sample used in g}}\right)$$

where (A_{468}/0.20) is the slope of a β-carotene calibration curve, the volume of the lipid extract used was 1.5 ml, the dilution factor was 6; and the weight of the sample used in grams was measured in g of lipid/g of oocytes. The β-carotene calibration curve used in this study was obtained from the literature (Kaur et al., 2012), in order to provide a reference point for intercomparison among species. Percent reflectance spectra were obtained using the same spectrophotometer.

2.3.7 Data Analysis

To detect differences in mean densities, percentage of lipid classes, and concentrations of pigment across species (*Lp, Hs, Hp, Cp, Se, Cf,* and *Pf*), a one-way ANOVA with Tukey post hoc test was performed. If the Normality test failed, a Kruskal-Wallis ANOVA on ranks with Dunn's post-hoc test was performed. If the results of the Dunn's test reported as 'Do Not Test', they were reported as non-significant and the data in the figure was described. Densities of >1.080 $g \cdot cm^{-3}$ were included as 1.080 $g \cdot cm^{-3}$. Only lipid classes important to buoyancy (i.e. GE and TAG) were tested.

Differences in colour among species were interpreted directly off the Figures (with no statistics conducted) based upon the colour region they occupied on the CIE 1931 Chromaticity Diagram and their position relative to each other. Results are described with the centre of the Chromaticity Diagram referred to as 'white' (chromaticity coordinates x and y: 0.33, 0.33) and colours near the edge of the perimeter of the colour space as pure spectral colours (e.g. pure spectral red; Choudhury, 2014). For inter-mother variability, the difference between mothers versus within mothers was compared and described as trends.

Percent reflectance was compared to the UV reflectance of β-carotene (a carotenoid known to display photoprotection) and the most harmful UV rays (UVA), as well as to the peak ultraviolet and visible wavelengths of the retinal spectral sensitivity of potential predators in the literature.

2.4 Results

2.4.1 Differences Across Oocytes of Different Species

A Kruskal-Wallis ANOVA on ranks was conducted and showed a statistically significant difference in mean density values among species (H_6=17.28, $p<0.05$); however, the Dunn's post-hoc test reported as 'Do Not Test' indicating that no pair comparisons were significantly different. Apart from that, the data in Figure 2.2 show that the oocytes of the external brooder *L. polaris* had the highest density with values >1.080 $g \cdot cm^{-3}$, while the oocytes of free spawning species had the least dense values and

hovered around 1.025 g·cm^{-3}. Contrary to expectation, the oocytes of the external brooder *H. sanguinolenta* were much less dense than those of congener *L. polaris*, resembling more closely the density values of free-spawned oocytes at 1.035 g·cm^{-3} (Figure 2.2).

The oocytes of the external brooder *L. polaris* had colour coordinates closer to white at 0.38, 0.41 (Figure 2.3), while the oocytes of free spawners were characterized by colour coordinates closer to pure spectral orange: (0.48, 0.37) for *H. perforata*; (0.55, 0.36) for *C. papposus*; (0.55, 0.36) for *S. endeca*, (0.49, 0.37) for *C. frondosa*; and (0.50, 0.38) for *P. fabricii* (Figure 2.3). Again, unexpectedly, the oocytes of external brooder, *H. sanguinolenta*, were much closer to pure spectral orange at (0.52, 0.39) than those of the other external brooder *L. polaris* and, resembled more closely the colour coordinates of free-spawned oocytes. Regarding inter-mother variability of oocyte colour, all species had greater variability within than between mothers except for the oocytes of *H. sanguinolenta*.

A one-way ANOVA was conducted and showed that there was a statistically significant difference in GE across species ($F_{2,9}$=87.38; $p<0.05$). A Tukey post-hoc test showed that the oocytes of the external brooder *L. polaris* had a statistically significantly low percentage of GE at 15% compared to that of all other species at ~58–77% ($p<0.05$; Figure 2.4, A2.2). An ANOVA on Ranks was conducted and showed that there was a significant difference in TAG across species (H_6=14.05; $p <0.05$); however, Dunn's reported as 'Do Not Test' and so the difference in TAG between pairs of species is not statistically significant. Otherwise, the data in Figure 2.4 showed that percentages of GE and TAG in the oocytes of *H. sanguinolenta* (GE 74%, no TAG) resembled the free spawners (GE 58-77%, no TAG-14%) more closely than those of the other external brooder *L. polaris* (GE 15%, TAG 36%).

Pigment analysis in lipid extracts showed that among the seven focal species, there were three types of absorption spectra, which did not follow the trend of three groupings of species: *L. polaris*, *H. sanguinolenta*, and all other focal species (Figure 2.5A). The absorption spectra of the oocytes of the external brooders *H. sanguinolenta* and *L. polaris*, as well as of free-spawner *H. perforata* was characterized by a 2-peak curve (major peak at ~480 nm and a minor peak at ~430 nm). The absorption

spectra of the oocytes of two of the free-spawners *C. papposus* and *P. fabricii* were characterized by a smooth curve with a single peak at the absorbance maximum (486 nm); however, *P. fabricii* showed a symmetric curve, whereas the curve in *C. papposus* was flattened in the lower wavelengths (from ~465 nm to 486 nm). The absorption spectra of the oocytes of free-spawners *C. frondosa* and *S. endeca* were characterized by a 3-peak curve (major peak at ~470 nm with minor peaks around 440 nm and 490 nm). The absorbance spectra of lipid extracts in the oocytes of the seven focal species were compared to those of known pigments in the literature and determined to match dark forms of a photoactive orange-carotenoid-protein (yellow/orange/red; Wenderoth et al., 1999, Tian et al. 2008), astaxanthin (red; Buckwald et al. 1968), and keto-carotenoids (a class of yellow/orange/red pigments; Sandmann et al., 2006, Solovchenko et al. 2008, Mochimaru et al., 2008), respectively. All pigments also reflected in the ultraviolet region of the electromagnetic spectrum (250-440 nm; Figure 2.5B).

The pigment concentration of the lipid extracts from the oocytes were statistically different across species ($F_{2,9}$=58.49, p<0.05). A Tukey post-hoc test showed that oocytes of the external brooder *L. polaris* had a significantly low pigment concentration (5–16 $\mu g \cdot g^{-1}$) compared to *H. perforata* (205 $\mu g \cdot g^{-1}$), *C. papposus* (175 $\mu g \cdot g^{-1}$), *C. frondosa* (237 $\mu g \cdot g^{-1}$), and *P. fabricii* (215 $\mu g \cdot g^{-1}$; all p<0.05), but not for *H. sanguinolenta* (140 $\mu g \cdot g^{-1}$, p=0.06) and *S. endeca* (137 $\mu g \cdot g^{-1}$; p=0.08; Figure 2.6). The data showed that *L. polaris* had the lowest pigment concentration out of all species. *H. sanguinolenta* still occupied a concentration level between that of *L. polaris* and the remaining species; however, oocytes of *S. endeca* had a similar pigment concentration to *H. sanguinolenta*, thus *H. sanguinolenta* does not appear to be a transitional between the oocytes of *L. polaris* and free-spawning species (Figure 2.6).

2.5 Discussion

This study provides important baseline information on the reasons for the drivers of colour and buoyancy of lecithotrophic oocytes from Phylum Echinodermata. The main trend amongst all metrics (oocyte density, chromaticity colour coordinates, percent lipid class composition, and pigment

concentration across species) showed that the species grouped as follows: *L. polaris*, *H. sanguinolenta*, and all remaining species (*H. perforata*, *C. papposus*, *S. endeca*, *C. frondosa*, and *P. fabricii*). Pigments in oocytes were tentatively identified as photoactive orange-carotenoid-protein (*L. polaris*, *H. sanguinolenta*, and *H. perforata*), astaxanthin (*C. papposus* and *P. fabricii)*, and keto-carotenoids (*C. frondosa* and *S. endeca*). All pigment in lipid extracts reflected in the ultraviolet portion of electromagnetic spectrum and had maxima in the red/orange/yellow portions of the visible portion of the electromagnetic spectrum.

2.5.1 Phylogenetic Relationships

The main trend found in the present study—that *L. polaris* consistently stood apart from all other species—is supported by the current knowledge on the phylogenetic relationships between the seven focal species. A number of studies have placed species of order Forcipulatida (which includes *L. polaris*) at one end of the phylogenetic tree, with superorder Valvatacea (which includes *C. papposus* and *S. endeca*) next, then order Dendrochirotida (which includes *C. frondosa* and *P. fabricii*) at the opposite end (Perseke et al., 2010; Schlegel et al., 2014). A recent study, in addition to supporting these findings, placed *Henricia* spp. between Forcipulatida and Valvatacea (Reich et al., 2015).

The developmental location (i.e. brooding versus free-spawning) could perhaps provide another explanation for the uniqueness of the oocytes of external brooder *L. polaris* compared with those of the free spawners (*H. perforata*, *C. papposus*, *S. endeca*, *C. frondosa*, and *P. fabricii*). However, the oocytes of the other externally brooding species (*H. sanguinolenta*) were not set apart from those of the free spawners, making developmental site an unlikely cause on its own. Both *L. polaris* and *H. sanguinolenta* are external brooders and it has been noted that sea stars in the orders Forcipulatida (which includes *L. polaris*) and Spinulosida (which includes *Henricia* spp.) have similar methods of positioning their egg masses during brooding in that they both arch upwards to create a bell-shaped brooding chamber using their arms (Gillespie et al., 2007). However, the characteristics of their oocytes are very different in that the oocytes of *L. polaris* are negatively buoyant and adhere to the substrate, while those of *H.*

sanguinolenta are positively buoyant and are held off the substrate, making them prone to slip out of the brood into the water column (Mortensen, 1927). In the present study, density, colour, GE/TAG, and pigment concentration findings confirmed this difference. *L. polaris* had the densest oocytes with colour closest to white, higher TAG levels than GE, and the lowest pigment concentration out of all species, whereas *H. sanguinolenta* had moderately dense oocytes, with moderate colour, more GE than TAG—but less so than the free spawners, and moderate pigment concentration. Thus, developmental site is not a plausible explanation because the characteristics of the oocytes of one of the external brooders is similar to the oocytes of the free spawners.

The two species in the genus *Henricia* differed in characteristics within the genus. *H. sanguinolenta* has externally brooded, positively buoyant, orange-coloured oocytes, while *H. perforata* has free-spawned, positively buoyant, maroon-coloured oocytes. Individuals of the genus *Henricia* are known for their extensive morphological variability (including at the oocyte level), which has made identification problematic (Knott et al., 2018; Madsen, 1987; Grainger, 1966; Eernisse et al., 2010). Variability in characteristics of oocytes is found in the deep-sea species of sea star *H. lisa*, which both broods negatively buoyant, grey-coloured oocytes and broadcasts positively buoyant, yellow-coloured oocytes from the same clutch (Mercier et al., 2008). The sea star *Pteraster militaris*, while phylogenetically unrelated to *Henricia* sp. (both are in class Asteroidea; however, *P. militaris* belongs to order Velatida, where *Henricia* sp. belongs to Spinulosida), also free-spawns its oocytes in addition to brooding them within aboral membranes (McClary et al., 1988). In both *H. sanguinolenta* and *P. militaris*, positively buoyant oocytes are being brooded and could accidentally escape. It is possible that the tendency of the oocytes of *H. sanguinolenta* to drift up and away from the brood chamber could act as an alternative strategy to ensure that genes are passed on in spite of threats (e.g. predation, deteriorating physical condition). Meanwhile, females of *H. perforata* free spawn and their oocytes are positively buoyant. Given the employment of various strategies and the extent of the diversity of oocyte characteristics within the genus, it is not surprising that positively buoyant oocytes of the external

brooding genus *Henrica* could represent an evolutionary transitional stage between negatively buoyant external brooding in *L. polaris* and positively buoyant free spawning in the five other focal species.

2.5.2 Trophodynamics May Affect Oocyte Pigment

The identities of the pigments found in the oocytes of the 7 focal species may not be linked by phylogeny, but by trophodynamics. *L. polaris*, *H. sanguinolenta*, and *H. perforata* shared the same pigment (tentatively identified as photosensitive orange carotenoprotein), *C. papposus* and *P. fabricii* shared the same pigment (astaxanthin), and *C. frondosa* and *S. endeca* shared the same pigment (ketocarotenoids). A cursory comparison may conclude that diet was not a factor because *L. polaris*, *H. sanguinolenta*, *H. perforata*, *C. papposus* and *S. endeca* are predators, and both *C. frondosa* and *P. fabricii* feed on phytoplankton. However, *L. polaris*, *H. sanguinolenta*, and *H. perforata* all feed on filter feeders, which consume a plankton size class that can include algae, bacteria and organic detritus. As well, *S. endeca* preys upon *C. frondosa*, and thus could be sequestering ketocarotenoids from its food. Also, *C. papposus* preys upon the echinoid *S. droebachiensis*, which feeds on algae, which is a similar diet to feeding in the plankton—as *P. fabricii* does. If all of the species sequester precursors for pigments as opposed to producing pigments *de novo*, then their specific diet—and even the diet of their prey—could be a likely explanation (e.g. de Carvalho et al., 2017; Pessier, 2014).

2.5.3 Effectiveness of Crypsis Throughout Depths

Colour can be measured from two different perspectives. In the present study, XYZ values were measured, converted to CIE xy coordinates, then plotted on the 1931 Chromaticity Diagram; however, these are based upon the range of vision of the human eye. In contrast, many animals are able to detect wavelengths beyond the 'visible' region of the electromagnetic spectrum (i.e. in the ultraviolet and infrared), which is important to consider when forming an ecological interpretation of results. In order to

determine if an organism (e.g. cuttlefish and fish: Chiao et al., 2011) fits within the range of vision of an animal, colour must be measured in units of percent reflectance (Johnsen, 2016).

Since we could not determine reflectance of the propagules themselves, we obtained reflectance of pigment in lipid extracts of propagules using spectrophotometry, which has been used to measure colour in units of percent reflectance (e.g. in human bruises; Hughes et al., 2003). Because of their dramatic change in buoyancy, fertilized oocytes and subsequent developmental stages of *C. frondosa* are exposed to the environment and a suite of benthic (Mercier et al., 2013a) and pelagic predators, some of which may rely on vision. The vivid red colour of echinoderm oocytes may act as camouflage under the blue light of the pelagic zone (Montgomery et al., 2017); however, the colour of propagules can also make them more conspicuous, depending the viewer (Johnsen, 2012).

Here, the percent reflectance of all propagules peaked in the ultraviolet (~350 nm) and in the yellow, orange, and red regions (>550 nm) of the electromagnetic spectrum. While specific information about the predators of the oocytes of the focal species is scarce, larval fish (e.g. herring) are known to consume crustacean larvae and to detect prey in the 600 nm range (red; Blaxter, 1968). Thus, they may be able to detect red propagules of *S. endeca*, *C. papposus*, *C. frondosa,* or *P. fabricii*. On the other hand, the red colour of these propagules may make them cryptic to crustacean predators such as midwater shrimp (e.g. Lindsay et al., 1999). As well, the visual sensitivity of most birds is in the red and ultraviolet regions (Shrestha et al., 2013); thus, sea birds may detect and ingest the red embryos of these species at the ocean surface.

2.5.4 Potential for Photoprotection Using Reflectance

There are three mechanisms by which photoprotection can be achieved in animals: 1) reflectance, 2) absorption with subsequent re-emittance at a longer wavelength (e.g. coral fluorescence), and 3) antioxidants (eliminate harmful free radicals). Certain pigments in adult echinoderms are known to be photoprotective due to their antioxidant properties (Lamare et al., 2004; Mamelona et al., 2007; Lamare et

al., 2011). In the present study, results showed that pigment in lipid extracts of oocytes from all species reflected in the ultraviolet region of the electromagnetic spectrum. This suggests that reflectance could be a potential mechanism of photoprotection.

2.6 Conclusion

This exploratory study has set the baseline for answering the broad question 'Why does diversity in the propagule characteristics of marine invertebrates exist?'. It has established that diversity of colour and buoyancy in oocytes of seven species of echinoderms were based on phylogeny, not developmental site, as well as identified possible visual predators and the potential for photoprotection. In addition to this, it has contributed a suite of reappropriated techniques for research on lecithotrophic oocytes. Future studies should be conducted to determine which aspects of species contribute to diversity in characteristics as well as their functional role in situations of predation, photoprotection, and dispersal.

2.7 Acknowledgments

We would like to thank Chris Parrish and Jeanette Wells for their help and guidance with the lipid analyses, as well as Derya Akkaynak for developing the custom MatLab algorithms for the colour analyses. Thank you to Guillaume Caulier for help with the pigment quantification and Emy Montgomery for laying the groundwork for this study and for enlightening discussions. Warmest thanks to the Ocean Sciences Field Services Unit for collection of the echinoderms for this study. This research was supported by the National Sciences and Engineering Research Council of Canada (grant to A.M.).

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2.9 Tables and Figures

Table 2.1 Description of samples used for the biophysical metrics (density, colour) and the biochemical analyses (lipids, pigments). 'Pooled' refers to samples where oocytes from all mothers were combined and processed as one sample. 'Averaged' values are where one value was obtained for each mother and the mean of these values is reported. 'Unknown' number of mothers is used in cases where the oocytes were spawned. *Lp=L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp=C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii*; — = non-applicable. For further information on the sampling, please see Appendix A.

Metric	Species	Number of Sample Replicates	Number of Mothers	Pooled or Not Pooled	Averaging
biophysical	*Lp*	4	4	—	—
biophysical	*Hs*	4	4	—	—
biophysical	*Hp*	1	1	—	—
biophysical	*Cp*	1	unknown	—	—
biophysical	*Se*	3	3	—	—
biophysical	*Cf*	5	5	—	—
biophysical	*Pf*	5	5	—	—
biochemical	*Lp*	4	4	—	all individuals averaged together for avg *Lp*
biochemical	*Hs*	1	3	orange oocytes from 3 mothers pooled	sample replicates for orange oocytes
biochemical	*Hp*	1	1	—	—
biochemical	*Cp*	1	unknown	—	—
biochemical	*Se*	3	3	—	all individuals averaged together for avg *Se*
biochemical	*Cf*	5	5	—	all individuals averaged together for avg *Cf*
biochemical	*Pf*	1	5	pooled	none

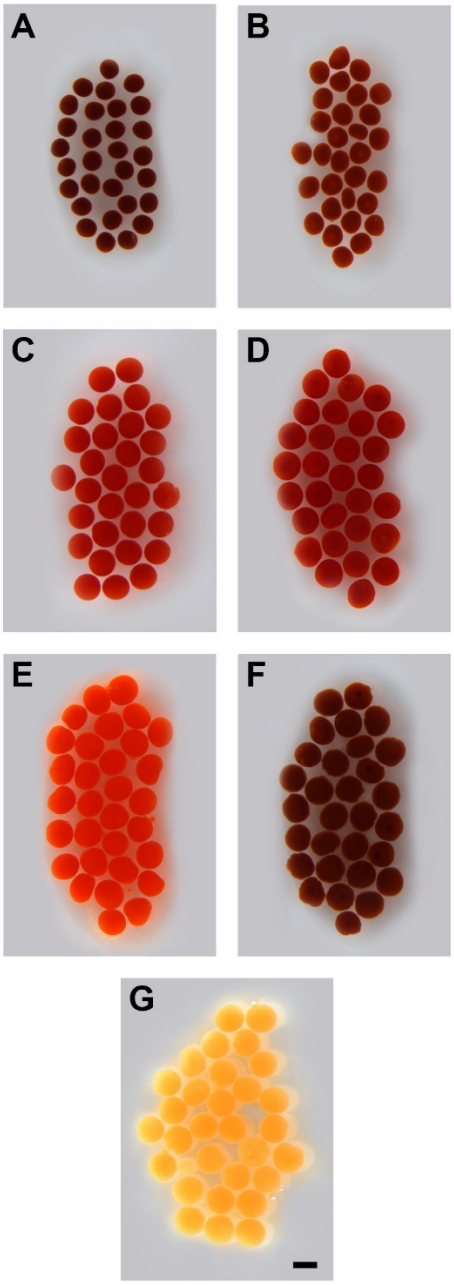


Figure 2.1 Oocytes from each of the seven focal species. A: *C. frondosa*, **B:** *P. fabricii*, **C:** *C. papposus*, **D:** *S. endeca*, **E:** *H. sanguinolenta* (dominantly coloured oocytes*)*, **F:** *H. perforata*, and **G:** *L. polaris*. Scale bar in panel G corresponds to 1000 µm and applies to all panels.

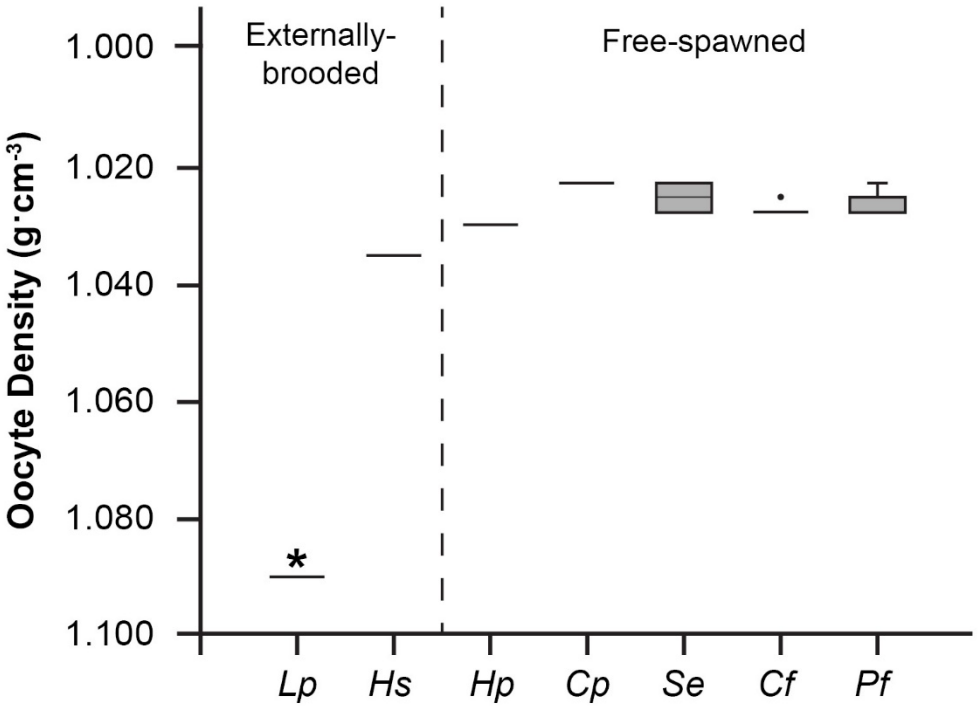


Figure 2.2 Comparison of oocyte density across the 7 focal species. The vertical dashed line separates species by development site. Note the reverse scale. Means that were underestimated (i.e. include density values above the tested range of 1.080 g·cm^{-3}) are indicated by an asterisk (*). *Lp=L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp=C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii.* The grey bars indicate those samples which fell between the 2nd and 3rd quartiles, the line within the boxes represents the median. Lines without boxes indicate those samples where there was no variability amongst mothers. Whiskers show the minimum and maximum values (5th and 95th percentile). The black dot indicates an outlier.

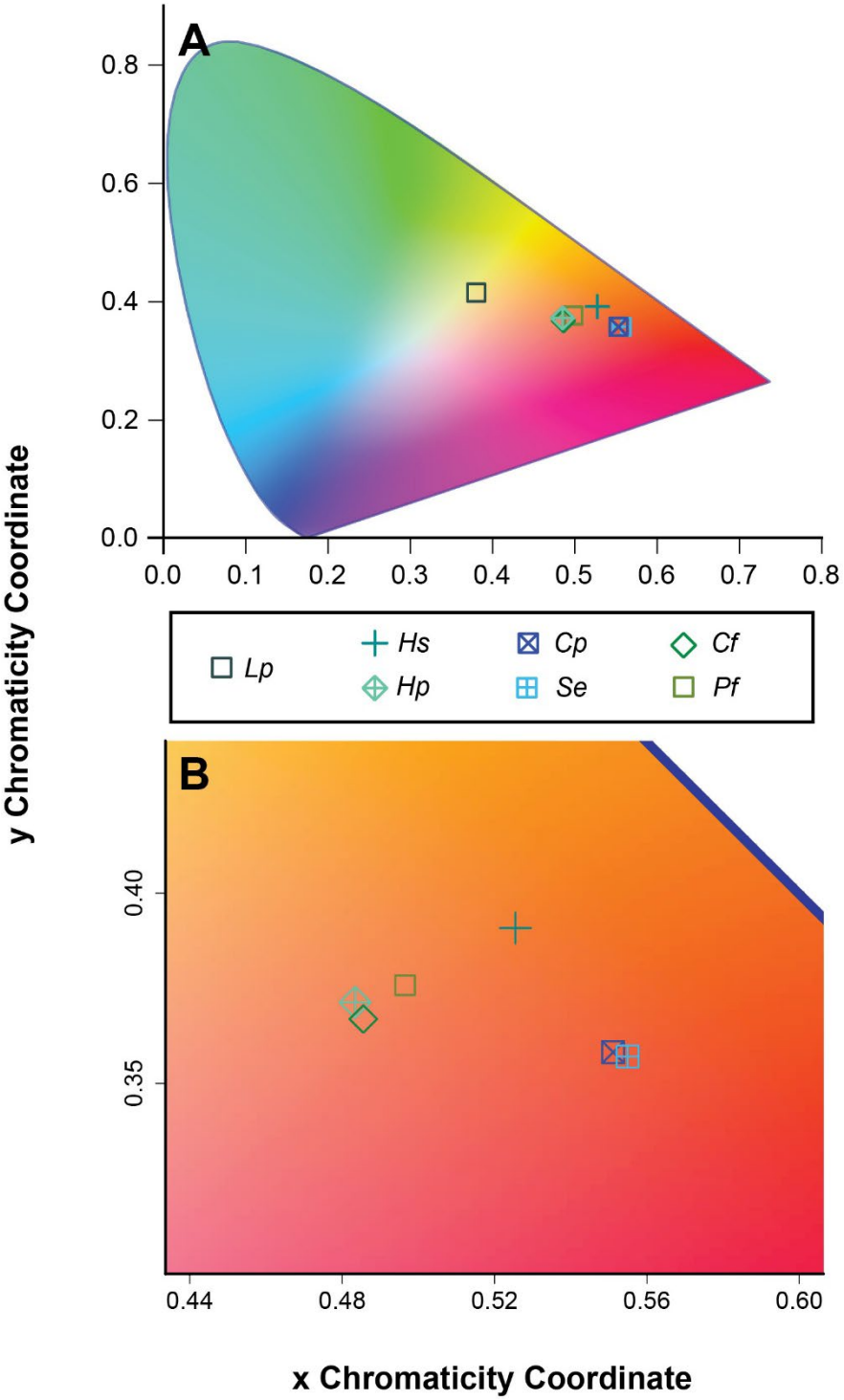


Figure 2.3 Comparison of oocyte colour across species. A: Species **B:** Zoom-in. Shown as xy values plotted onto CIE's 1931 Chromaticity Diagram. *Lp=L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp=C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii.*

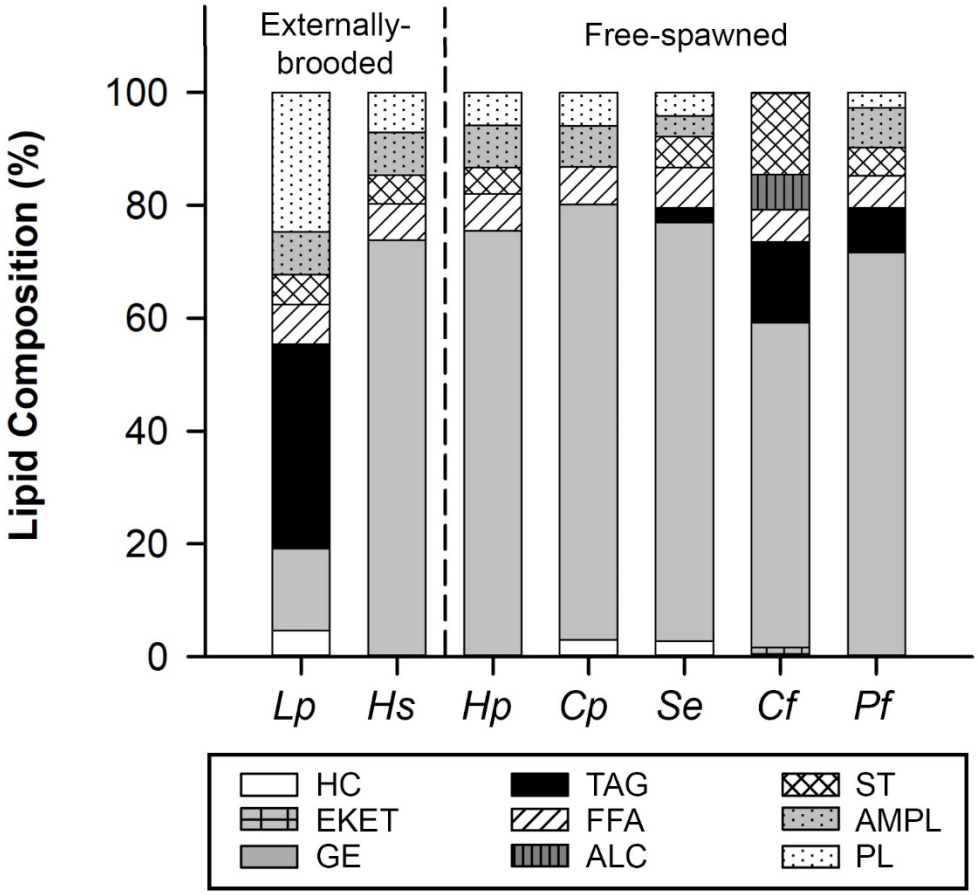


Figure 2.4 Comparison of lipid composition across the 7 focal species. The vertical dashed line separates species by development site. *Lp=L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp=C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii*; HC= hydrocarbons, EKET= ethyl ketones, GE=glyceryl ethers, TAG=triacylglycerols, FFA=free fatty acids, ALC=alcohols, ST=sterols, AMPL=acetone mobile polar lipids, and PL= phospholipids.

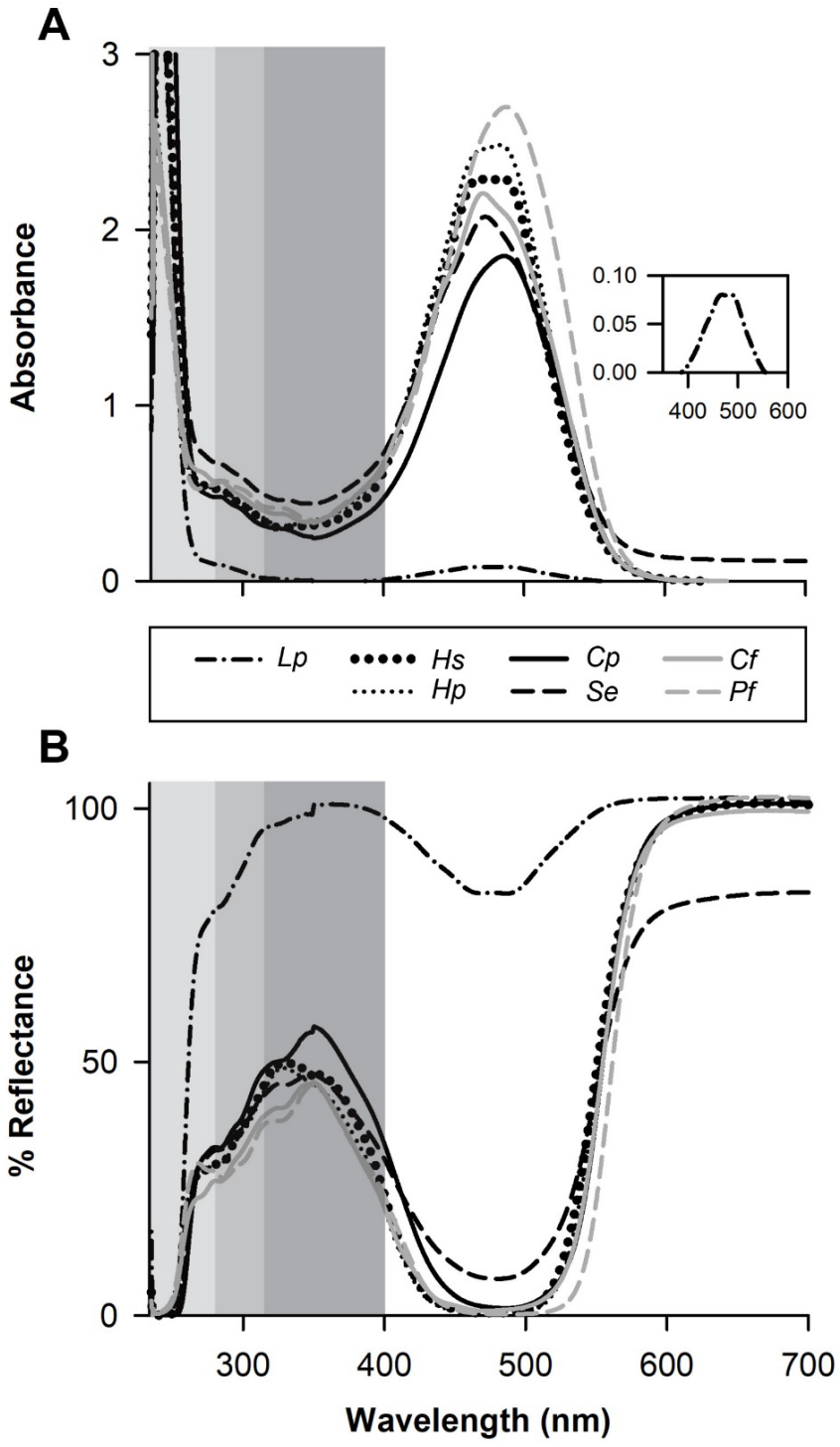


Figure 2.5 Comparison of spectrophotometric metrics between species. A: Absorbance. **B:** Reflectance. Grey shading indicates the UV portion of the electromagnetic spectrum: dark grey=UVA, medium grey=UVB, light grey=UVC. *Lp= L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp= C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii*.

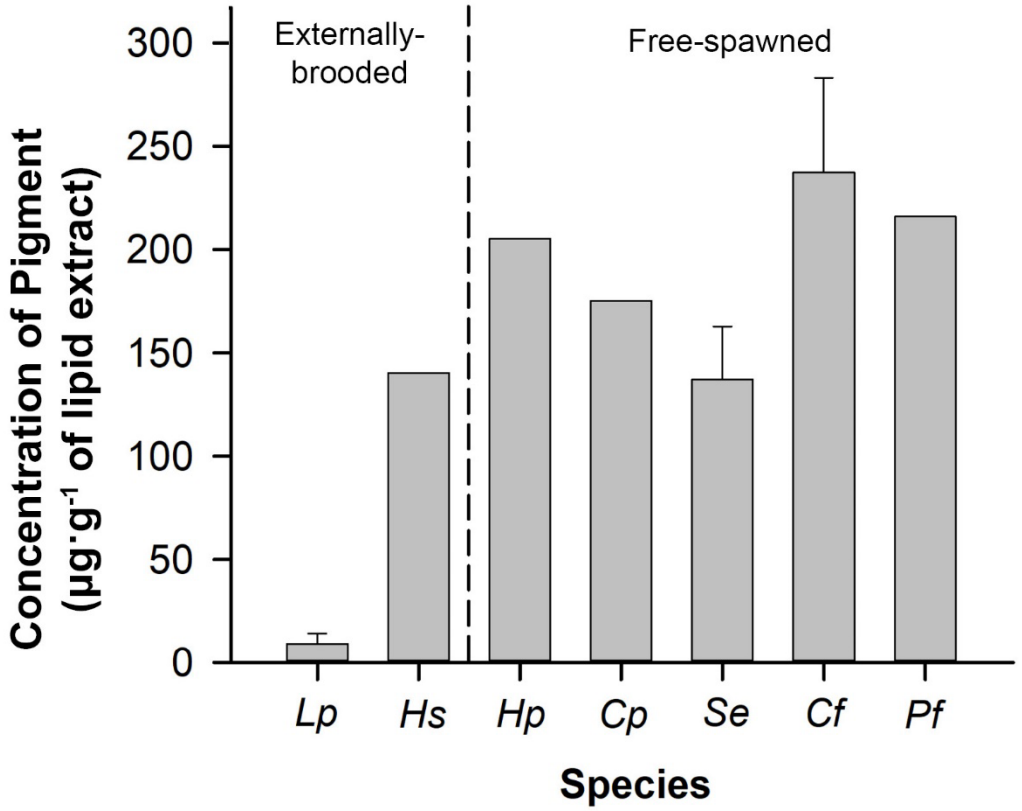


Figure 2.6 Comparison of pigment concentration (as β-carotene) across oogenic and early developmental stages in *C. frondosa*. The vertical dashed line separates species by development site. *Lp=L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp=C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii.* Error bars are absent for most species because not enough material was able to be obtained for a biochemical sample for each mother; therefore, all oocytes from all mothers were pooled. Error bars indicate standard deviation.

2.10 Appendix Chapter 2

A2.1 Description of the oocytes of the species used in this study. In cases where the collection date is unknown, individuals were obtained from the Ocean Sciences animal holding. All animals induced to spawn were done so with phytoplankton.

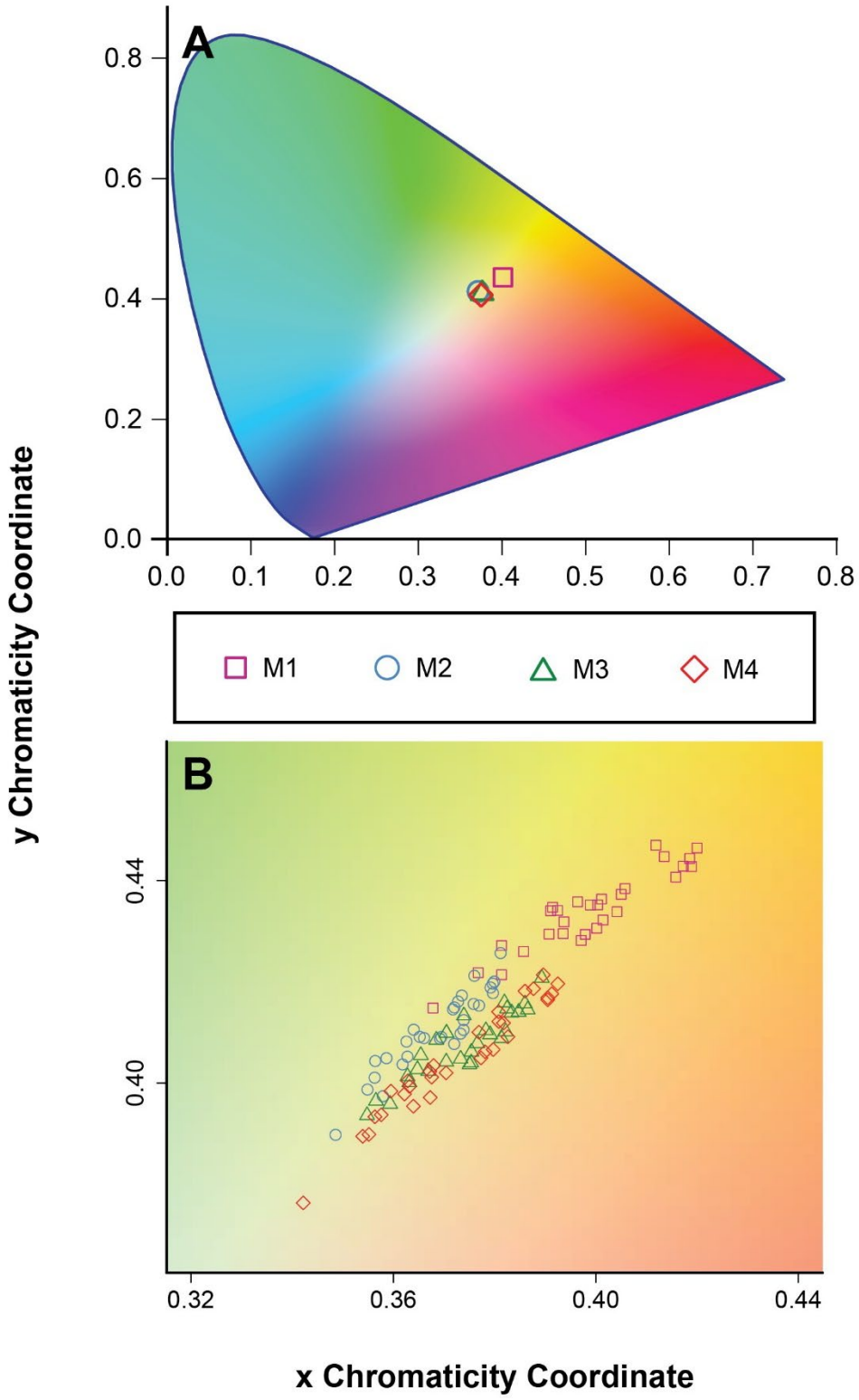
Species	Species Abbreviation	Annual Spawning Time	Oocyte Extraction from Ovary	Figure
Leptasterias polaris	*Lp*	late November–early December 2017	1-MA	2.1G
Henricia sanguinolenta	*Hs*	unknown	1-MA	2.1E
Henricia perforata	*Hp*	unknown	1-MA	2.1F
Crossaster papposus	*Cp*	late November–early December 2017	naturally-spawned	2.1C
Solaster endeca	*Se*	unknown	1-MA	2.1D
Cucumaria frondosa	*Cf*	late February 2017	mechanical	2.1A
Psolaster fabricii	*Pf*	July 2017	mechanical	2.1B

A2.2 Values for lipid composition (percent lipid class composition). 'Averaged' values are where one value was obtained for each mother and the mean of these values is reported (see Table 2.1). *Lp=L. polaris*, *Hs=H. sanguinolenta*, *Hp=H. perforata*, *Cp=C. papposus*, *Se=S. endeca*, *Cf=C. frondosa*, and *Pf=P. fabricii*. Avg=average; HC=hydrocarbons, EKET= ethyl ketones, GE=glyceryl ethers, TAG= triacylglycerols, FFA=free fatty acids, ALC=alcohols, ST=sterols, AMPL=acetone mobile polar lipids, and PL= phospholipids. SD for all species that had only one sample is 0.00.

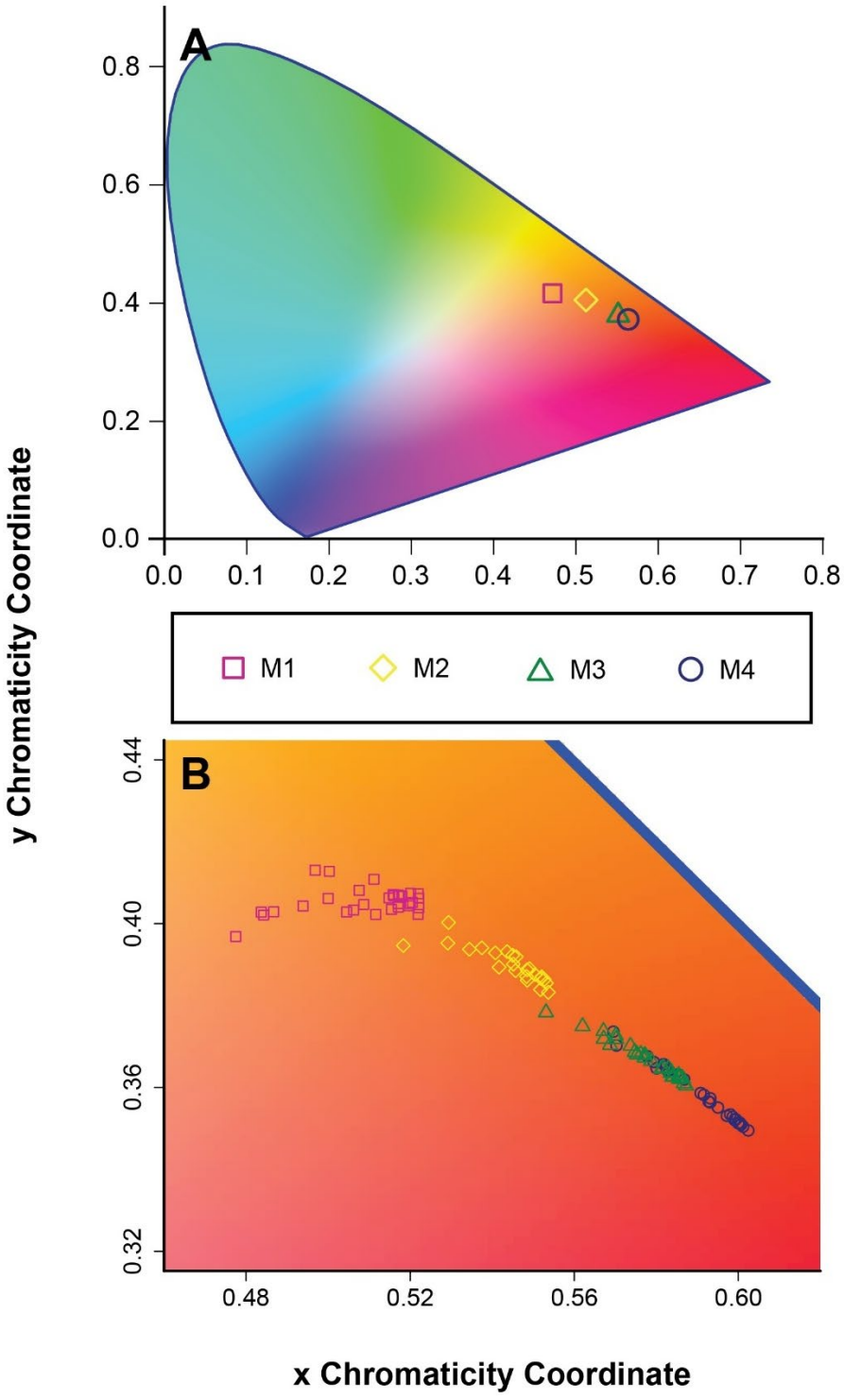
Species	**HC**	**EKET**	**GE**	**TAG**	**FFA**	**ALC**	**ST**	**AMPL**	**PL**
Lp (1)	7.71	0.00	12.47	29.82	9.71	0.00	0.00	16.73	23.57
Lp (2)	4.89	0.00	11.49	31.12	7.74	0.00	8.69	13.27	22.79
Lp (3)	0.00	0.00	20.67	49.29	0.00	0.00	0.00	0.00	30.04
Lp (4)	6.11	0.00	13.49	34.40	10.81	0.00	12.62	0.00	22.58
Avg *Lp*	4.68	0.00	14.53	36.16	7.06	0.00	5.33	7.50	24.74
SD *Lp*	3.33	0.00	4.17	8.96	4.88	0.00	6.36	8.77	3.56
Hs	0.00	0.00	73.79	0.00	6.41	0.00	5.10	7.59	7.11
Hp	0.00	0.00	75.47	0.00	6.51	0.00	4.70	7.46	5.87
Cp	3.00	0.00	77.16	0.00	6.67	0.00	0.00	7.22	5.94
Se (1)	5.94	0.00	58.74	7.85	10.75	0.00	9.54	0.00	7.18
Se (2)	2.35	0.00	82.50	0.00	5.83	0.00	3.70	5.62	0.00
Se (3)	0.00	0.00	81.20	0.00	5.02	0.00	3.27	5.27	5.25
Avg *Se*	2.76	0.00	74.15	2.62	7.20	0.00	5.50	3.63	4.14
SD *Se*	2.99	0.00	13.36	4.53	3.10	0.00	3.50	3.15	3.71
Cf (1)	2.77	0.00	55.27	14.90	4.63	6.05	15.72	0.00	0.66
Cf (2)	0.00	0.00	51.93	19.61	5.44	6.74	16.27	0.00	0.00
Cf (3)	0.00	0.00	62.08	13.93	5.32	5.55	13.12	0.00	0.00
Cf (4)	0.00	0.00	60.35	12.46	6.64	7.02	13.53	0.00	0.00
Cf (5)	0.00	5.51	57.88	10.99	6.19	6.13	13.29	0.00	0.00
Avg *Cf*	0.55	1.10	57.50	14.38	5.64	6.30	14.39	0.00	0.13
SD *Cf*	1.11	2.20	3.61	2.93	0.70	0.52	1.33	0.00	0.26
Pf	0.00	0.00	71.69	7.89	5.66	0.00	5.01	7.01	2.74

A2.3 Average oocyte colour coordinates on the 1931 Chromaticity Diagram for each mother and morphometrics of mothers of *L. polaris*, *H. sanguinolenta*, *S. endeca*, *C. frondosa*, and *P. fabricii*. M1=Mother 1, M2=Mother 2, M3=Mother 3, M4=Mother 4, M5=Mother 5.

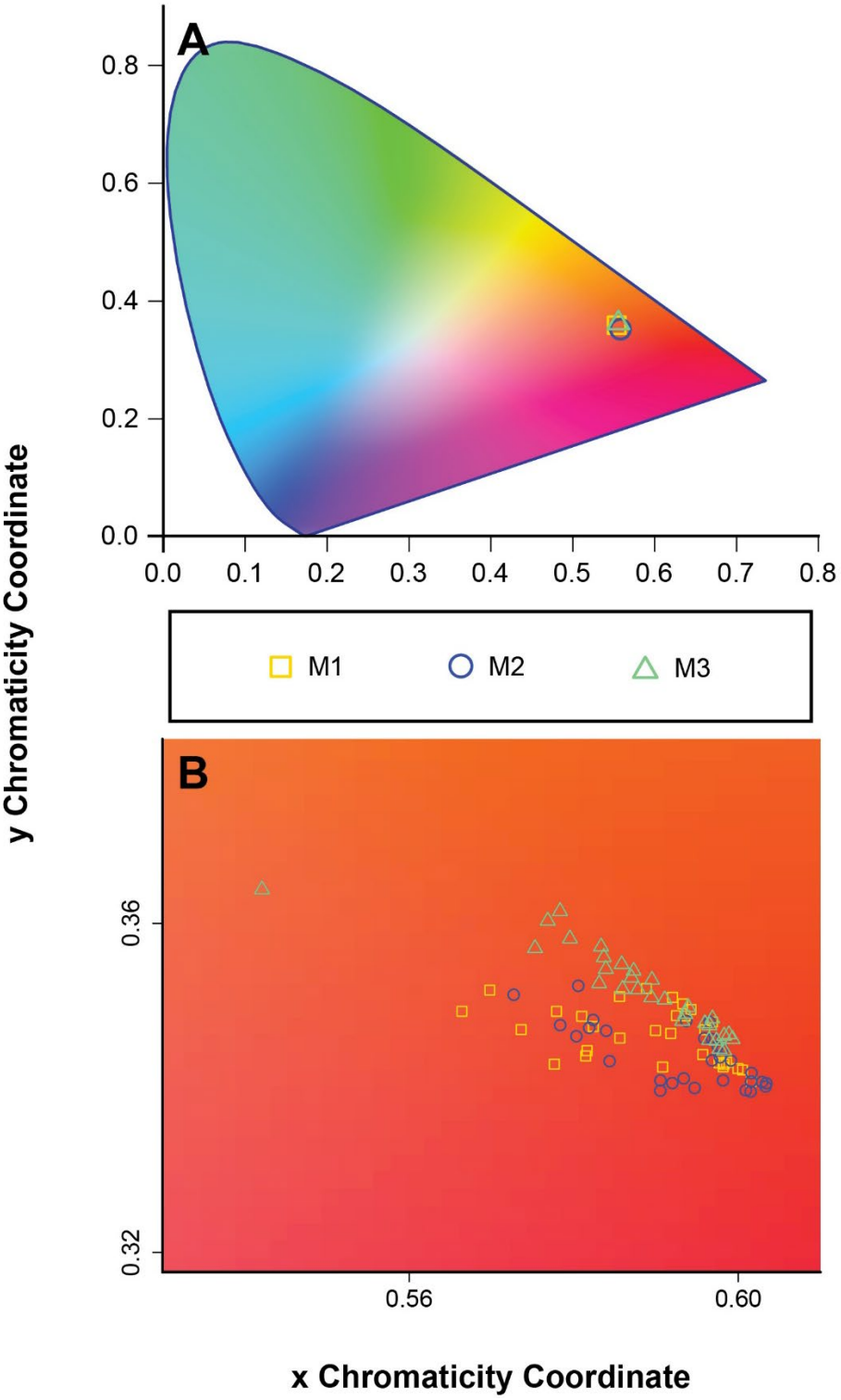
		Mothers	Oocytes					
Species	Mother No.	Avg Chromaticity Coordinate	Closest to White	Closest to Pure Spectral Orange	Diameter or Contracted Length (cm)	Total Mass (g)	Gonad Mass (g)	Rest of Body Mass (g)
Lp	M1	(0.40, 0.43)	(0.37, 0.41)	(0.42,0.45)	18.2	102.2	2.4	75.9
Lp	M2	(0.37, 0.41)	(0.35, 0.39)	(0.38, 0.43)	19	146.8	5.8	115.1
Lp	M3	(0.37, 0.41)	(0.35, 0.39)	(0.39, 0.42)	11	50.6	1.3	39.1
Lp	M4	(0.37, 0.40)	(0.34, 0.38)	(0.39, 0.42)	16	102	2.8	83.5
Hs	M1	(0.47, 0.41)	(0.48, 0.40)	(0.52, 0.41)	5.8	3.2	too light to weigh	no data
Hs	M2	(0.56, 0.37)	(0.57, 0.35)	(0.60, 0.37)	7.4	6.6	too light to weigh	4.5
Hs	M3	(0.55, 0.38)	(0.55, 0.36)	(0.59, 0.38)	6	5	too light to weigh	3.5
Hs	M4	(0.51, 0.40)	(0.52, 0.38)	(0.55, 0.40)	5	2.2	too light to weigh	1.7
Se	M1	(0.55, 0.36)	(0.57, 0.34)	(0.60, 0.35)	20.5	334.5	9.7	181.3
Se	M2	(0.56, 0.35)	(0.57, 0.34)	(0.60, 0.35)	24	599.1	47.1	380.9
Se	M3	(0.55, 0.36)	(0.54, 0.34)	(0.60, 0.36)	31	688.4	34.1	405.2
Cf	M1	(0.50, 0.37)	(0.49, 0.36)	(0.51, 0.38)	12.3	300.9	14.6	185.5
Cf	M2	(0.50, 0.37)	(0.48, 0.36)	(0.51, 0.38)	11.5	276.7	15.1	126
Cf	M3	(0.45, 0.36)	(0.44, 0.35)	(0.47, 0.36)	11	222.9	11.5	121.6
Cf	M4	(0.49, 0.37)	(0.48, 0.36)	(0.50, 0.38)	10	297.7	11.8	145.3
Cf	M5	(0.48, 0.37)	(0.47, 0.37)	(0.49, 0.38)	10.5	236.9	11	149
Pf	M1	(0.54, 0.37)	(0.51, 0.36)	(0.56, 0.37)	5.5	46.1	3.7	37.7
Pf	M2	(0.54, 0.37)	(0.51, 0.36)	(0.55, 0.38)	7.7	74.6	4.7	50.4
Pf	M3	(0.55, 0.37)	(0.53, 0.36)	(0.56, 0.37)	7.2	50.5	1.3	31.3
Pf	M4	(0.53, 0.37)	(0.51, 0.36)	(0.54, 0.38)	6.2	44.7	2.3	26.8
Pf	M5	(0.55, 0.37)	(0.53, 0.36)	(0.56, 0.37)	7	44.4	1.3	27.5



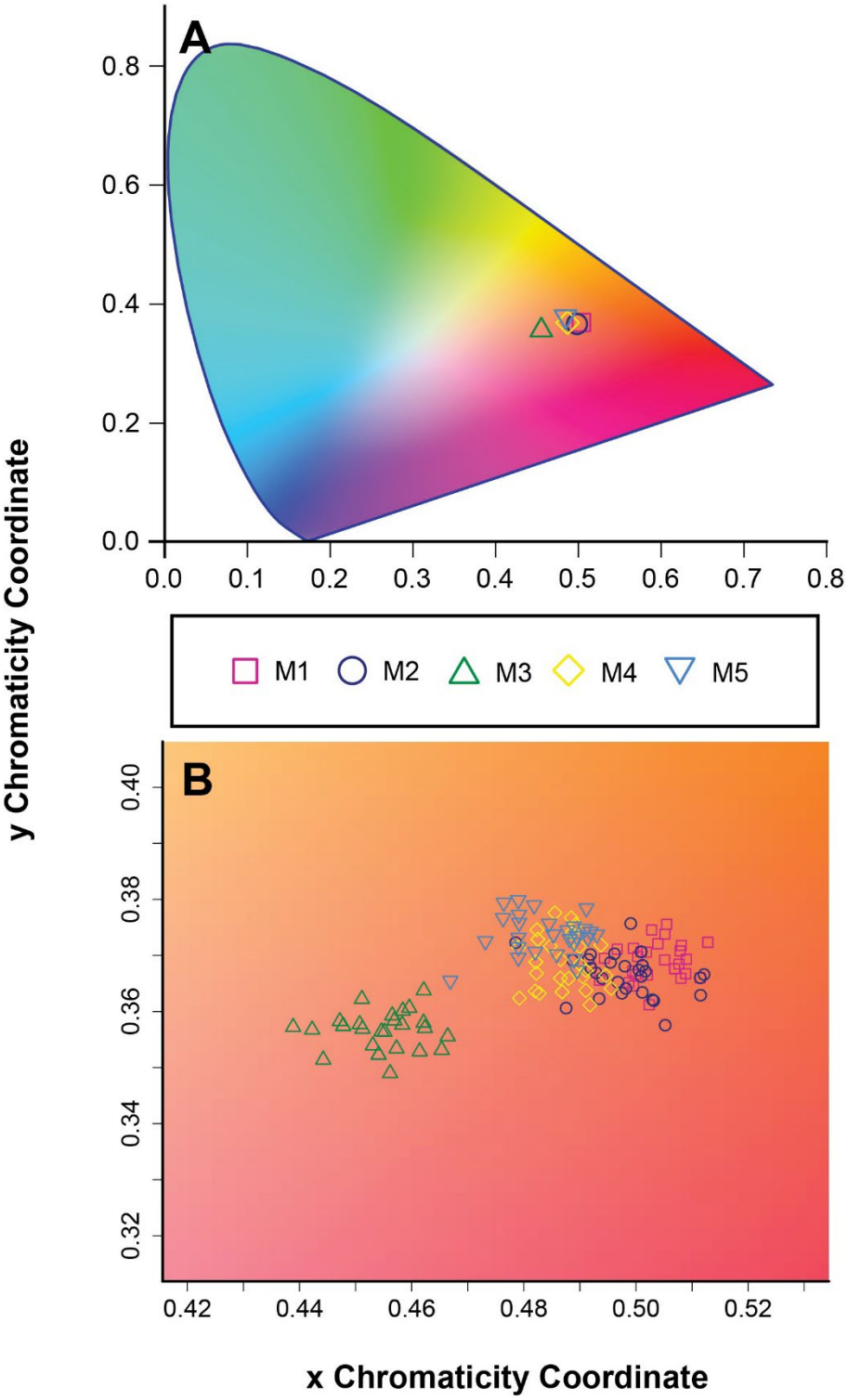
A2.4 Comparison of colour in oocytes across mothers in *L. polaris*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



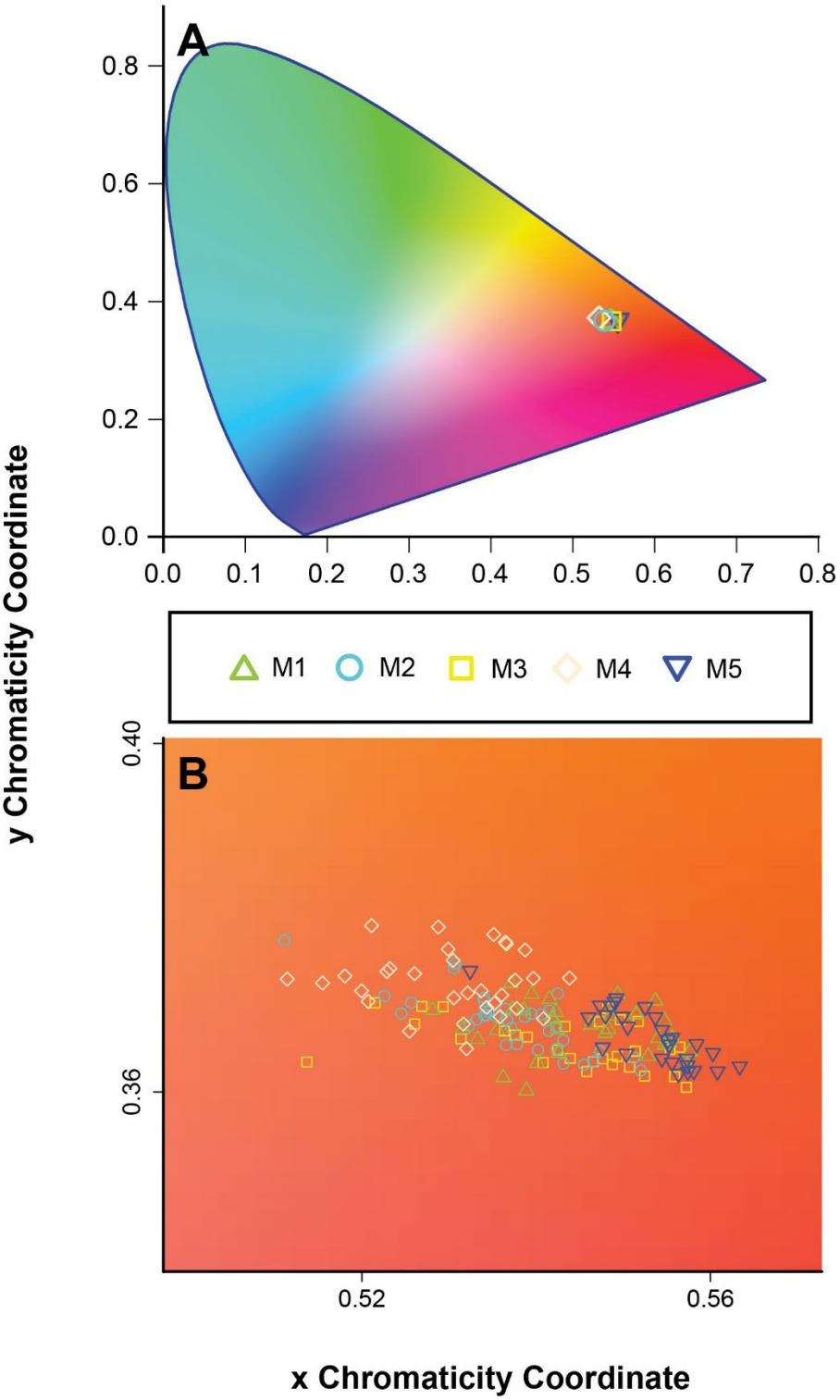
A2.5 Comparison of colour in oocytes across mothers in *H. sanguinolenta*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



A2.6 Comparison of colour in oocytes across mothers in *S. endeca*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



A2.7 Comparison of colour in oocytes across mothers in *C. frondosa*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



A2.8 Comparison of colour in oocytes across mothers in *P. fabricii*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.

Chapter 3: Shifts in colour and buoyancy across oogenic and developmental stages in the lecithotrophic sea cucumber *Cucumaria frondosa*

3.1 Abstract

Characteristics of marine lecithotrophic propagules (oocytes, embryos, larvae, and early juveniles) may allow them to survive in dynamic ocean conditions. In echinoderms, pre-zygotic and early ontogenetic stages undergo marked changes in buoyancy and colour. The present study explored the ties between the biophysical properties and chemical composition of the propagules of the sea cucumber *Cucumaria frondosa* over a year, from the onset of oocyte provisioning to 6-month-old juveniles. The colour, density, lipid classes, and pigment contents were examined. The highest density and lightest, least saturated colours were found at both ends of the life-history stages examined, i.e. in early vitellogenic oocytes (5 months before spawning) and in 6-month-old benthic juveniles. Mid-vitellogenic to mature oocytes and all post-spawning stages until the vitellaria larva showed the lowest density and most saturated colours. Lipid analysis revealed elevated levels of glyceryl ethers in all stages of oogenesis and development up until the juvenile, which exhibited a decrease in glyceryl ethers and an increase in triacylglycerols. Spectrophotometric analysis of lipid extracts suggests that all pre- and post-zygotic stages of *C. frondosa* harbour keto-carotenoids, but in varying quantities. This study confirms existing knowledge on the life cycle of *C. frondosa*, a species that can be used as a model organism for further studies on the colour and buoyancy of oocytes of other invertebrates and externally fertilizing species.

3.2 Introduction

Phenotypic plasticity helps propagules (eggs, embryos, larvae) of benthic marine animals disperse to and survive in various marine environments. Modulating their eco-physiological requirements allows them to exploit available resources optimally (George et al., 1990), to avoid being ingested (Mercier et al., 2013), and to stay within their optimal physiological range of tolerance when faced with changing ambient conditions (Thomas et al., 1999). Overall, undergoing morphological and physiological changes

through early ontogeny makes propagules more flexible, 'adaptable', and resilient, and may allow them to survive when confronted with a dynamic and unpredictable environment. Precursor phases of these key propagule features likely develop during oogenesis through maternal provisioning.

Depending on the species, the propagules of echinoderms can undergo either lecithotrophic development, whereby non-feeding larvae receive nutrition through maternal yolk stores, or planktotrophic development, whereby larvae acquire nutrition by feeding on plankton (Strathmann, 1985). Lecithotrophic propagules, in particular, are known to display striking interspecific variations in colour and buoyancy (Montgomery et al., 2017a; Chapter 2). However, limited knowledge exists on how colour and buoyancy change throughout ontogenesis, partly due to the many challenges associated with measuring these properties at the level of the reproductive cells. Most of the existing research covers only one stage (e.g. egg only, larva only) and does not compare across ontogeny (e.g. colour: Montgomery et al., 2017a; Dimelow, 1958; buoyancy: Young et al., 1987, Hamel et al, 1993; Villinski et al., 2002; Byrne et al., 1999) and most often colour or buoyancy is only mentioned in passing (e.g. colour: Byrne et al., 2003; Contins et al., 2011; Falkner et al., 2013; buoyancy: Olsen et al., 1993; Falkner et al., 2015; Falkner et al., 2013; Byrne et al., 2003) or used in a different context such as describing the localization of pigment in certain parts of the body during embryogenesis (e.g. Hendler, 1982; Byrne 2005).

General descriptions of colour across egg, embryo, larva, and juvenile stages of asteroid *Ophidaster granifer* have been made (Yamaguchi et al., 1984) and changes in colour across embryogenesis through to larva have also been qualitatively described in a handful of species of crinoids (Nakano et al., 2003; Barbaglio et al., 2011; Obuchi et al., 2010). Buoyancy has been quantified from eggs to larvae in the asteroid *Stegnaster inflatus* (Zamora et al., 2018); ascent rates were used as a proxy for buoyancy across both eggs and larvae of asteroid *Pteraster tesselatus* (Kelman et al., 2000), and recent work has looked at the change in lipids across life stages in urchin *Heliocidaris erythrogramma*, without commenting on its effects on buoyancy (Byrne et al., 2019). As well, a recent study described qualitative change in both colour and buoyancy from larva to juvenile stages in the holothuroid

Cucumaria frondosa (Gianasi et al., 2018). To date, no study has attempted to quantify the colour or examine pigment through ontogeny in echinoderms; there has been no quantification of buoyancy in ophiuroids, and crinoids across ontogeny; and no studies had been conducted on lipids across ontogeny for crinoids (Byrne et al., 2019) and holothuroids with lecithotrophic development.

The ubiquity of the sea cucumber *Cucumaria frondosa* in the North Atlantic (Hamel et al., 2008) and the fact that its propagules undergo pelagic lecithotrophic development makes it an appropriate candidate for the present study. Oogenesis unfolds in four main phases: recovery (oocytes under 200 µm), growth (onset of yolk addition or vitellogenesis, oocytes 200-400 µm), advanced growth (oocytes between 400-600 um), and maturity (oocytes > 800 µm; Hamel et al., 1996a). Following fertilization, which occurs externally, successive cleavages lead to embryonic development, comprised of the unhatched blastula (spinning counter-clockwise within fertilization membrane), the hatched blastula (emerging from the fertilization membrane), and the gastrula (elongated along animal-vegetal axis, swimming with cilia). The larval stages include the vitellaria (rudiments of 5 tentacles appearing), and the pentactula (with tentacles), which is the stage at which settlement occurs, followed by metamorphosis (Hamel et al., 1996b). Early juveniles undergo a switch in nutrition from maternal provisions (yolk) to autonomous suspension feeding (Hamel et al., 1996b). Tolerance to light intensity and water flow increases with age along with a change in body-wall colour was observed (Gianasi et al., 2018). The oocytes of *C. frondosa* are red and have been observed to be positively buoyant (Hamel et al., 1996b); their colour and buoyancy have recently begun to be quantified (Chapter 2).

The present study seeks to 1) quantify colour and buoyancy across stages in the lecithotrophic sea cucumber *Cucumaria frondosa*, and 2) link the external characteristics of colour and buoyancy to the chemical composition across early life stages. It is expected that all metrics will vary according to the needs of *C. frondosa* at the time of each respective life stage.

3.3 Materials and Methods

3.3.1 Collection and Maintenance of Adults

Adult individuals of *Cucumaria frondosa* were collected from three areas of the coastal Avalon Peninsula, Newfoundland (eastern Canada): Fortune Bay (47° 12' 46.72" N, 56° 33' 8.36" W), Admiral's Cove (52°53'34.4" W 47°06'05.9" N), and Tors Cove (52°50'40.4" W, 47°12'44.0" N). Collections in Fortune Bay were made in June 2017 by licensed, commercial trawlers at a depth of ~240 m. Individuals were collected in Admiral's Cove (late November 2017) and Tors Cove (early February 2018) by SCUBA divers (Memorial University's Field Services Unit) at depths between 6 and 8 m. All sea cucumbers were transported back to the laboratory in containers that held bags filled with seawater and ice (Fortune Bay) or coolers filled with seawater only (Admiral's Cove and Tors Cove). At the laboratory, they were held in tanks (20-L), supplied by a flow-through seawater system at ambient temperature (~3°C), which provided natural phytoplankton as food. The sea cucumbers were exposed to natural photoperiod conditions through large windows (maximum light intensity of 300 lux) throughout their time in the laboratory (until April 2018).

3.3.2 Sampling of Oogenic and Developmental Stages

Oocytes at various stages of oogenesis were sampled over 21 weeks, at time points determined from the known cycle of gonad maturation for this species in eastern Canada, as per Hamel et al. (1996a). Each stage was categorized according to mean size of oocytes, percentage of visible yolk present, and location within the gonad tubules (Hamel et al., 1996a). The focal oogenic stages are detailed in A3.1 and Figure 3.1. Translucent portions of the early vitellogenic 1 oocytes (EV1) were used as a proxy for the colour of previtellogenic oocytes. Previtellogenic oocytes were not quantitatively analyzed for any other metric (density, lipids, pigments) due to their transparency, small size, and entanglement within follicular cells, and to the minimal amount of material they provided, which was insufficient for biochemical analyses. Pre-vitellogenic oocytes (PV) were sampled in November, early vitellogenic oocytes (EV1 and

EV2) in early November; mid-vitellogenic (MV) oocytes between late December and early January; and late vitellogenic (LV; mature) oocytes in mid to late February, when gametes reached full maturity (Hamel et al., 1996a). Specific stages were collected from separate females (Table 3.1, Appendix A) using a syringe (12 gauge) for biophysical samples and by sacrifice and gently stroking oocytes out of gonadal tubules using a blunt probe for biochemical samples. Stages of oocyte were then separated using a pipette (100-µl) and filtered through Nitex mesh where necessary (450 µm for only mid-vitellogenic stage).

A embryo/larval culture was set up following the spawning of adults of *C. frondosa* collected from Tors Cove. Mature individuals were kept together and were induced to spawn by adding live phytoplankton (Shellfish Diet 1800, Reef Mariculture; 150 000 cells·mL^{-1}; Gianasi et al., 2018) for 5 consecutive days prior to the first full moon of the spawning season (March 31, 2018; period known to be the natural time of spawning; Mercier et al., 2010). Communal oocytes from many different mothers were opportunistically collected from the tanks during spawning events that occurred from early to mid April. Fertilization occurred naturally and larval cultures were kept in black culture baskets closed with 300-µm mesh. Flow was supplied through the seawater system described in Chapter 2 (at flow rates of ~22 mL·s^{-1}). Temperature of the ambient seawater was 3°C and salinity was 35 psu. Cultures were exposed to natural lighting conditions, with maximum intensity varying from ~200–300 lux, through large windows. The following developmental stages were sampled: fertilized spawned oocytes (S), blastula (just hatched; B), gastrula (G), vitellaria (V), pentactula (P), and early juvenile (J) (Figure 3.1) as per Hamel et al. (1996b) and Gianasi et al. (2018). Biophysical characteristics were determined in all post-zygotic stages. Biochemical samples were obtained for all stages except the blastula and pentactula, because natural mortalities were depleting the culture and a choice had to be made to maintain enough propagules alive, representing enough tissue, to complete the last samplings at the juvenile stage.

3.3.3 Propagule Density (Buoyancy)

Propagule density (the biophysical property that underlies buoyancy) was measured using the classic method described in Kenkel (2003) i.e. by measuring the density of seawater in which propagules reach hydrostatic equilibrium (i.e. neutral buoyancy, see Appendix B). Eleven glass vials (11 mL) were each filled with a different density of artificially mixed seawater (Instant Ocean ® mix), i.e. 1.000, 1.010, 1.015, 1.020, 1.025, 1.030, 1.040, 1.050, 1.060, 1.070, and 1.080 $g \cdot cm^{-3}$). They were maintained in a water bath (Boekel Scientific Inc.; heater: GD120L, chiller: IC-400) at the temperature at which spawning occurred (3°C). Densities higher than 1.080 $g \cdot cm^{-3}$ could not be tested because of the difficulty to dissolve the quantity of salt required at such a cold temperature and because of the osmotic shock that could damage the propagules. Each vial was divided into 4 equal vertical strata by placing a visual guide behind the vial.

In this study, a single measurement was performed using 20 live propagules at the same oogenic or developmental stage (except 5 for the pentactula 2 and 3, based on availability); the number of replicates and the number of mothers sampled for each comparison are provided in Table 3.1 (for further information see Appendix A: Sampling Diagrams). The 20 propagules were released in the middle of each density-specific vial using a 100-µl pipette (Eppendorf Research) with truncated tip and allowed 5 min to immobilize. The density was determined to be that of the vial in which the propagules reached hydrostatic equilibrium (i.e. evenly spread in suspension, not in contact with the surface of the water or bottom of the vial). In cases where all propagules sank in one density and floated in the next highest density, the intermediate density value was scored. Motile stages (B, G, V, P, and J) were anesthetized with 0.37 M magnesium chloride (Hart, 1995) prior to measurement in seawater to ensure the observed changes in buoyancy were due to composition only, and not to mobility (Montgomery et al., 2017b).

3.3.4 Propagule Colour

A sample consisted of 30 live propagules for each stage (except 5 for the P2 and P3, based on availability), with stage-specific number of replicates and mothers detailed in Table 3.1. The propagule sample was placed on a white background next to a small colour standard (Pico ColorGauge, matte; Edmund Optics Inc., NJ, USA) and photographed using an automated stereomicroscope (Leica M205 with DFC7000T camera) equipped with a ring light and diffuser dome (Leica LED5000 HDI).

Colour values were obtained for each propagule in the photo using a customized Matlab code (Akkaynak et al., 2014). The reference values for the Pico ColorGauge were obtained from the Image Science Associates website under 'ISA Products' in the Excel spreadsheet named 'Micro/Nano Target Colorimetry Data' (http://www.imagescienceassociates.com/mm5/merchant.mvc?Screen=PROD&Store_Code=ISA001&Product_Code=CGNT&Category_Code=TARGETS). XYZ values were used, as opposed to RGB values, because they can be compared accurately across different cameras (Choudhury, 2014). One set of values was obtained for each individual propagule by averaging the pixels within the area in the photograph that it occupied. For the EV1 oocytes, which were in the process of being provisioned during vitellogenesis, only the area occupied by the yolk was sampled in order to measure the colour of the yolk. In cases where the ingested food or internal organs could be viewed by transparency (i.e. in the juvenile), these areas were excluded from the analysis. XYZ values were converted to 'x' and 'y' chromaticity coordinates as per Mortimer et al. (2011), then plotted on the Commission Internationale de L'éclairage's (CIE) 1931 Chomaticity Diagram using the 'pavo' package in R.

Results are described with the centre of the Chromaticity Diagram referred to as 'white' (chromaticity coordinates x and y: 0.33, 0.33) and colours near the edge of the perimeter of the colour space as pure spectral colours (e.g. pure spectral orange; Choudhury, 2014).

Inter-mother variability in colour of propagules was examined. Colour coordinates of oocytes from each mother were plotted on the CIE 1931 Chromaticity Diagram.

3.3.5 Propagule Lipids

A sample consisted of 0.1–0.5 g of tissue obtained from each of the oogenic and developmental stages, except the blastula and pentactula (see above), following the replication (and pooling, where necessary) detailed in Table 3.1. As much seawater as possible was removed from live propagule samples before they were weighed. Samples were then preserved in 100% chloroform under nitrogen gas at -80°C until lipid extraction and analysis.

Total lipids were assessed using standard procedure detailed in Parrish (1999). Briefly, lipids were extracted from samples using a chloroform:methanol (2:1) mixture. Then, lipid classes were determined using thin-layer chromatography with flame ionization detection (TLC-FID). For this, lipid classes were first separated using TLC in a three-stage development system (Parrish, 1987). Chromatograms of lipid classes were obtained using an Iatroscan MK6 (Iatron Laboratories, Tokyo, Japan) and recorded using PeakSimple Chromatography software (4.54, SRI Inc). The ionization profile of a prepared standard composed of 9 lipids [hydrocarbons (HC), steryl esters/wax esters (SE/WE), ethyl ketones (EKET), glyceryl ether (GE), triacylglycerols (TAG), free fatty acids (FFA), alcohols (ALC), sterols (ST), acetone mobile polar lipids (AMPL) and phospholipids (PL)] was also obtained for lipid class identification. TAG and GE identities were confirmed by comparing to a second standard that only contained saturated TAG. The same development systems were used except four times the amount was spotted in the second system (‘intermediately polar lipids’; which includes both TAG and GE) which was held for 50 min in 79.9:20:0.1 hexane:diethyl ether:formic acid. In the second development, a peak that ran before TAG was considered to be GE. Peaks that ran with the saturated TAG standard were considered saturated TAG; if a peak ran slightly after the saturated TAG standard were considered unsaturated TAG. Identification of lipid classes was performed by comparing scanned lipid chromatograms to that of the prepared standard. Percentages of each lipid class per sample were calculated using ionization peaks. The percentage of each lipid class in a sample was calculated as relative weight (g).

3.3.6 Propagule Pigments

To circumvent the small quantities of material naturally available, the lipid extracts used for the total lipid analysis detailed above were also used for determination of pigments, and chloroform was used as the solvent. Pigments are typically extracted with acetone, which does not affect absorbance profiles of pigments; however, it has been shown that using chloroform has negligible effects (Wellburn, 1994). Absorbance spectra were obtained using a UV-VIS spectrophotometer (Cary 6000i) and 3-mL quartz cuvettes supplied through the Core Research Equipment and Instrument Training (CREAIT) Network at Memorial University. Lipid extracts were scanned from (200–800 nm), including in the ultraviolet (200–400 nm), visible (400–700 nm), and infrared range (700–800 nm) using chloroform as a blank. All lipid extracts were scanned to obtain initial absorption spectra. When the peaks were below or beyond the absorption scanning range (0-3), the extract with the highest concentration of pigment was diluted to fit within the absorption scanning range. All other lipid extracts containing pigment were brought to the same dilution, then re-scanned to fit within the appropriate range.

Identification of pigments was attempted by comparing the shape of the curves and the wavelength of the absorbance maxima with that of the absorbance spectra of known pigments in the literature (e.g. Mochimaru et al., 2008; Sandmann et al., 2006; Buckwald et al., 1968). Concentration of pigment (μg of pigment · g of lipid^{-1}) in the various lipid extracts was obtained using the formula in Abuzaytoun (2017), adjusted from Takeungwongtrakul et al. (2012):

$$\text{Concentration of pigment in lipid} = \left(\frac{A_{468}}{0.20}\right)\left(\frac{\text{volume of extract} * \text{dilution factor}}{\text{weight of lipid sample used in g}}\right)$$

The ratio $A_{468}/0.20$ was determined following Abuzaytoun (2017): β-carotene was used as a standard (i.e. the shape of the curve and the λ_{max} of the unknown pigment were assumed to be the same as those for β-carotene); the concentration of the pigment was calculated using the linear equation y=mx+b, where y=the λ_{max} of the unknown pigment, m=the slope from the calibration curve for β-carotene obtained

from Kaur et al. (2001), x=concentration of pigment in the lipid in $\mu g \cdot ml^{-1}$, and b was considered to be negligible. The volume of extract was 1.5 ml, the dilution factor was 6, and the weight of lipid sample used (in g) was obtained from the total lipid analysis (i.e. number of µg per replicate calculated by multiplying the value in $\mu g \cdot g^{-1}$ by the weight of the sample, and dividing by the estimated number of propagules in the sample).

3.3.7 Data Analysis

To detect differences in mean densities, percentage of lipid classes, and concentrations of pigment across life stages (EV1, EV2, MV, LV, S, B, and G), a one-way ANOVA with Tukey post hoc test was performed. If the Normality test failed, a Kruskal-Wallis ANOVA on ranks with Dunn's post-hoc test was performed. If the results of the Dunn's test reported as 'Do Not Test', they were reported as non-significant and a description of the data in the figure was provided. Densities of >1.080 $g \cdot cm^{-3}$ were included as 1.080 $g \cdot cm^{-3}$. Only lipid classes important to buoyancy (i.e. GE and TAG) were tested.

Differences in colour among stages (EV1, EV2, MV, LV, S, B, G, V, P1, P2, P3, and J) were interpreted directly from the figures (with no statistics conducted) based upon the colour region they occupied on the CIE 1931 Chromaticity Diagram and their position relative to each other. Results are described with the centre of the Chromaticity Diagram referred to as 'white' (chromaticity coordinates x and y: 0.33, 0.33) and colours near the edge of the perimeter of the colour space as pure spectral colours (e.g. pure spectral red; Choudhury, 2014). For inter-mother variability, between mother versus within mother differences were interpreted directly off Chromaticity Diagram plots.

Changes in the identity of the pigment across lipid extracts from oogenesis and development, were interpreted directly from the graph based on the absorbance spectra (shape of the curve and absorbance maxima). It was also noted whether reflectance curves of each stage peaked in the ultraviolet region of the electromagnetic spectrum or not.

3.4 Results

3.4.1 Differences Across Oogenic and Post-Zygotic Developmental Stages

A Kruskal-Wallis ANOVA on ranks was conducted and showed a statistically significant difference in densities across stages (H_6=32.1, p<0.001); however, a Dunn's post-hoc test reported 'No' and 'Do Not Test' for all the pairwise comparisons, thus there is no statistical difference between density values of pairs of life stages. Otherwise, the data in Figure 3.2 revealed a bell-shaped curve as follows: EV1>EV2>MV=LV=S=B=G=V=P1<P2 <P3=J, i.e. density decreased during oogenesis (means of EV1:1.075 and EV2: 1.049 g·cm^{-3}) until the mid-vitellogenic oocytes, the first of the stages with the minimum densities observed, and remained low after fertilization and throughout embryonic and larval stages (MV:1.028; LV: 1.023 S: 1.025; B: 1.026; G: 1.025; V: 1.030; P1: 1.028) and increased in late larval stages (P2:1.045 P3: >1.080 g·cm^{-3}) until the juvenile stage, with the highest values (J: >1.080 g·cm^{-3}).

This bell-shaped curve was echoed in the colour values of the propagules, with proximity to pure spectral orange (as opposed to white) fluctuating as follows: PV < EV1 < MV = LV = S = B = G = V = P1 = P2 = P3 > J (Figure 3.3A and B). Specifically, the analysis of colour showed that most propagules occupied a similar area of the Chromaticity Diagram in the orange range, except for the previtellogenic (PV) and earliest vitellogenic oocytes (EV1), which ranged from translucent to light yellow. The orange colour characteristic of vitellogenic oocytes and of all embryonic and larval stage attenuated when the propagules reached the juvenile stage, which were orange closer to white (Figure 3.3A). Regarding inter-mother variability, there was greater variability within mothers than between mothers for all stages of oogenesis and development (Figure 3.7 and A3.4–A3.7).

A one-way ANOVA showed clear differences in the percent GE across groups of life stages ($F_{2,6}$=85.3, p<0.05). A Tukey post-hoc test showed that there was a difference between EV and the stages with the highest percentages (LV, p=0.02 and S, p=0.03). Likewise, a one-way ANOVA showed that

there were differences in the percent TAG across groups of life stages ($F_{2,6}$=195.3, $p<0.05$). A Tukey test was conducted and showed that there was a difference between J and all other stages (all $p<0.05$). Results showed that percentages of GE progressed through the stages as follows: EV<MV=LV=S=G>V>J (Figure 3.4A and B, and A3.2). The total lipid analysis showed that proportions of (GE) were low in the early oocytes combined (EV, 34%), then increased and remained constant in the oogenic and embryonic stages near fertilization (MV, 56%; LV, 58%; S, 58%; and G, 55%), decreased again at the vitellaria stage (V, 43%) and was not detected in the juvenile. TAG remained low in all oogenic and developmental stages (EV, 20%; MV 15%; LV, 14%; S, 10%; G, 9%; V, 12%) until the juvenile, where values peaked (76%; Figure 3.4A and B, A3.2).

Pigment analysis in lipid extracts showed that all stages of oogenesis and post-zygotic developmental stages shared one type of absorption spectrum (Figure 3.5A), characterized by a 3-peak curve in the visible portion of the electromagnetic spectrum (minor peaks at ~440 and 490 nm and major peak at 471 nm). The absorbance spectra of lipid extracts in the early developmental stages of *C. frondosa* were compared to those of known pigments in the literature and determined to match keto-carotenoids, a class of yellow/orange/red pigments. This keto-carotenoid pigment also reflected in the ultraviolet region of the electromagnetic spectrum (250-440 nm; Figure 3.5B). Both the early vitellogenic oocyte (EV) and the juvenile (J) samples had the flattest curves, reflecting at all wavelengths. All other stages (MV, LV, S, B, G and V) examined reflected in the red portion of the visible spectrum (>590 nm).

A one-way ANOVA was conducted and showed that there were statistically significant differences in pigment concentration across life stages ($F_{2,6}$=14.8, $p<0.05$). A Tukey post-hoc test showed that there was a difference between LV and J ($p=0.05$). The data from Figure 3.6 showed that pigment concentrations progressed as follows: EV<MV<LV>S=G>V>J, i.e. increasing from the early to the late vitellogenic oocyte stages (from 65.7 to 226.1 $\mu g \cdot g^{-1}$), then experienced a decrease at spawning (177.5 $\mu g \cdot g^{-1}$), remaining stable until the gastrula stage (177.6 $\mu g \cdot g^{-1}$), and gradually decreasing again at the vitellaria (129.1 $\mu g \cdot g^{-1}$) until the minimum values were reached at the juvenile stage (37.2 $\mu g \cdot g^{-1}$).

3.5 Discussion

The present study took an integrative, multi-metric (density, colour, lipids, and pigments) approach to compare differences in the physical and chemical properties of propagules in the holothuroid *C. frondosa*. Results showed that colour and buoyancy as well as lipid composition and pigment concentration vary across oogenesis and development, with direct implication on the eco-physiological constraints in pre- and post-zygotic stages.

3.5.1 Life Cycle and Eco-Physiological Needs of C. frondosa

In the present study, the bell-shaped pattern observed in the density and pigment concentration values from early oocytes to juveniles, and indirectly in the propagule colour and in the GE and TAG levels (Figure 3.8), seems to echo the life cycle of *C. frondosa* as described by Hamel et al. (1996b). The positively buoyant oocytes give rise to embryos (blastula, gastrula) that develop close to the water surface until the vitellaria stage (Hamel et al. 1996 b, c; So et al. 2010; Gianasi et al. 2019). At the pentactula stage, offspring begin to attach to the substrate for short periods of time until settlement occurs, a time at which they hover with tentacles periodically touching the bottom; and the reddish colour is replaced by a brown, translucent colour (Hamel et al., 1996d).

3.5.2 Switch in Larval Feeding

In maternally provisioned lecithotrophic propagules, yolk is a substantial component of the egg. It is made up of proteins and lipids (Byrne et al., 1999) as well as carbohydrates which can vary in proportion depending on the ecological requirements of the embryo. For example, echinoid, asteroid, and ophiuroid embryos that develop in the water column have been proposed to have an enhanced 'lipogenic program' as opposed to those on the benthos with an enhanced 'vitellogenic program' (Byrne et al., 2003). As well, propagules with small as opposed to large lipid droplets show selection for different buoyancy in eggs of ophiuroids (Falkner et al., 2013). The main lipid in lecithotrophic propagules is GE (Villinski et al., 2002; Prowse et al., 2009) and it has been proposed that its main function is as long-term

nutrition in asteroids and ophiuroids (Prowse et al., 2009) and buoyancy has been mentioned as a potential function in asteroids, ophiuroids, and echinoids (Villinski et al., 2002; Prowse et al., 2009; Falkner et al., 2013; Falkner et al., 2015; Zamora et al., 2018). GE has been shown to play a role in buoyancy (Emlet et al., 1997; Villinski et al., 2002; Byrne et al., 2000) and has been shown to be more buoyant and have greater lift due to a lower specific gravity than other energetic lipids such as TAG (Lewis, 1970). GE is responsible for positive buoyancy as in the small lecithotrophic eggs of ophiuroids (Falkner et al., 2015); however, it has also been suggested that it may not be the cause of buoyancy because some eggs high in GE are negatively buoyant because of high protein content (Prowse et al., 2009; Prowse et al., 2008). Little is known about lipids across life stages in holothuroids (Byrne et al., 2019, Peters-Didier et al., 2017). The results of the present study suggest that GE is associated with positive buoyancy in *C. frondosa*. It seems that oocytes of *C. frondosa* may become progressively richer in lipids like GE (developing buoyancy) throughout vitellogenesis, as yolk deposits build up. After fertilization, the embryo would start to deplete (or otherwise modify/process) the yolk reserves and buoyancy would decrease as the larva progresses towards settlement at the juvenile stage. The present study showed that the juvenile stage of *C. frondosa* lacked GE, whereas the short-term storage lipid TAG spiked at that stage in *C. frondosa*, suggesting that it is used for energy in replacement of GE. This shift in the dominant lipid class could potentially be due to the juvenile switching nutrition from maternal stores to suspension feeding on plankton.

3.5.3 Consumption of Lipid Through Development

Along with driving buoyancy, the lipids within the yolk also hold the pigment(s) that give the propagules their colour. Findings in the present study reveal apparent thresholds of pigment concentration at which a change in colour can be observed externally. The main pigment across the propagules was identified as a (keto-)carotenoid, a group that is known to be lipid soluble (Fox et al., 1960). Pigment and colour data suggest that metabolization or transformation of yolk may have already begun within the propagule before becoming observable externally and that there is a lag before the quantity of pigment

reaches the threshold at which it is detectable. The late vitellogenesis (mature oocyte), the post-zygotic gastrula, and the vitellaria stages had mild decreases in pigment that did not cause a change in colour externally; however, the transition between the vitellaria and juvenile showed a drop in pigment two times larger and did cause a change in external colour. This suggests that the colour change across stages, examined here as the concentration relative to body weight (size), is likely passive, with no active initiation from the propagule itself. However, this does not mean that the colour is unimportant.

3.5.4 Oocytes Mature at Late Vitellogenic Stage

It was clear that the peaks in all metrics (density, colour, lipids, pigments) occurred at the late vitellogenic oocyte stage, suggesting that the oocytes of *C. frondosa* may be physically and chemically mature (ready to be spawned) at this stage, in respect to ecology of colour and buoyancy. The major changes between mid and late stages of vitellogenesis pertain mainly to colour, while lipids and pigments are already comparable to that of the mature oocyte.

3.6 Conclusion

This exploratory study has set a baseline for our understanding of the characteristics of lecithotrophic propagules and is intended to stimulate future research and has contributed quantitative techniques for the measurement of the physical properties in small propagules. It has also established a clear pattern of changes in buoyancy and colour across life stages and their associated biochemical composition, supported by long-standing knowledge on oogenesis and development in a lecithotrophic holothuroid. The external appearance and biophysical properties are tied to the ecological and energetic needs of each life stage. This study has additionally confirmed existing knowledge of the characteristics

of propagules of *C. frondosa* through ontogeny, which can act as a model/representative and first point of understanding for other species of echinoderms with lecithotrophic development.

3.7 Acknowledgments

We would like to thank Chris Parrish and Jeanette Wells for their help and guidance with the lipid analyses, as well as Derya Akkaynak (Harbour Branch Oceanographic Institute, Florida Atlantic University) for developing the custom Matlab code used in the colour analyses. Thank you to Guillaume Caulier for help with the pigment quantification and Emaline Montgomery for laying the groundwork for this study and for enlightening discussions. Warmest thanks to the Ocean Sciences Field Services Unit for collection of the echinoderms for this study. This research was supported by the National Sciences and Engineering Research Council of Canada (grant to A.M.).

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3.9 Tables and Figures

Table 3.1 Description of samples used for the biophysical metrics (density, colour) and the biochemical analyses (lipids, pigments). 'Pooled' refers to samples where oocytes from all mothers were combined and processed as one sample. 'Averaged' values are where one value was obtained for each mother and the mean of these values is reported. EV= early vitellogenic oocytes (combined), EV1=early vitellogenic oocytes 1, EV2=early vitellogenic 2 oocytes 2, MV=mid-vitellogenic oocytes, LV=late vitellogenic oocytes, S=spawned oocytes, B=blastula, G=gastrula, V=vitellaria, P1= early pentactula, P2=intermediate pentactula, and P3=late pentactula; — = non-applicable. For further information on the sampling, please see Appendix A.

Metric	Stage	Number of Sample Replicates	Number of Mothers	Pooled or Not Pooled	Averaging
photos only	PV	5	—	—	—
biophysical	EV1	5	5	—	—
biophysical	EV2	5	5	—	—
biophysical	MV	15	15	—	—
biophysical	LV	5	5	—	—
biophysical	S	3	—	—	—
biophysical	B	3	—	—	—
biophysical	G	3	—	—	—
biophysical	V	1	—	—	—
biophysical	P1	1	—	—	—
biophysical	P2	1	—	—	—
biophysical	P3	1	—	—	—
biophysical	J	1	—	—	—
biochemical	EV	1	6	pooled	none
biochemical	MV	2	6	pooled	both sample replicates averaged together for avg MV
biochemical	LV	5	1	not pooled	all individuals averaged together for avg LV
biochemical	S	2	—	—	both sample replicates averaged together for avg S
biochemical	G	1	—	—	none
biochemical	V	1	—	—	none
biochemical	J	1	—	—	none

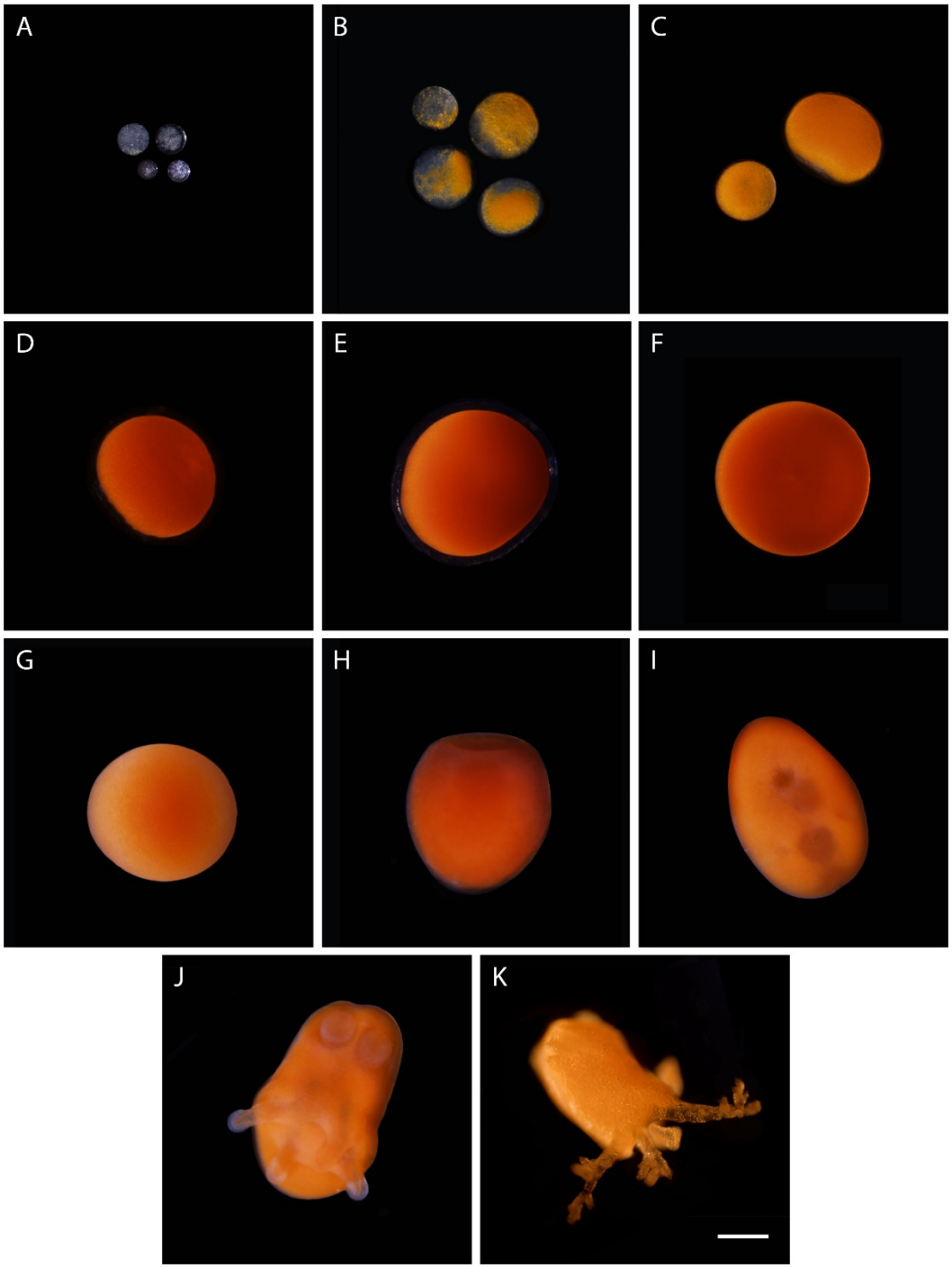


Figure 3.1 Stages of oogenesis and development in *Cucumaria frondosa*. Scale bar in panel K corresponds to 200 µm and applies to all panels. **A:** previtellogenic oocytes (PV). **B:** early vitellogenic 1 oocytes (EV1). **C:** early vitellogenic 2 oocytes (EV2). **D:** mid-vitellogenic oocyte (MV), **E:** late vitellogenic oocyte (LV), **F:** spawned oocyte (S), **G:** blastula (B), **H:** gastrula (G), **I:** vitellaria (V), **J:** pentactula (P), and **K:** juvenile (J). Colour not representative of values in quantitative analysis.

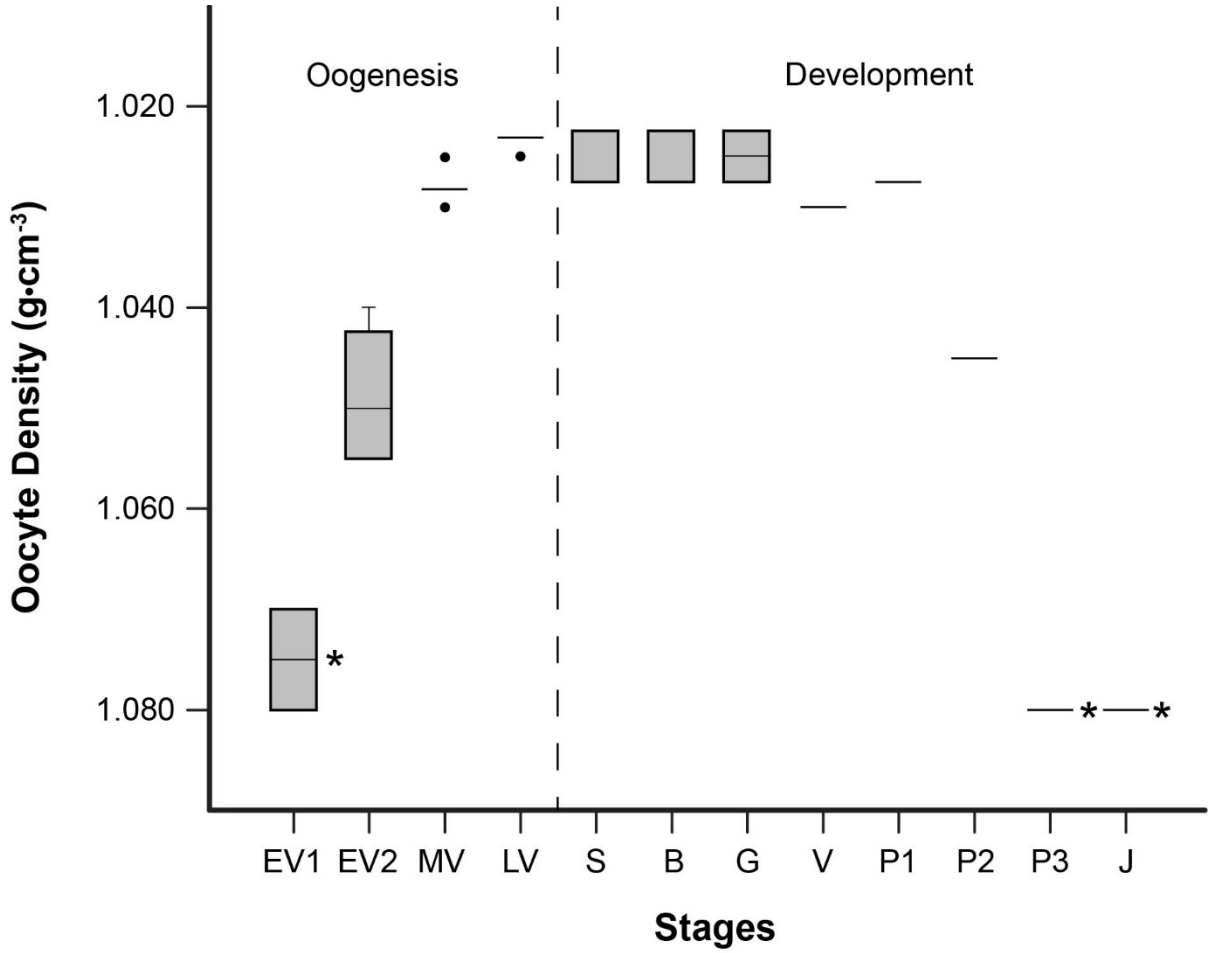


Figure 3.2 Comparison of density across oogenic and early developmental stages of *C. frondosa*. The vertical dashed line separates oogenesis from post-zygotic development. Note the reverse scale. Means that were underestimated (i.e. include density values above the tested range of 1.080 g·cm^{-3}) are indicated by an asterisk (*). EV1=early vitellogenic 1 oocytes, EV2=early vitellogenic 2 oocytes, MV=mid-vitellogenic oocytes, LV=late vitellogenic oocytes, S=spawned oocytes, B=blastula, G=gastrula, V=vitellaria, P1= early pentactula, P2= intermediate pentactula, and P3=late pentactula. The grey bars indicate those samples which fell between the 2nd and 3rd quartiles, the line within the boxes represents the median. Lines without boxes indicate those samples where there was no variability amongst mothers. Whiskers show the minimum and maximum values (5th and 95th percentile). Black dots indicate outliers.

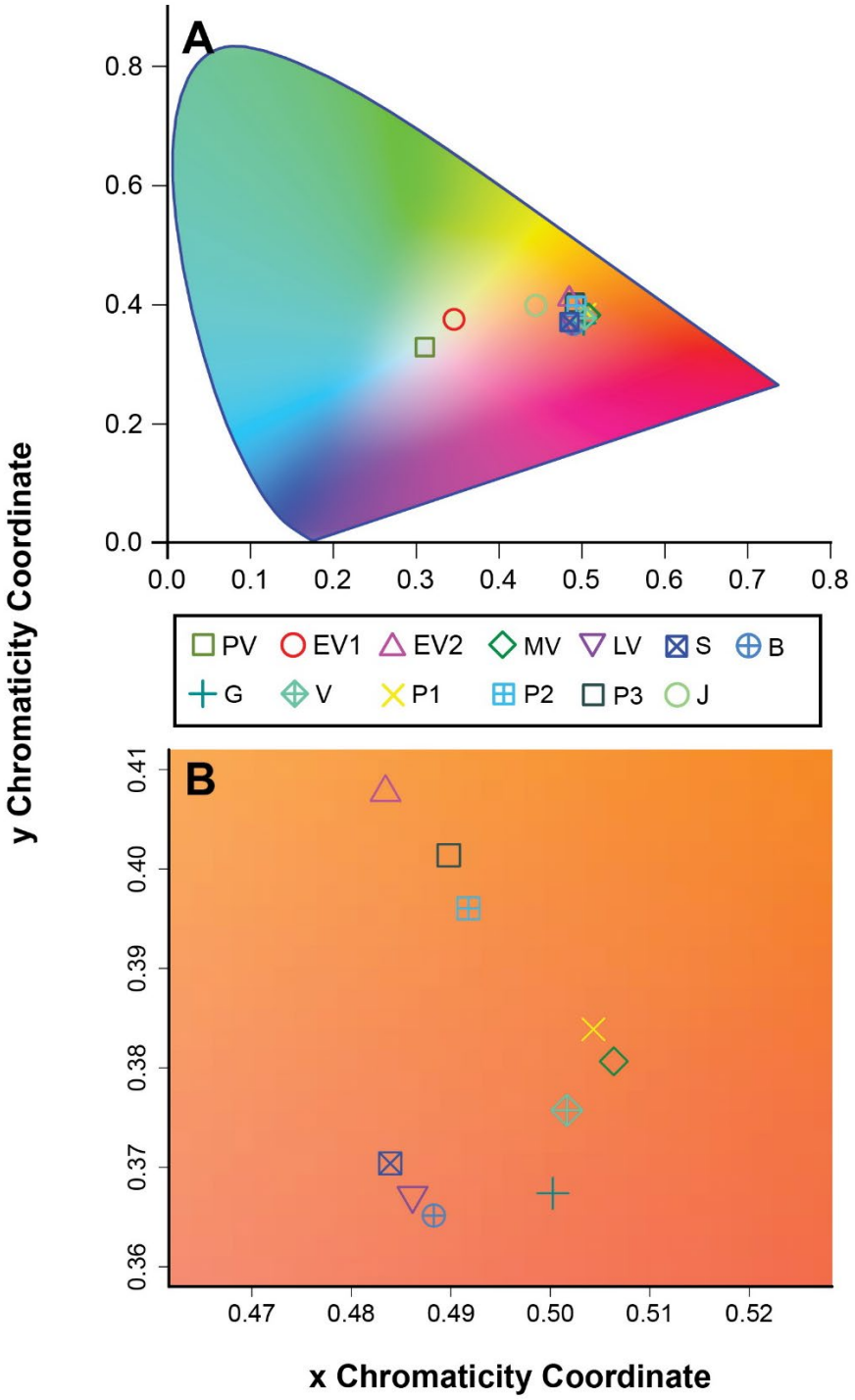


Figure 3.3 Comparison of colour across oogenic and early developmental stages of *C. frondosa*. A: All stages. **B:** Zoom-in on all stages except EV1 and J. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. EV1=early vitellogenic 1 oocytes, EV2=early vitellogenic 2 oocytes, MV=mid-vitellogenic oocytes, LV=late vitellogenic oocytes, S=spawned oocytes, B=blastula, G=gastrula, V=vitellaria, P1= early pentactula, P2= intermediate pentactula, and P3=late pentactula.

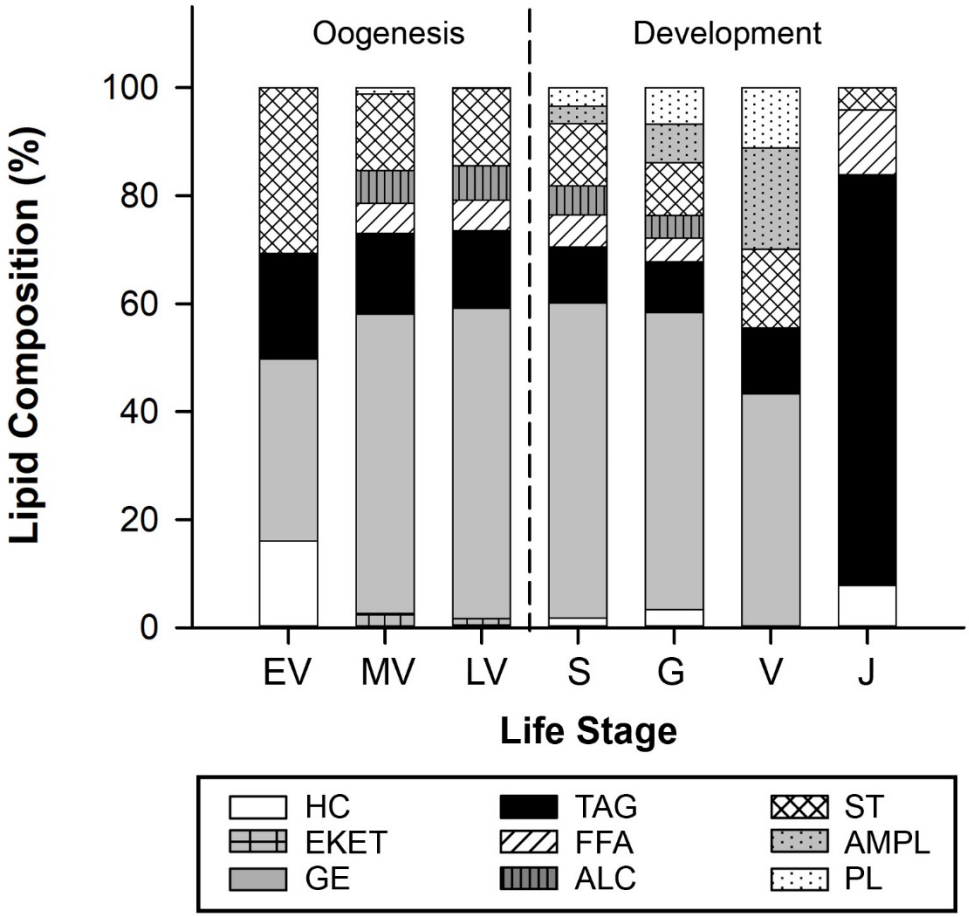


Figure 3.4 Comparison of lipid composition across oogenic and early developmental stages of *C. frondosa*. The vertical dashed line separates oogenesis from post-zygotic development. EV=early vitellogenic oocytes (combined), MV=mid-vitellogenic oocytes, LV=late-vitellogenic oocytes, S=spawned oocytes, G=gastrula, V=vitellaria, J=juvenile; HC=hydrocarbons, EKET= ethyl ketones, GE=glyceryl ethers, TAG= triacylglycerols, FFA=free fatty acids, ALC=alcohols, ST=sterols, AMPL=acetone mobile polar lipids, PL= phospholipids.

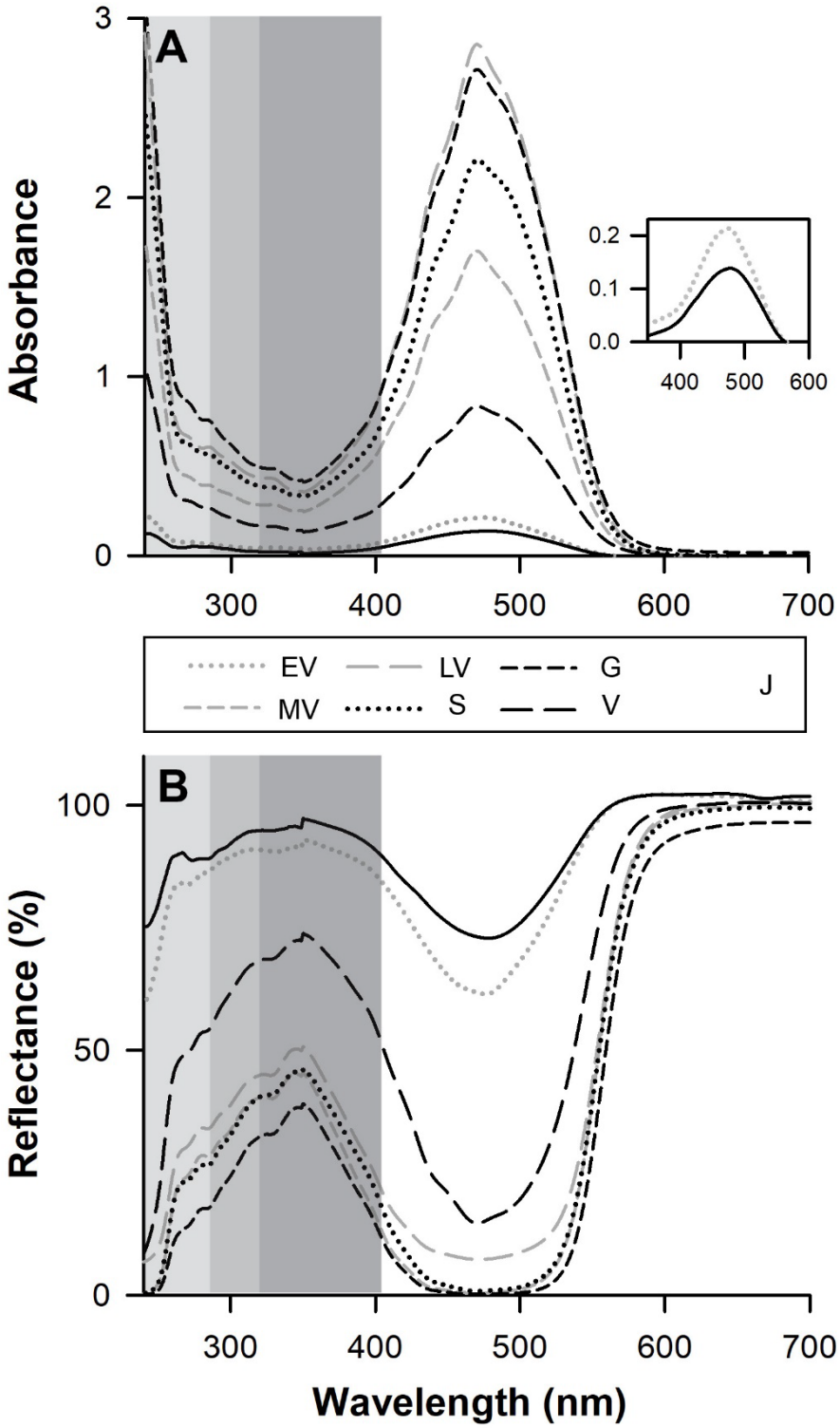


Figure 3.5 Comparison of spectrophotometric metrics across oogenic and early developmental stages of *C. frondosa*. A: Absorbance. **B:** Reflectance. Grey shading indicates the UV portion of the electromagnetic spectrum: dark grey=UVA, medium grey=UVB, light grey=UVC. EV= early vitellogenic (combined) oocytes, MV=mid-vitellogenic oocytes, LV=late vitellogenic oocytes, S= spawned, G=gastrula, V=vitellaria, and J=juvenile.

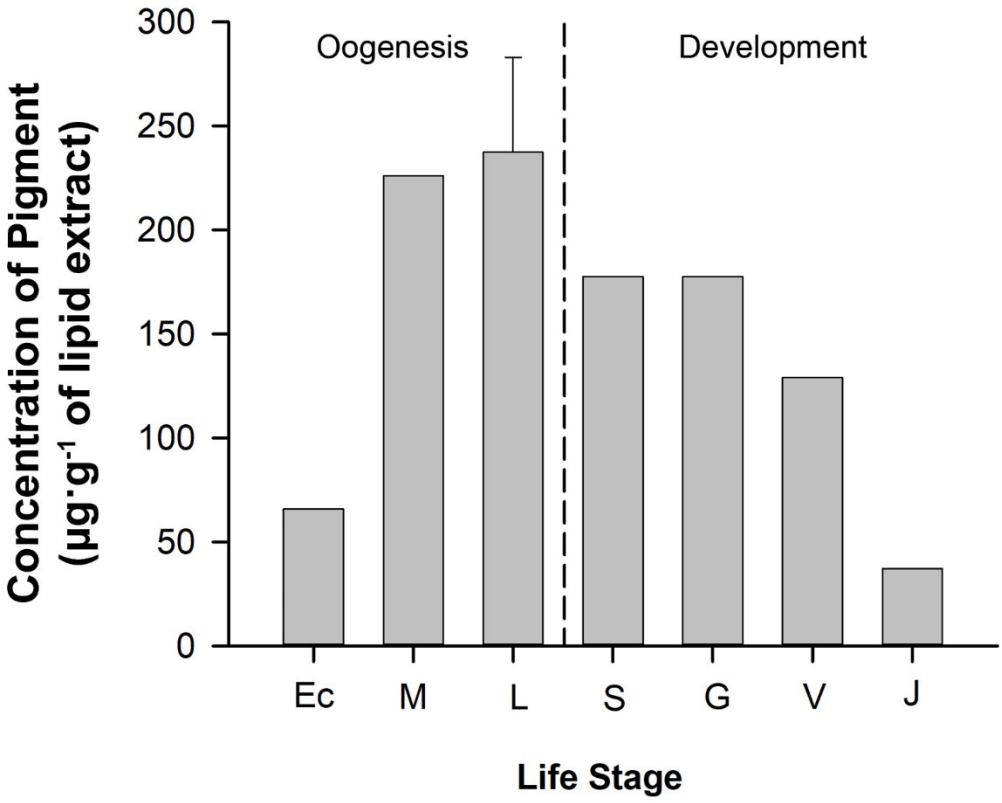


Figure 3.6 Comparison of pigment concentration across oogenic and early developmental stages in *C. frondosa*. EV= early vitellogenic (combined), MV=mid-vitellogenic, LV=late vitellogenic, S= spawned, G=gastrula, V=vitellaria, and J=juvenile. The vertical dashed line separates oogenesis from post-zygotic development. Error bars indicate standard deviation among mothers.

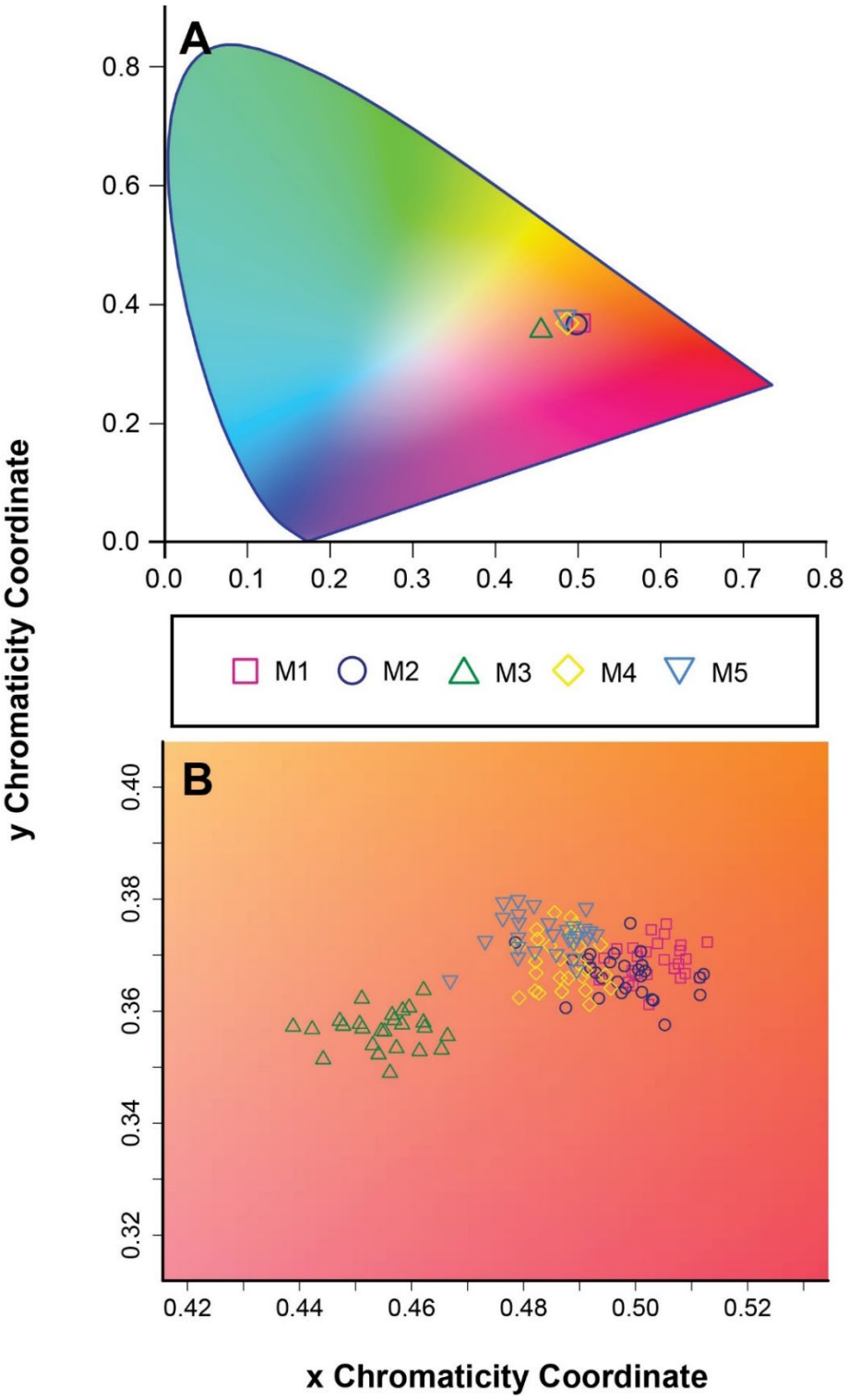
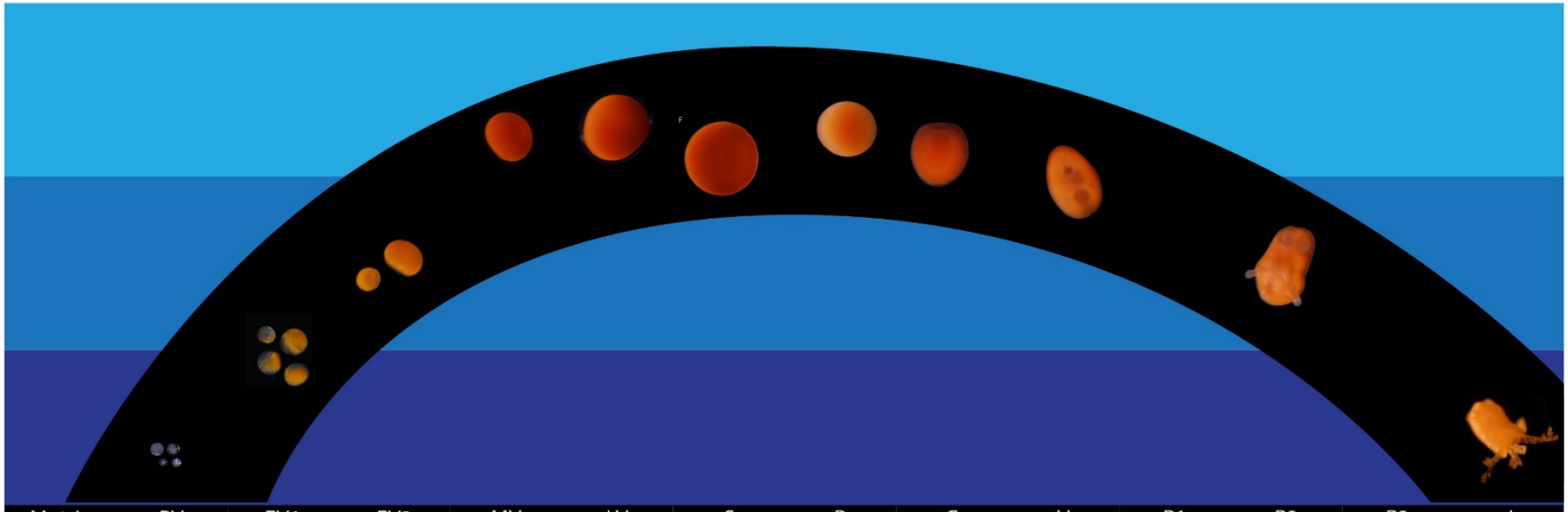


Figure 3.7 Comparison of colour in late vitellogenic oocytes across mothers in *C. frondosa*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. **A:** Averaged values. **B:** Zoom-in across mothers showing all oocytes sampled.



Metric	PV	EV1	EV2	MV	LV	S	B	G	V	P1	P2	P3	J
Density	—	1.075	1.049	1.028	1.023	1.025	1.026	1.025	1.030	1.028	1.045	1.080	>1.080
Colour	(0.31, 0.33)	(0.34, 0.37)	(0.48, 0.41)	(0.51, 0.38)	(0.49, 0.37)	(0.48, 0.37)	(0.49, 0.37)	(0.50, 0.37)	(0.50, 0.38)	(0.50, 0.38)	(0.49, 0.40)	(0.49, 0.40)	(0.44, 0.40)
GE (%)	—	34		56	58	58	—	55	43	—	—	—	0
TAG (%)	—	20		15	14	10	—	9	12	—	—	—	76
Pigment	—	6.75	—	226.11	237.42	177.49	—	177.63	129.12	—	—	—	37.19

Figure 3.8 Conceptual and results summary of findings. Different shades of blue denote different regions of the bell-shaped curve in respect to buoyancy and colour. EV1=early vitellogenic 1 oocytes, EV2=early vitellogenic 2 oocytes, EV= early vitellogenic (combined), MV=mid-vitellogenic oocytes, LV=late vitellogenic oocytes, S=spawned oocytes, B=blastula, G=gastrula, V=vitellaria, P1= early pentactula, P2= intermediate pentactula, and P3=late pentactula; GE=glyceryl ethers, TAG= triacylglycerols. Dashes indicate no data.

3.10 Appendix Chapter 3

A3.1 Description of the stages of oogenesis and development of *Cucumaria frondosa* used in this study.

Stage of Oogenesis	Abbreviation	Time of Year Collected	Size Range (µm)	Appearance	Figure
previtellogenic	none	early November	50 – 60	· located along the germinal epithelium · stuck together in clumps (similar to grape vine), some early vitellogenic and the odd advanced oocyte also found in these clumps · yolk occupying 0% of the cytoplasm	3.1A
early vitellogenic 1	EV1	early November	50 – 330	· located along the germinal epithelium · stuck together in clumps (as described in *previtellogenic appearance*) · yolk occupying 1-99% of the cytoplasm	3.1B
early vitellogenic 2	EV2	early November	270 – 430	· located along the germinal epithelium · stuck together in clumps (as described in *previtellogenic appearance*) · completely full of yolk (light orange or orange yolk either concentrated more on one side, or evenly dispersed) · yolk occupying 100% of the cytoplasm · larger orange oocytes appear bean-shaped as opposed to spherical · follicular cell layer absent in most	3.1C
mid-vitellogenic	MV	late December	370 – 490	· located closer to the middle of tubule lumen · free in the lumen (not stuck together in clumps) · red yolk · yolk occupying 100% of the cytoplasm · follicular cell layer 17-40 µm thick	3.1D
late vitellogenic	LV	late February	500 – 650	· located in the middle of tubule lumen · free in the lumen · red yolk · yolk occupying 100% of the cytoplasm · follicular cell layer 31-47 µm thick	3.1E
spawned	S	late March to early April	580–750	· released into the water column · red yolk · yolk occupying 100% of the cytoplasm · lacks follicular cell layer · forms fertilization envelope after fertilization	3.1F
blastula	B	mid to late April	520–720	· individual cells from cleavage begin to become visually indistinguishable · unhatched still has fertilization envelope and not movement · once “hatched”, rotates around central axis	3.1G
gastrula	G	late April	520–770	· conical shape · archenteron visible · directional movement traveling with apical side forward	3.1H
vitellaria	V	late April to early May	670–900	· ovoid shape · vestibule and podial pit formed, podia visible within	3.1I
pentactula	P	early to late May	800–1090	· ovoid shape · podia emerge, 5 tentacles present · begins crawling	3.1J
juvenile	J	early October	830–1540	· shape resembling adult form · tentacle ramifications and ossicles form · digestive tract functional with open anus	3.1K

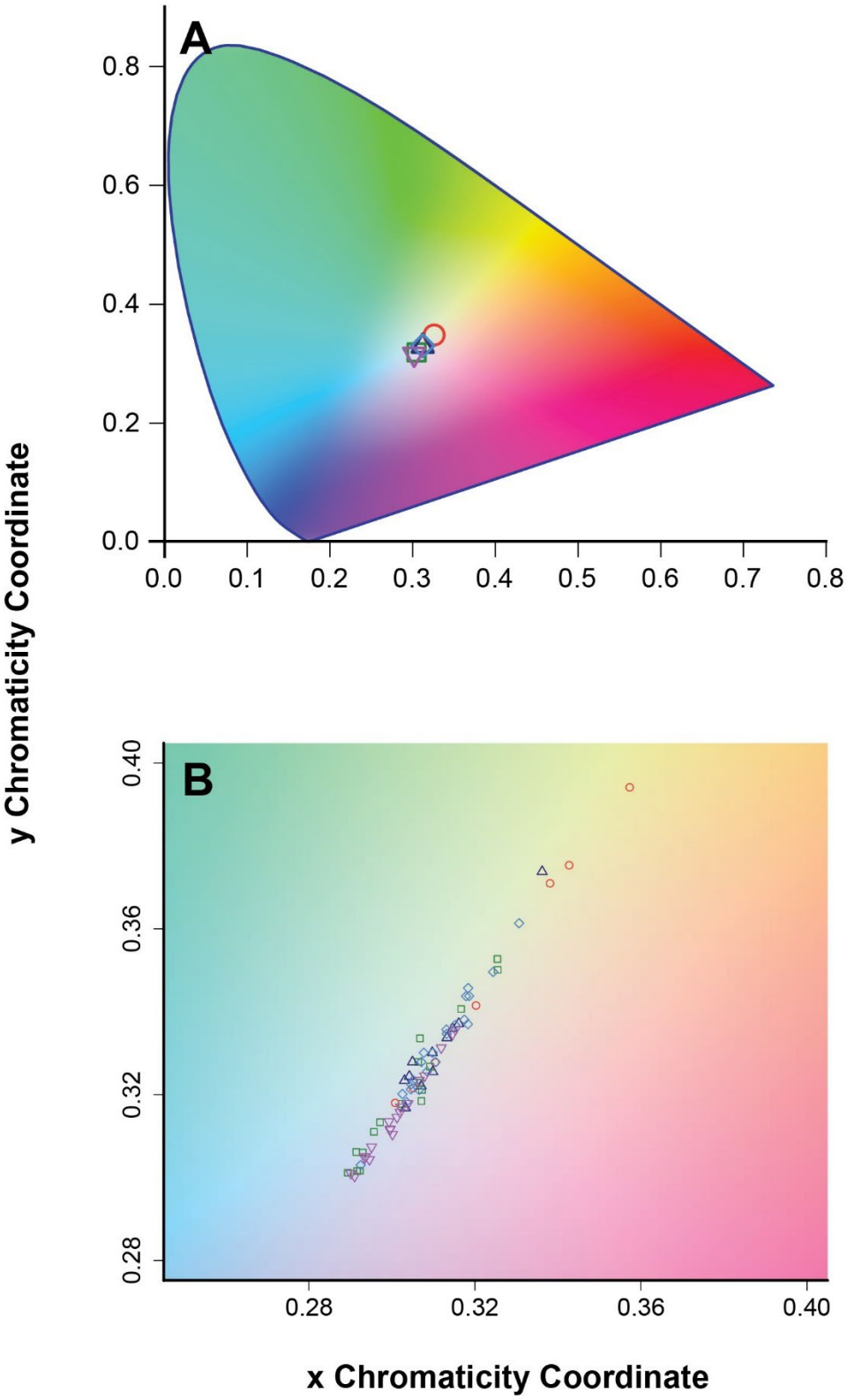
A3.2 Values for lipid composition (percent lipid class composition). Early-vitellogenic oocytes (combined) included a mix of previtellogenic, early vitellogenic 1, and early vitellogenic 2 oocytes. 'Averaged' values are where one value was obtained for each mother and the mean of these values is reported (see Table 3.1). EV=early vitellogenic oocytes (combined), MV=mid-vitellogenic oocytes, LV=late vitellogenic oocytes, S=spawned oocytes, G=gastrula, V=vitellaria, J=juvenile; Avg=average; HC=hydrocarbons, EKET= ethyl ketones, GE=glyceryl ethers, TAG=triacylglycerols, FFA=free fatty acids, ALC=alcohols, ST=sterols, AMPL=acetone mobile polar lipids, and PL= phospholipids. SD for all species that had only one sample is 0.00.

Developmental Stage	HC	EKET	GE	TAG	FFA	ALC	ST	AMPL	PL
EV	16.00	0.00	33.72	19.56	0.00	0.00	30.72	0.00	0.00
MV (1)	0.00	0.00	53.59	17.01	5.59	6.05	15.66	0.00	2.09
MV (2)	0.00	5.23	57.43	12.71	5.59	6.11	12.60	0.00	0.33
Avg MV	0.00	2.62	55.51	14.86	5.59	6.08	14.13	0.00	1.21
SD MV	0.00	3.70	2.72	3.04	0.00	0.04	2.16	0.00	1.24
LV (1)	2.77	0.00	55.27	14.90	4.63	6.05	15.72	0.00	0.66
LV (2)	0.00	0.00	51.93	19.61	5.44	6.74	16.27	0.00	0.00
LV (3)	0.00	0.00	62.08	13.93	5.32	5.55	13.12	0.00	0.00
LV (4)	0.00	0.00	60.35	12.46	6.64	7.02	13.53	0.00	0.00
LV (5)	0.00	5.51	57.88	10.99	6.19	6.13	13.29	0.00	0.00
Avg LV	0.55	1.10	57.50	14.38	5.64	6.30	14.39	0.00	0.13
SD LV	1.24	2.46	4.04	3.28	0.79	0.58	1.49	0.00	0.30
S (1)	0.00	0.00	63.05	11.82	5.73	6.18	13.22	0.00	0.00
S (2)	3.46	0.00	53.68	8.91	6.31	4.55	9.79	6.54	6.76
Avg S	1.73	0.00	58.37	10.37	6.02	5.37	11.50	3.27	3.38
SD S	2.45	0.00	6.63	2.06	0.41	1.15	2.43	4.62	4.78
G	3.34	0.00	54.98	9.47	4.31	4.25	9.79	7.06	6.80
V	0.00	0.00	43.36	12.20	0.00	0.00	14.51	18.79	11.15
J	7.82	0.00	0.00	76.04	12.06	0.00	4.08	0.00	0.00

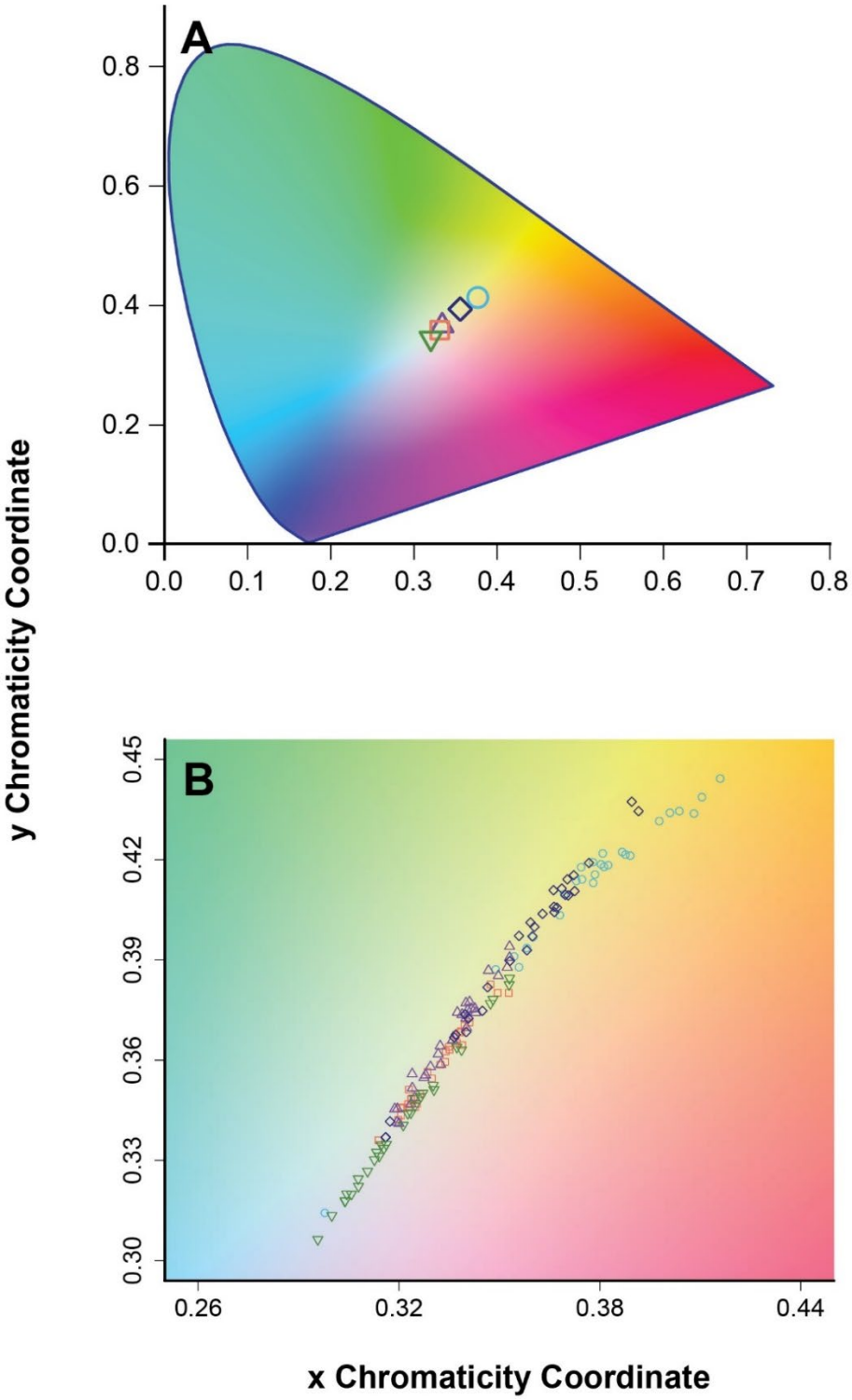
A3.3 Morphometrics of mothers for the maternal effects check (colour) of early vitellogenic 1, early vitellogenic 2, and mid-vitellogenic oocytes. M1=Mother 1, M2=Mother 2, M3=Mother 3, M4=Mother 4, M5=Mother 5, M6=Mother 6, M7=Mother 7, M8=Mother 8, M9=Mother 9, M10=Mother 10, M11=Mother 11, M12=Mother 12, M13=Mother 13, M14=Mother 14, and M15=Mother 15.

Stage	Mother	Relaxed Length (cm)	Contracted Length (cm)	Total Mass (g)	Gonad Mass (g)	Rest of Body Mass (g)
EV1	M1	12.5	11	286.6	13.7	147.2
EV1	M2	13	10.5	322.8	6.4	202.6
EV1	M3	13	10.5	338.8	8.4	189.1
EV1	M4	11.5	10.5	288.2	8.3	130.2
EV1	M5	16.5	13.2	432.6	14	290.3
EV2	M1	12.5	11	286.6	13.7	147.2
EV2	M2	13	10.5	322.8	6.4	202.6
EV2	M3	13	10.5	338.8	8.4	189.1
EV2	M4	11.5	10.5	288.2	8.3	130.2
EV2	M5	16.5	13.2	432.6	14	290.3
MV	M1	11.5	9.5	238.8	18.7	162.2
MV	M2	10	9	172.4	12.7	103.3
MV	M3	20	13	370.2	9.4	134.7
MV	M4	13	9.4	216.5	23	110.3
MV	M5	16	11.5	213	10.8	120.5
MV	M6	10.9	14.5	328.5	17.4	145.2
MV	M7	13	12	283.1	18.6	178.6
MV	M8	14	9.5	250.6	20.5	158.5
MV	M9	11.5	11	143.2	7.2	94.4
MV	M10	13	11.5	382.3	14.4	215.1
MV	M11	11.5	9	222.9	7.6	135.6
MV	M12	10	8.5	237.9	4.8	126.5
MV	M13	11	10	249.5	7.9	135.8
MV	M14	11.7	10.5	254.2	8.7	133.3
MV	M15	12	10	237.7	9.2	138.7

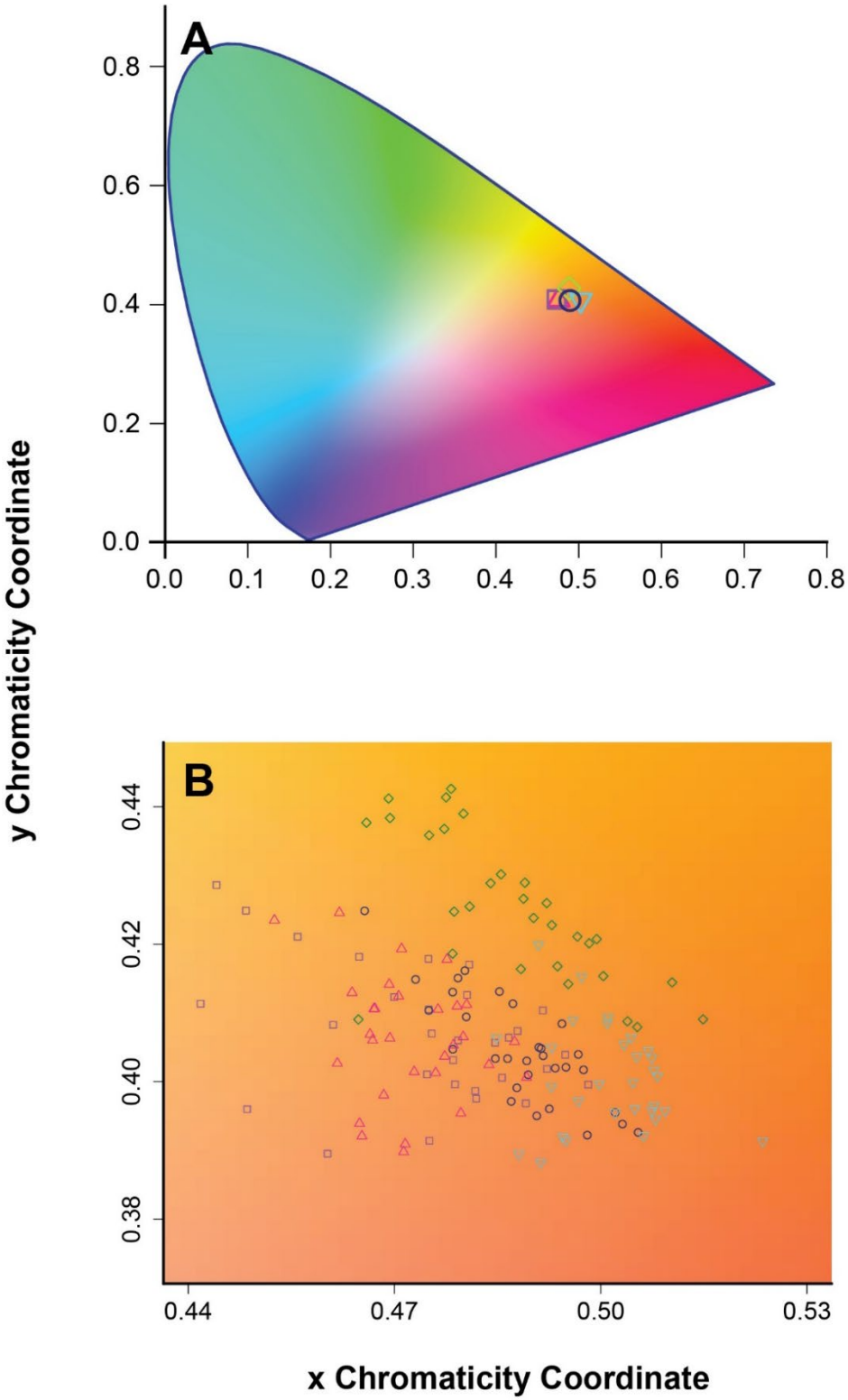
LV	M1	15.5	12.3	300.9	14.6	185.5
LV	M2	14.5	11.5	276.7	15.1	126
LV	M3	13.5	11	222.9	11.5	121.6
LV	M4	11.5	10	297.7	11.8	145.3
LV	M5	11.5	10.5	236.9	11	149



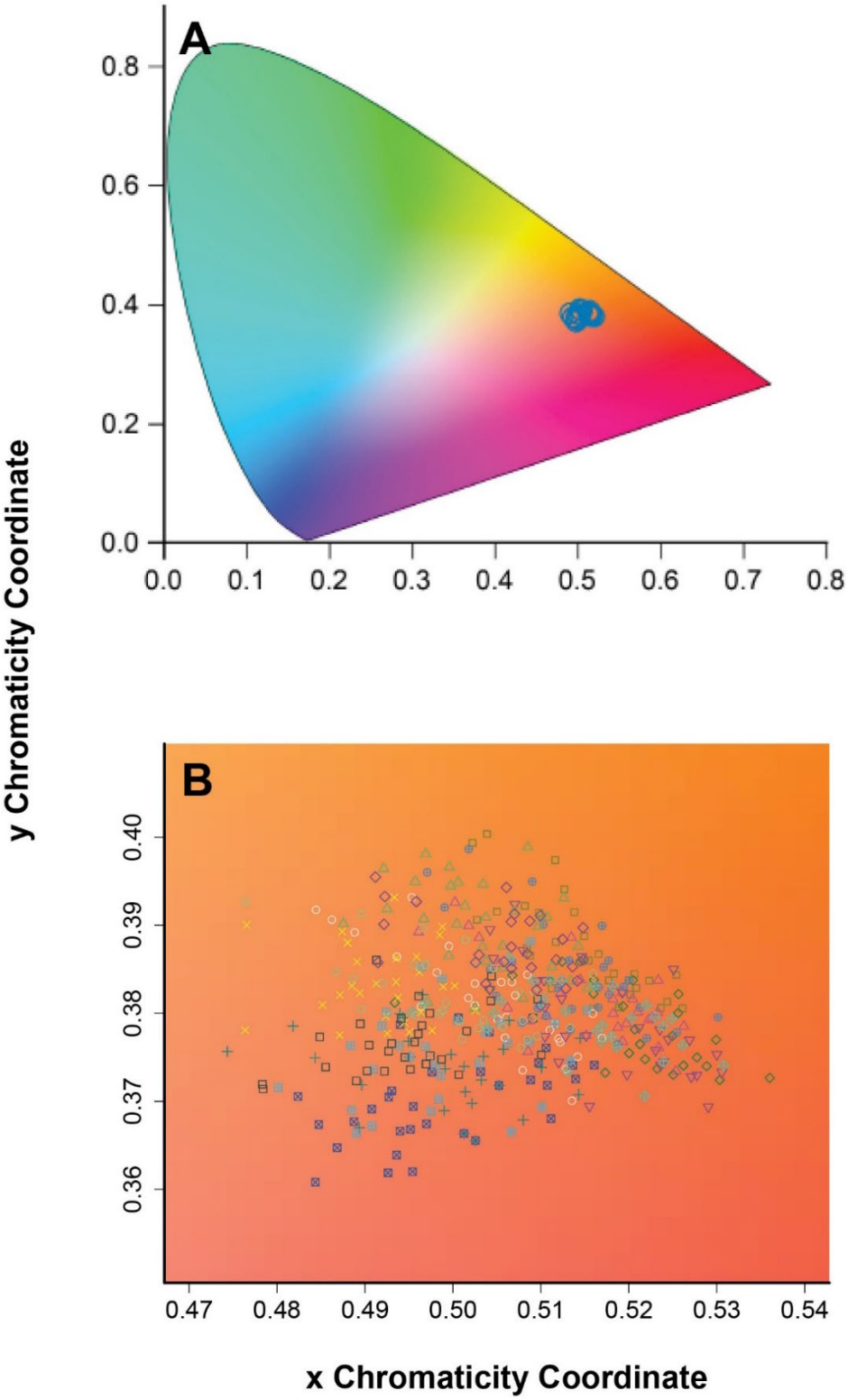
A3.4 Comparison of colour in PV oocytes across mothers in *C. frondosa*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



A3.5 Comparison of colour in EV1 oocytes across mothers in *C. frondosa*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



A3.6 Comparison of colour in EV2 oocytes across mothers in *C. frondosa*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.



A3.7 Comparison of colour in MV oocytes across mothers in *C. frondosa*. Shown as 'x' and 'y' coordinates on CIE's 1931 Chromaticity Diagram. A: Averaged values. Different symbols indicate oocytes from each mother. B: Zoom-in across mothers showing all oocytes sampled.

Chapter 4: General Discussion

4.1 Introduction

Propagules (e.g. spores, seeds and fruit, fertilized eggs, embryos, and larvae) are often dispersed in air or water and occur across biological kingdoms. Invertebrate animals display countless reproductive strategies, described in part by propagule characteristics (e.g. size, shape, colour, viscosity, composition), which influence propagule viability and consequently the survival of externally fertilizing individuals. This raises the broader question 'Why does diversity in propagule characteristics exist in marine invertebrates?'. Lecithotrophic (non-feeding) propagules of echinoderms display a fascinating array of buoyancies and colours, which made them fitting study subjects. More knowledge exists on the biochemical composition of propagules in relation to the ecology of lecithotrophic echinoderms. Thus, in my thesis, I quantified and revealed trends in the external, biophysical properties (of density and colour) and linked them to trends found in lipid and pigment content across: 1) oocytes of different echinoderm species with lecithotrophic development: *Leptasterias polaris*, *Henricia sanguinolenta*, *Henricia perforata*, *Crossaster papposus, Solaster endeca*, *Cucumaria frondosa*, and *Psolus fabricii*, and 2) across stages of oogenesis and development in the sea cucumber *Cucumaria frondosa*. I also provide some reflections on challenges (including opportunistic sampling) and new quantitative methods that will enable substantial advancements in characterizing the physical and chemical characteristics of oocytes to determine potential drivers of these differences.

4.2 Major Findings

I found two key initial mechanistic trends: 1) differences across species were associated with phylogeny (Chapter 2) and 2) differences across life stages of *C. frondosa* were associated with their respective eco-physiological requirements (Chapter 3).

4.2.1 Chapter 2: Interspecific variations in colour, buoyancy, and composition of oocytes of lecithotrophic echinoderms

In the first part of the study (Chapter 2) I found a trend in oocyte density, colour, and percentage of GE and TAG across species that was associated with phylogeny, with the species grouping as follows: *L. polaris*, *H. sanguinolenta*, all remaining species (*H. perforata*, *C. papposus, S. endeca*, *C. frondosa*, and *P. fabricii*) and potential for photoprotection and potential visual predators. Pigment identity and concentration was not associated with phylogeny.

Oocytes of *L. polaris* had the highest density, the lowest percentage of GE and the highest percentage of TAG (all other lipid classes did not show discernable trends related to density), occupied areas on the Chromaticity Diagram between white and pure spectral yellow and had the lowest concentration of its identified pigment. Oocytes of *H. sanguinolenta* had intermediate density between *L. polaris* and all other species, higher percentage of GE than TAG, occupied areas of the Chromaticity Diagram spanning pure spectral oranges and had the second lowest concentration of its identified pigment. The oocytes of the remaining five focal species (*H. perforata*, *C. papposus, S. endeca*, *C. frondosa*, and *P. fabricii*) had the lowest densities, highest percentage of GE and lowest percentage of TAG, and the highest concentrations of their identified pigments, with the exception of low pigment concentration in the oocytes of *S. endeca*.

Unusually high variability in the buoyancy and colour of oocytes was found within the genus *Henricia*. Adults in the genus *Henricia* are characterized by their morphological variability, making it difficult to distinguish between species. Our findings supported existing knowledge on the variability in characteristics of oocytes within the genus (i.e. the oocytes of *Henricia lisa;* Mercier et al., 2008). Given where species of *Henricia* are positioned in the phylogeny of the focal species, oocytes of species in this genus could likely represent an evolutionary transitional stage between negatively buoyant external brooding in *L. polaris* and positively buoyant free spawning in the five other focal species (*H. perforata*, *C. papposus*, *S. endeca, C. frondosa*, and *P. fabricii*).

Some pigments were common across species, but did not vary according to phylogeny (photo-sensitive caroteno-protein: *L. polaris*, *H. sanguinolenta*, and *H. perforata;* astaxanthin: *C. papossus* and *P. fabricii;* and ketocarotenoid: *S. endeca* and *C. frondosa*). Instead, pigments common across certain species may be linked with the diet of the prey: *L. polaris*, *H. sanguinolenta*, and *H. perforata* feed upon filter feeding prey that consumes a certain size class of organisms (algae, bacteria, organic detritus), *S. endeca* consumes *C. frondosa*, and *C. papposus* feeds upon *S. droebachiensis* which consumes algae and *P. fabricii* consumes phytoplankton.

The maximum percent reflectance of the oocytes of all species occurred in the yellow, orange, and red regions (>550 nm) of the visible portion of the electromagnetic spectrum. The colour red is often used as camouflage by pelagic animals, which may render oocytes invisible to crustacean predators such as midwater shrimp (e.g. Lindsay et al., 1999). However, the colour of propagules can also make them more conspicuous to potential predators such as larval fish (e.g. herring) which are known to consume crustacean larvae and to detect prey in the 600 nm range (red; Blaxter, 1968) as well as sea birds, which can detect prey that fall within the red and ultraviolet regions of the spectrum (Shrestha et al., 2013).

In both parts of the study (Chapters 2 and 3), oocytes of *L. polaris*, *H. sanguinolenta*, *H. perforata*, *C. papossus, S. endeca, C. frondosa*, *P. fabricii* as well as all of stages of *C. frondosa* had peak reflectance in the ultraviolet region of the electromagnetic spectrum. Photoprotection can be achieved by three methods: 1) reflection and 2) by absorbing and re-emitting at a longer wavelength (e.g. coral fluorescence; Salih et al., 2000), and 3) through the use of antioxidants (which remove harmful free radicals). Carotenoids are known to be photoprotective in cyanobacteria and plants, and they may have photoprotective abilities via antioxidant properties in echinoid eggs (Lamare et al., 2011). Photoprotection by the reflectance has been suggested in certain acids in berries (Diaz-Barradas et al., 2016). My findings suggest that in addition to antioxidant properties, the propagules of my study species may use reflectance as physical protection from UV radiation as well.

4.2.2 *Chapter 3: Shifts in colour and buoyancy across oogenic and developmental stages of the lecithotrophic sea cucumber* Cucumaria frondosa

In the second part of the study (Chapter 3), I found a trend in the metrics appearing as a bell-shaped curve across oogenic and developmental life stages, echoing the eco-physiological needs of each stage.

Early vitellogenic oocytes had the highest density, the lowest percentages of GE and the highest percentages of TAG, occupied an area on the Chromaticity Diagram between white and pure spectral yellow, and had the lowest concentration of pigment (identified in Chapter 2 as a keto-carotenoid). Mid-vitellogenic oocytes had density values just higher than stages near spawning, high percentages of GE and low percentages of TAG, and high pigment content. Late vitellogenic oocytes, spawned oocytes, and gastrulae had the lowest density of all stages, had the highest percentages of GE and the lowest percentages of TAG, occupied an area on the Chromaticity Diagram in the pure spectral red region, and had the highest pigment concentrations. Vitellaria were amongst those with the lowest density, occupied an area on the Chromaticity Diagram in the pure spectral red region, had slightly lower percentages of GE and slightly higher percentages of TAG, and had a pigment concentration intermediate between the gastrulae and early juvenile. Pentactulae progressively increased in density and were in the pure spectral red region. Early juveniles were amongst the densest stages, occupying an orange space, had no GE and the highest percentages of TAG, and had the lowest pigment concentration.

As expected, trends found across oogenesis and development matched the eco-physiological constraints of each stage. The patterns followed the lifecycle of *C. frondosa* (Hamel et al., 1996): as oocytes are free-spawned and fertilized, they are unprotected by the mother and their positive buoyancy can assist in evading benthic predators. As they begin to ascend, they go through the resulting cell cleavages to become a blastula and gastrula. It is useful to have camouflage from visual predators during their ascent, later protection from ultraviolet radiation at the surface, and buoyancy needs to be enough to allow them to travel to the general area of an appropriate habitat. After that, vitellaria become negatively

buoyant at the time of settlement, then they begin to feed on the substrate as a pentactula, and eventually on phytoplankton as an early juvenile.

There was a gradual increase in GE through oogenesis, then a gradual decrease after fertilization. This likely represents the process of vitellogenesis (adding of yolk to the oocytes) as well as the consumption or modification of yolk through development. The tentatively identified pigment was a keto-carotenoid, which is lipid soluble, so this use of lipid could also be responsible for the change in pigment concentration and colour observed in the post-zygotic stages.

An abrupt switch in use of GE to TAG occurred in the transition from vitellaria to early juvenile. This is likely explained by the switch in feeding that occurs at the late pentactula/early juvenile stage from receiving nutrition from maternal yolk stores to feeding on the substrate, to feeding on phytoplankton in the early juvenile.

In addition, the peak of the bell-shaped curve across metrics (lowest density, closest to pure spectral red, highest percentages of GE and lowest percentages of TAG, and highest pigment concentration) occurred at the late vitellogenic stage, suggesting that the oocytes of *C. frondosa* may be physically and chemically ready to be spawned at this stage.

Taken together, the results suggest that colour and buoyancy are either mandatory or are closely tied to factors that are mandatory during this period of development (early vitellogenic oocyte to early juvenile).

4.3 Difficulty of the Study

4.3.1 Opportunistic Sampling

From my sampling, I was able to conclude general overall mechanistic patterns between biophysical and chemical factors, as well as get a preliminary idea of pigment identity and of the types of animals that could potentially visually detect oocytes. I can also make an educated guess at

photoprotection against UV radiation. Greater sample sizes would definitely have helped solidify the conclusions of Chapter 2. In the absence of logistical and processing time constraints, samples of 5 per group (species or stage) would have been ideal for species that were collected by scuba (e.g. female *S. endeca*, *H. sanguinolenta*, and *C. frondosa*: Ross et al., 2013). Sampling over consecutive years would also have been beneficial. As the Avalon Peninsula was the area of focus in my study, and it is more likely to obtain the desired species from multiple sites, equal numbers of animals from each site in each group (e.g. if 5 sites, have one individual from each site for each species) would have helped to control for random variation (Ruxton et al., 2003). I collected my animals through Memorial University's Field services unit by SCUBA. In the past, a sample size of 5 females has been possible to obtain (e.g. female *S. endeca*, *H. sanguinolenta*, and *C. frondosa*: Ross et al., 2013). Trawl collections may yield 10-50 females of certain species (e.g. female *Leptasterias muelleri*, *S. endeca*, *C. frondosa* in Norway: Falk-Petersen et al., 1982; both sexes *S. endeca* in St. Pierre, NL: So et al., 2010); however, it is not always possible since these collections require ship time and complex and costly logistics. Furthermore, indiscriminate and potentially destructive collections methods are not encouraged.

As for Chapter 3, in order to properly separate propagules from mothers, a mesh barrier isolating each individual (of perhaps 500 μm) would have been useful, where chemical cues and sperm would be able to pass through but not spawned oocytes. This would have allowed me to collect propagules from each mother separately, be able to tell from which mother they came and maintain embryos of separate lineages in different culture baskets as they developed. The propagules from each female would also have to be hearty enough to survive through the hatching of the blastula and metamorphosis, a time where propagules typically experience high mortality. If the embryos from each mother did not survive until early juvenile stage, then there would be 'high drop out' of individuals in the study (Ruxton et al., 2003). This is likely to be the case, as despite the large initial cultures, only 105 individuals (approximately 0.13 g for lipid analysis; minimum weight needed for analysis is 0.1 g) survived to the early juvenile stage at six months old.

Other logistical challenges existed. Different species have different times of the year that the oocytes mature and must be collected and processed fresh from the field (to retain any effects associated with exposure to ecological variables). So, my work had to be scheduled around spawning. Obtaining adults is challenging because out of the individuals obtained, approximately half of them will be male and not contain oocytes. Several individuals will have also not yet reached sexual maturity. Once in the lab, it may not be possible to identify certain individuals as male or female and some females may be too small to offer a sample. Some of the species are solitary, top predators (*S. endeca*, *C. papposus*) and occur only sparsely on the benthos, i.e. one every kilometre or so, making encounters less likely. For more common species, it is always possible to experience a poor year for collections.

Sampling of propagules was challenging for the biochemical analysis because to obtain a large enough mass to be analyzed, oocytes from multiple individuals had to be combined or a large enough mass of propagules of a particular stage had to survive to be used. The mass of oocytes available to sample in the gonad (e.g. at the correct stage of oogenesis) also depended on where the mother was in her reproductive cycle. Moreover, the oocyte sampling for both biophysical and compositional metrics had to be done on the same mother within the same day. So for colour and density measurements, the oocytes had to be obtained in a way that did not harm the mother (via syringe). Then, those same mothers had to be sacrificed later that day to be able to obtain enough oocytes for lipid extracts, which were used in both lipid and pigment analyses.

4.3.2 Methods Contribution

New techniques developed from scratch can contribute immensely to a scientific field but can also be reappropriated from one scientific field for use in another. Such was the case for stable isotope analysis, which was adapted from tracking trace elements in the field of biogeochemistry to studying food webs and tracing seasonal movement of animals in ecology (Martinez del Rio et al., 2009). This led to a boom in ecology publications in the early 2000s (Martinez del Rio et al., 2009). In my thesis, I

successfully reappropriated techniques from the fields of colour science and analytical chemistry to measure the colour and density of the propagules of lecithotrophic echinoderms.

The biophysical metrics in particular are considered unfamiliar to many biologists. I chose to reappropriate a technique from analytical chemistry for measuring the density of samples of material (Kenkel, 2003) to measure the density of the echinoderm propagules. The most sophisticated option is the density gradient column which is a specialized instrument and is mainly used in polymer research. It has been used with seawater to measure the density of cod eggs (Jung et al., 2012) and is able to provide a density value for each egg. It is also possible to calculate the buoyancy using the ascent velocity, as was done in Young et al. (1987); however, this is a much more mathematical approach. Velocity can also be used as a proxy for buoyancy (e.g. Hamel et al., 1993) and is appropriate in cases where a general idea of the buoyancy in a certain context is desired.

To accurately assess colour, photos must be shot in the camera RAW setting so the values remain device-independent. With this being said, it is still unclear as to whether photos can be taken in RAW using a microscope camera. It is possible to do so with an off-the-shelf camera (Akkaynak et al., 2014) if many propagules are collected in a dish, which has been done with capelin and salmon eggs (personal communication Brittany Palm; PhD student in C. Purchase Lab); however, the propagules of my focal species were more prone to bursting in the air as they lack a chorion. Because my photos were not shot in RAW, my findings are not comparable with other studies, but because I used a highly controlled lighting setup and a colour standard, they are still comparable across the samples presented in my thesis. Another improvement could be made in the colour analysis, which was only conducted to the level of describing the colour coordinates but did not go as far as to be able to report easily comparable values within the text of the results. To do this, I would have had to do a calculation to be able to describe the hue (termed 'dominant wavelength') and saturation (termed 'excitation purity') as described in Goodman (2012). Statistics could potentially be performed on these values using the methods as described in Nadal et al. (2011), but it is unclear as to what approach could be taken. Current statistical analysis is used in industry,

largely for the purpose of quality control by comparing the colour of a product to a standard. These steps might have been applied to the data in my thesis with additional time and resources.

Biochemical techniques are more familiar and are regularly used by biologists. I chose to identify the pigments using spectrophotometry, which is a widely accepted method. A more accurate technique would be to separate the pigments using high-performance liquid chromatography (HPLC) together with mass spectrometry to determine the ratio of elements within each pigment. Since pigments were obtained from the lipid extracts in this study, only those that were lipid soluble could be identified. For each species, the spectra seemed to mirror existing pigment profiles in the literature, this made it seem as if there was just one pigment present per species while, in actuality, there could be multiples. If I conducted HPLC, in addition to non-polar molecules, I would be able to separate out polar molecules; determine whether multiple pigments were present; and be able to more accurately identify the pigments. This would significantly refine our understanding of pigments in propagules but was not feasible to achieve within the timeline of my thesis and sample volume available.

The technique I used to identify lipid classes (Parrish, 1987; Parrish, 1999) is considered standard practice and has been successfully used in studies of echinoderm propagules (e.g. Prowse et al., 2009; Falkner et al., 2015; and Ross et al., 2013). A limitation of this technique for use on propagules was obtaining enough material for analysis. In the future, it would be useful if this technique could be adapted to analyze smaller masses (<0.1 g).

Given the exploratory nature of this project, there were many challenges: the metrics were difficult to analyze in relation to one another because they varied widely and were measured at different resolutions and were not directly quantitatively comparable; small and uneven sample sizes had to be addressed; and the colour values had to be interpreted directly off the plots. Despite these challenges, my findings show clear patterns amongst propagule: density, chromaticity colour coordinates, percent lipid class composition, and pigment concentration, all factors which contribute to the colour and buoyancy of lecithotrophic propagules in echinoderms.

4.4 Future Research

This study was formed to begin to answer the broader question 'Why does diversity in invertebrate propagule characteristics exist?'.

My findings suggest that several directions may be taken when conducting more statistically robust or causal studies on this topic going forward. Efforts should be directed towards determining: 1) Which attribute of species with lecithotrophic development (e.g. habitat, predators, diet) affects the buoyancy and colour the most and how, 2) How widespread the diversity of egg colour and buoyancy is within the morphologically variable genus *Henricia*, 3) How effective the various pigments of lecithotrophic propagules are at photoprotection and in what amounts, and 4) To what extent the colour of lecithotrophic propagules help them to evade visual predators, and if the pigment in the propagules is toxic to potential predators.

Lastly, my thesis focused on the propagules of widely distributed lecithotrophic echinoderms collected around Newfoundland. However, it would be desirable to conduct similar studies on the same species collected in other regions, and in other lecithotrophic echinoderms across the globe. This latter approach would allow us to determine if the causes of diversity in colour and buoyancy of propagules is true not only for echinoderms, but for other invertebrates. Answering the broader question of what causes differences in characteristics of invertebrate propagules will allow us to determine the ecological factors that affect the characteristics — consequently, the viability of propagules — which ultimately influences the survival of individuals of externally fertilizing species.

4.5 References

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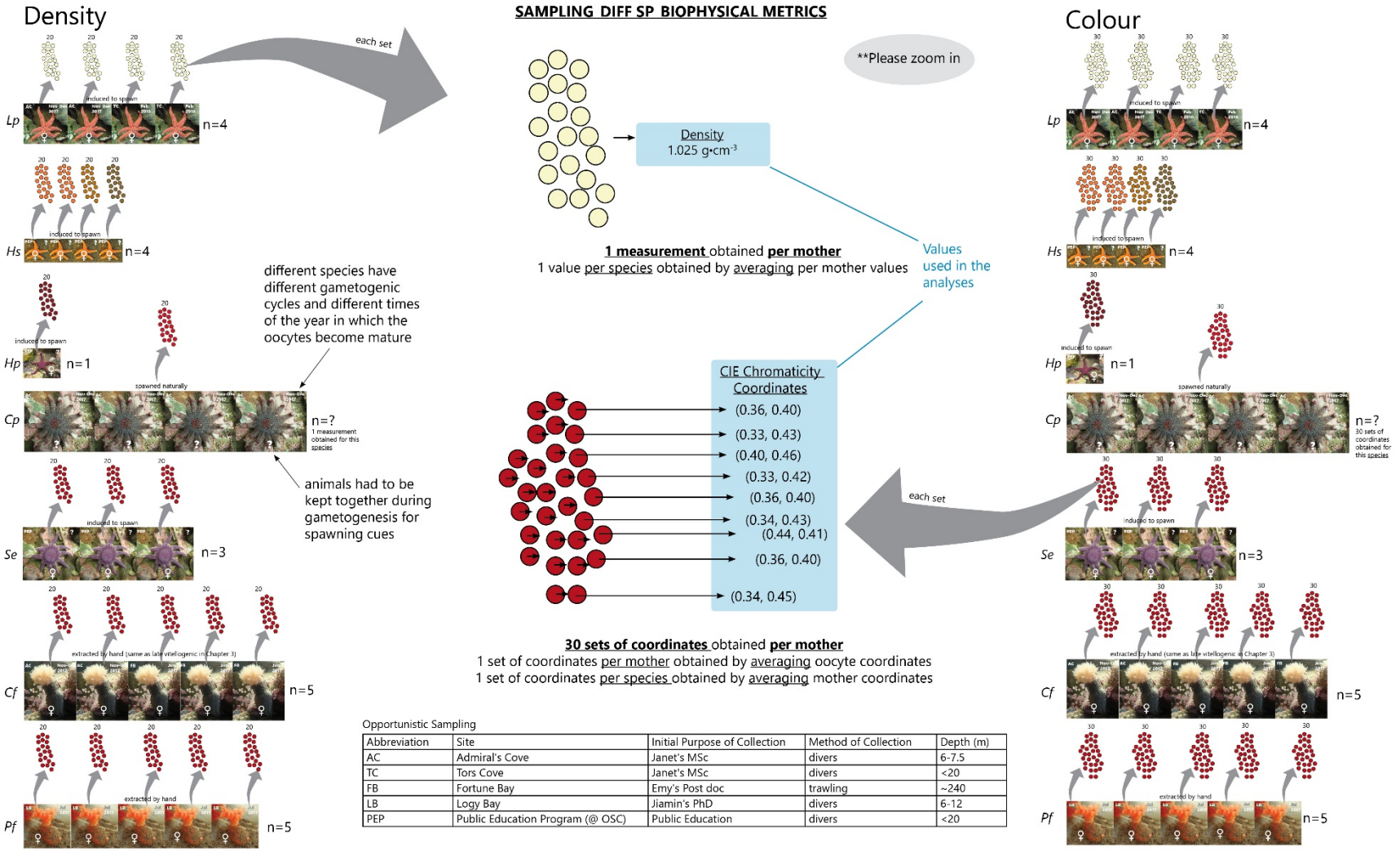
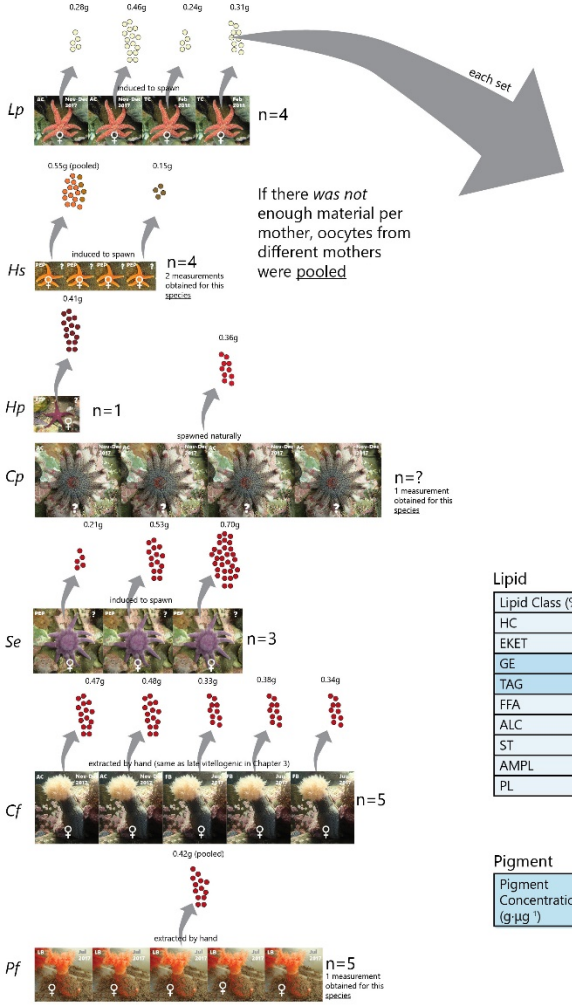
Appendix A: Sampling Diagrams



Opportunistic Sampling

Abbreviation	Site	Initial Purpose of Collection	Method of Collection	Depth (m)
AC	Admiral's Cove	Janet's MSc	divers	6-7.5
TC	Tors Cove	Janet's MSc	divers	<20
FB	Fortune Bay	Emy's Post doc	trawling	~240
LB	Logy Bay	Jiamin's PhD	divers	6-12
PEP	Public Education Program (@ OSC)	Public Education	divers	<20

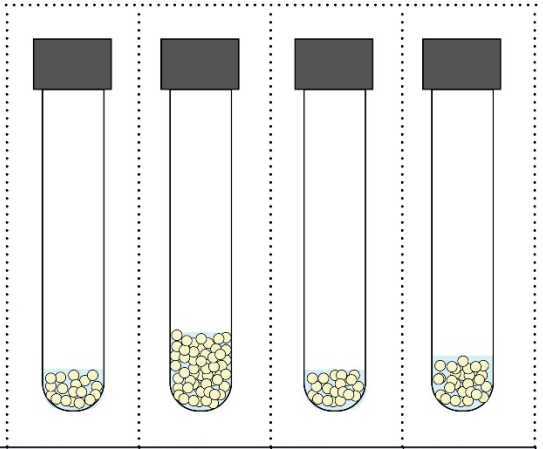
Lipid/Pigment



SAMPLING_DIFF SP_BIOCHEMICAL METRICS

the same animals were used for biophysical and biochemical analyses

If there *was* enough material per mother,



**Please zoom in

0.1 g was enough to do analysis

could not weigh 100% accurately because of seawater in test tube

Lipid

Lipid Class (%)	Mother 1	Mother 2	Mother 3	Mother 4
HC	8	5	0	6
EKET	0	0	0	0
GE	12	11	21	13
TAG	30	31	49	34
FFA	10	8	0	11
ALC	0	0	0	0
ST	0	9	0	13
AMPL	17	13	0	0
PL	24	23	30	23

1 measurement obtained **per mother**
1 value per species obtained by averaging per mother values

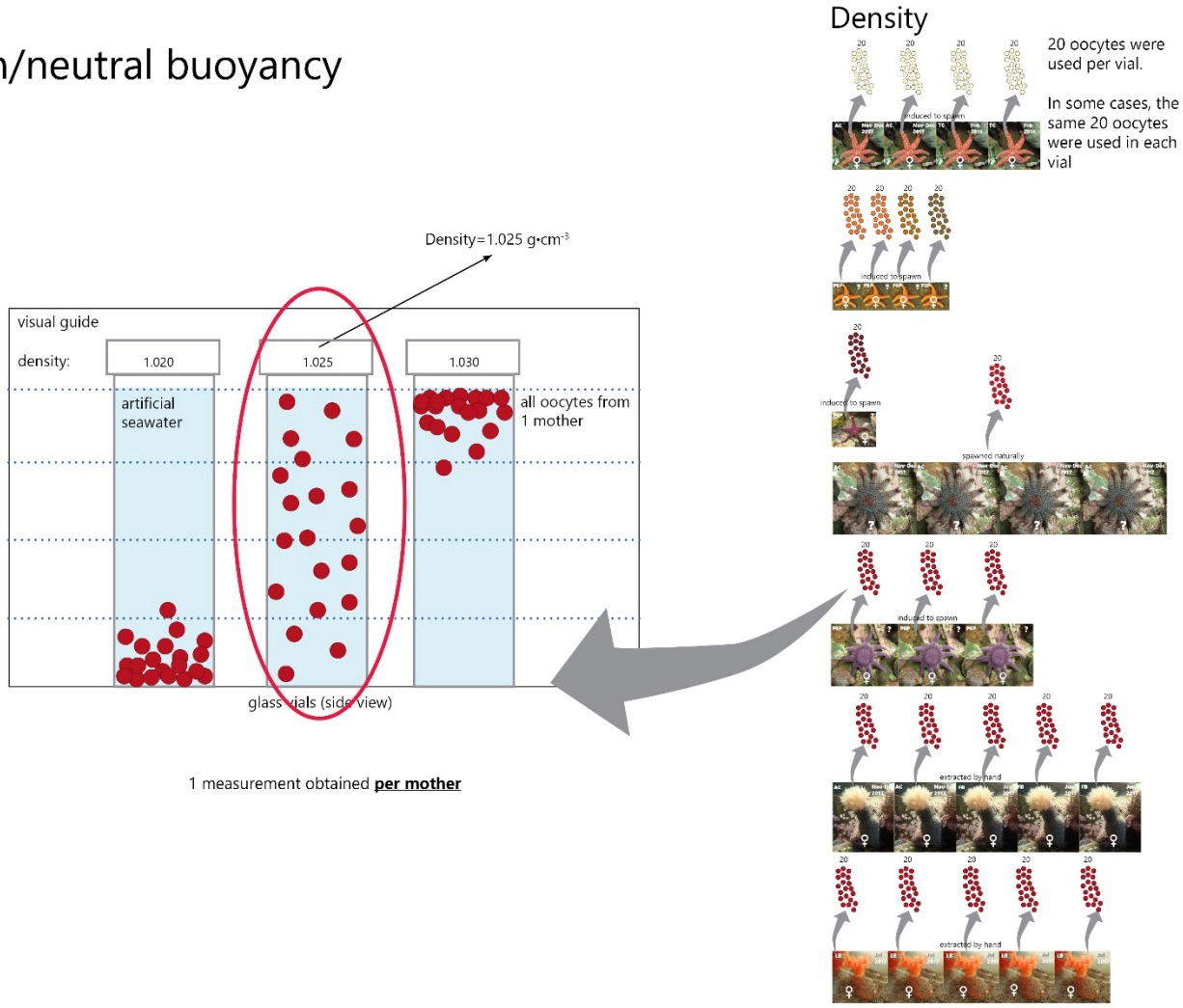
Values used in the analyses

Pigment

Pigment Concentration ($g \cdot \mu g^{-1}$)	16.18	5.23	9.45	5.90

1 measurement obtained **per mother**
1 value per species obtained by averaging per mother values

Appendix B: Hydrostatic Equilibrium/Neutral Buoyancy



Appendix C: Functional significance and characterization of sexual dimorphism in holothuroids

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A version of this manuscript was published in the journal Invertebrate Reproduction & Development in July 2018 (DOI: https://doi.org/10.1080/07924259.2018.1491898).

Abstract

Sexual dimorphism has been reported in all extant echinoderm classes except crinoids, but has never been examined from a phylogenetic, biogeographical, or life-history perspective. This review provides a literature survey of sexual dimorphism in holothuroids and uses this dataset to analyze putative drivers. Sexually dimorphic genital papillae were found in Persiculida and Dendrochirotida but not in other holothuroid taxa. No planktotrophic species (feeding larvae) had known genital dimorphism, though many lecithotrophs (non-feeding larvae) displayed clear morphological differences between male and female papillae. Males with genital dimorphism had either digitate or extensible papillae while females had unbranched papillae. Extensible papillae were common among males in brooding species, suggesting an adaptive advantage for certain reproductive strategies such as sperm transfer or pseudo-copulation. Digitate papillae bearing many gonopore openings were common among free-spawning, lecithotrophic males (~80% of species) and may serve to disperse sperm into the water column. Finally, we found that external dimorphism of the genital papillae in a case study of the dendrochirotid *Cucumaria frondosa* (Gunnerus, 1767) provided a reliable method of sex determination. These results suggest that genital dimorphism among dendrochirotid sea cucumbers is widespread and may facilitate determination of sex in the field where sacrificing animals is not practical.

Key Words: Sea cucumbers, biogeography, genital papillae, echinoderm, sex determination

Introduction

Dimorphic traits are widespread in the animal kingdom and may encompass differences in body size, morphology, cellular chemistry and behaviour (Shine 1989; Bonduriansky 2006; Williams and Carroll 2009; Cooper et al. 2011). Sexual dimorphisms can be classified as primary (specifically related to reproduction), secondary (to enhance reproductive success) and ecological (different ecological roles among the sexes; Williams and Carroll 2009).

Records of sexual dimorphism among dioecious echinoderms go back many decades (reviewed by Hyman 1955; Lawrence 1987). These sex-based differences can include variation in: (i) body size (e.g. *Amphipolis linopneusti*; Stohr 2001, *Ophiodaphne* spp.; Tominaga et al. 2004, Parameswaran et al. 2013, Tominaga et al. 2017, *Arcaster typicus*; Ohshima and Ikeda 1934); (ii) body shape (e.g. *Echinarachius parma*; Hamel and Himmelman 1992, some fossil echinoids; Neraudeau 1993); (iii) internal skeletal organization (e.g. *Urechinus mortenseni*; Mooi and David 1993); (iv) gonad weight (e.g. *Acanthaster planci*; Stump 1994); (v) gonad length/diameter (e.g. *Holothuria leucospilota*; Gaudron et al. 2008) and/or (vi) genital papillae and gonopores (e.g. some holothuroids; O'Loughlin 2001, some echinoids; Chia 1977, Pawson and Miller 1979, *Antedon mediterranea*; Barbaglio et al. 2009).

Sexual dimorphism in echinoderms is presumed to be related to reproductive functions, such as increased female body size to house developing eggs (e.g. ophiuroids and echinoids) or modifications of the papillae in males to enhance spawning efficiency (e.g. holothuroids and echinoids, O'Loughlin 2001). However, the prevalence and practicality of using sexual dimorphism to distinguish sex among dioecious echinoderm species has not been explored in any depth. The significance of sexual dimorphism in echinoderms relative to phylogeny, biogeography and life history also remains relatively unexplored with only a few authors

discussing these topics in a small number of taxa (e.g. Chia 1977; Pawson and Miller 1979; O'Loughlin 2001). Notable examples of holothuroid species with external sexual dimorphisms in the genital papillae were described (e.g. cold-water cucumariids: Hamel and Mercier 1996b; Levin and Stepanov 2005, other dendrochirotids: O'Loughlin 2001), suggesting that holothuroids are an ideal group to further explore the presence and purpose of sexual dimorphism in this class and echinoderms in general.

This study surveys the presence of external and internal sexual dimorphisms in the echinoderm order Holothuroidea and assesses the practicality of using sexual dimorphisms to determine sex among individuals in a case-study species (*Cucumaria frondosa*; Gunnerus, 1767). A comprehensive dataset of external and internal sexual dimorphisms from the holothuroid literature was gathered including gonad size and colour, along with characterization of the genital papilla. Genital papillae have been previously reported to be dimorphic in holothuroids (e.g. O'Loughlin 2001), but the presence of sexual dimorphism among holothuroids has not been examined from a phylogenetic or life-history perspective. Finally, the commercial holothuroid *Cucumaria frondosa* was used to test the reliability of characterization using internal and external sexual dimorphism.

Consistent and accurate non-lethal means to sex individuals are particularly valuable in the context of fisheries management, aquaculture and scientific studies that may want to separate males and females into different tanks or treatments. Finally, determination of sex in the field can be extremely valuable for ecological and survey studies, when sacrificing animals is not always practical, possible or ecologically responsible.

Methods

Dataset collection

Gonad size and distinct gonad colour differences were considered internal dimorphisms, while variation in shape and size among genital papillae were considered external dimorphisms. Genital papillae were defined as finger-like outgrowths from the body wall, with one or many gonopore openings that connect the gonoduct to the external environment. The dataset included representatives from seven orders of holothuroids: Dendrochirotida, Elasipodida, Holothuriida, Molpadida, Persiculida, Synallactida and Apodida. Taxonomic delineations were confirmed via the World Registry of Marine Species (WoRMS) and a recently updated phylogeny (Miller et al. 2017). Information provided on WoRMS was also used to determine global distribution patterns of each species. Life-history traits (Poulin et al. 2001) included larval nutritional mode (planktotrophic vs. lecithotrophic) and larval development location (brood-protected vs. unprotected). We gathered a dataset (n=77 species) from the literature on species with and without sexual dimorphism to assess the types and frequency of sexual dimorphism. Studies were only included in the analysis if they explicitly discussed the presence (or absence) of external and/or internal sexual dimorphisms. We were specifically interested in the presence or absence of confirmed dimorphic genital papillae and focused on studies that clearly discussed these features rather than those that made non-specific claims about the presence/absence of overall morphological differences between males and females (often related to the size, not the genital papilla morphology).

Case study – Cucumaria frondosa

We explored the sexual dimorphism present in the commercial sea cucumber *Cucumaria frondosa* (Dendrochirotida: Cucumariidae) in order to determine the practicality of using external morphological differences to determine sex. This free-spawning, lecithotrophic species

can be found in the North Atlantic and Arctic from ~5 to 300 m (Hamel and Mercier 1996a). It is exploited by a commercial fishery in several countries (USA, Iceland, Russia, Canada) and has been identified as a candidate for multi-trophic aquaculture (Nelson et al. 2012). Therefore, a reliable and accurate method for determining sex in *C. frondosa* will be beneficial for both field-based studies and population management.

To better understand the reliability of the sexual dimorphism of the genital papillae and its accuracy in telling the difference between males and females in a gonochoric species, we examined the external and internal morphology of male and female *C. frondosa* (already determined to exhibit a clear sexual dimorphism of the gonopore; Hamel and Mercier 1996b) collected from two different geographic areas in Newfoundland (eastern Canada; n ~ 200 per area); Logy Bay/Bay Bulls (47°40'00.3"N 52°41'54.4"W, inshore population), and Fortune Bay/Grand Banks (47°14'28.0"N 57°08'35.2"W, offshore population). Individuals from inshore and offshore populations typically have different body lengths and body wall colours (pers. observ. E. Montgomery 2018). Individuals were collected during the main fishing season (June-September 2017), coinciding with early to mid-gametogenesis. They were first examined alive for characterization of the genital papilla (see method below), followed by assessment of gonad colour from surgical inspection, and of sex using a gonad smear. Permanently-visible papillae do not seem to change in shape or size during the reproductive cycle except during spawning, where the female papilla stretches out as eggs are released (pers. observ. E. Montgomery 2018),

Two main morphological features were compared between males and females, based on previous reports of sexual dimorphism in sea cucumbers (O'Loughlin 2001), other species of *Cucumaria* spp. (Levin and Stepanov 2005), and the first report in *C. frondosa* (Hamel and Mercier 1996b). Genital papillae (external) and gonad colour (internal) were compared between

males and females to assess any differences between sexes and locations (e.g. inshore versus offshore). Papillae length (mm), width (mm) and the number of branches per cluster of papillae were measured in individuals (~9-15 cm contracted length) with naturally-deployed tentacles. A comparison of sexing reliability between external and internal methods was also made after confirming the presence or absence of oocytes, a feature indicative of females in dioecious species (n~200 individuals). The presence of sperm was used as a feature indicative of males.

Results

General morphology and types of papillae

Genital papillae, when present, were finger-like projection(s) or a bulge of tissue outside of the body wall, with one or many gonopore openings at the tip. These papillae were commonly found between the oral tentacles (or close to them), often on the dorsal side of the individual (the side that typically faces up). Genital papillae were permanent and visible externally in some species (i.e. present throughout the year, 30% of species), transient in others (i.e. only clearly visible during spawning events, 51% of species) and entirely absent in some species (i.e. some brooders, 19% of species). External sexual dimorphism in the genital papillae, when present, was only found in species with permanently visible papillae (n = 23 species, Table 1, 30% of species). Two main appearances of permanent genital papillae have previously been reported for male sea cucumbers: digitate (branching) and extensible (elongated/flexible) forms (O'Loughlin 2001). In contrast, female papillae, where present, were smooth and not elongated. We identified 16 species with digitate male papillae and 7 species with extensible male papillae (Table 1). Of these species with sexual dimorphism, male papillae (overall) were double the length of female papillae (from the base on the sea cucumber body wall to tip of the papillae; 4 ± 2 mm vs. 2.2 ± 2.1 mm respectively; Table 2). In males, digitate papillae were longer on average than extensible

papillae (4.6 ± 1.3 vs. 2.2 ± 0.5 mm), though this difference could be confounded by overall body size since whole groups of sea cucumbers (e.g. brooders) tend to be smaller overall.

Phylogeny and biogeography

Two of the six orders of sea cucumbers in the dataset possessed external sexual dimorphism in their genital papillae. Approximately 62% of all dendrochirotid species in the dataset (total n =45) displayed external sexual dimorphism (Fig. 1A). Two families contained nearly all of the species in the dataset with external dimorphism: Cucumariidae and Psolidae (Fig. 1B). Approximately 62% and 67% of species belonging to these two families displayed sexual dimorphism of the genital papillae, respectively (Fig. 1B). Moreover, external sexual dimorphism was only reported for one species of Persiculida (Fig. 1A). No sexual dimorphism was described in Elasipodida, Holothuriida, Molpadida, Synallactida or Apodida.

The dataset (n = 77 total species) contained records from all of the world's oceans. There was a slight geographic trend among species displaying sexual dimorphism of the genital papillae; these species were mostly located in temperate-cold waters in both the northern and southern hemispheres of the Pacific and Atlantic oceans, the Antarctic and in the Arctic (Fig. 2). However, there are examples of tropical species with sexual dimorphism including *Pseudocolochirus violaceus, P. unica, Colochirus robustus* and *Psolus lawrencei*, all of which are dendrochirotids. While members of the order Dendrochirotida occur across a broad latitudinal gradient, most research in lower latitudes focuses on commercial species in the order Holothuriida, potentially biasing the relationship between sexual dimorphism and latitudinal gradient.

Larval nutritional mode and location of larval development

The presence and morphological type of sexually dimorphic genital papillae varied among species with different larval nutritional modes. Representatives with sexual dimorphism were found in lecithotrophic species (with larvae relying on maternal provision), but not in planktotrophic species (with larvae feeding in the plankton; Fig. 3). This pattern was consistent across all taxa examined. External sexual dimorphism was also present in species with different types of lecithotrophy: in both brood-protected and pelagic development. Approximately 44% of internal brooders, 50% of external brooders and 78% of species with pelagic lecithotrophic larvae displayed external sexual dimorphism (Fig. 3). Interestingly, external sexual dimorphism was found in 100% of cucumariid species and 60% of psolid species with pelagic lecithotrophic larvae in the dataset (from studies that explicitly assessed sexual dimorphism of the genital papillae).

Male-specific morphology and location of larval development

Digitate papillae were found in species that displayed both brood-protected and pelagic lecithotrophic development (Fig. 4). Approximately 50% of brooding and 100% of free-spawning species had males with digitate genital papillae (Fig. 4). In contrast, extensible papillae were only found among species with internal and external brooding, never in free spawners (Fig. 4). Approximately 50-60% of brooding species had extensible male papillae (Fig. 4). While most species had either digitate or extensible papillae, males of two internally-brooding species displayed papillae that were digitate and extensible (e.g. *Echinopsolus charcot* and *Echinopsolus splendidus*).

Case study – Cucumaria frondosa

Males and females of *Cucumaria frondosa* displayed clear external sexual dimorphism of the genital papillae regardless of their provenance (inshore vs. offshore populations). Male and

female papillae were located between the oral tentacles and commonly were on the “dorsal” side of the animal (the side typically facing up; Fig. 5). Examination of the genital papillae in both sexes was possible when the tentacles were naturally deployed (or after provoking the extrusion of the aquapharyngeal bulb with a pressure on the mid-body). Identification of the sex on the basis of the genital papilla was consistently successful in our experience (Fig. 5A-D). An investigator with knowledge of the dimorphism showed full accuracy with this technique (n~200).

Males had digitate papillae, consisting of clusters of two or more (up to 15) protrusions or branches, each with its own opening at the apex (Fig. 6A-D). Male papillae ranged in length between 1-4 mm and in width between 2-4 mm for individuals that ranged in contracted length from 9 to 15 cm (Table 3). The width of male papillae increased with the number of branches in the cluster but the size of the papillae did not display any relationship to the size of the animal. Papillae clusters of 2-5 and 9 branches were the most common when the frequencies were examined in a subset of male individuals (Table 4). In comparison, females had smooth, non-digitate papillae between their oral tentacles (Fig. 6E-F). Female papillae were ~2 mm long and 2 mm wide (Table 3). The morphological differences between these papillae types are clear during natural spawning events with multiple streams of sperm visible from digitate male papillae (Fig. 7A) and a single string of eggs being released from smooth, non-digitate female papillae (Fig. 7B).

In comparison, gonad colour (internal sexual dimorphism) was highly variable among sexes and geographic locations. Males collected inshore (protected coastal areas) consistently had pale peach/orange gonads (Fig. 8A, B). Males collected offshore (exposed coastal areas) had dark red gonads, very similar in colour to females from both areas (Fig. 8C, D).

Discussion

Distinct patterns emerged between the presence of external sexual dimorphism and phylogeny and life-history strategies in holothuroids. External sexual dimorphism of the genital papillae is typical of species with permanently visible papillae, and almost exclusive to Dendrochirotida, whereas internal sexual dimorphism of the gonad is common in species from most taxa. Of the dendrochirotids, species with external sexual dimorphism belong to two main families, Cucumariidae and Psolidae. This trend may partially be explained by the relationship between phylogeny and life-history strategies. In the dataset, there was a strong link between lecithotrophic development (maternally provisioned larvae) and external sexual dimorphism. There was also some correlation with geographic distribution since polar and cold waters are hot spots for lecithotrophy. Many representatives of Cucumariidae and Psolidae are lecithotrophic, which makes it difficult tease out phylogenetic relatedness from larval nutritional mode. However, not all lecithotrophic species reproduce the same way (e.g. free-spawning vs. brooding) and possess the same type of sexual dimorphism in their genital papillae.

We found two types of permanently visible male papilla and one type of permanently visible female papilla in lecithotrophic species. Males had either digitate (branching) or elongated papillae that extended out from their body walls. In contrast, females typically had a cone-shaped papilla that was unbranched or shorter on average than the papillae of males, though size data were only available for a few species (13 of 23 species with sexual dimorphism). O'Loughlin (2001) reported that the presence of papillae was common in males of brooding species from New Zealand; however, he did not establish a strong connection between papillae types and types of brood protection. Here, we found that males of both brooding and free-spawning lecithotrophic species could have digitate papillae, but that elongated/extensible forms

of papillae were restricted to brooding species. These morphological differences could therefore have evolved to support the different spawning strategies in sea cucumbers. Importantly, the dataset also contained numerous reports of species with visible papillae but no mention of sexual dimorphism that could be explored further (see Table S1 for examples), which might eventually provide additional examples of dimorphic papillae.

Digitate papillae may benefit both brooders and free-spawners by increasing the volume and spread of sperm during spawning events. *Cucumaria frondosa*, for example, can display as many as 27 different branches, each with their own openings (Levin and Stepanov 2005). In contrast, brooding species may rely on internal fertilization / copulation that requires different sperm-emitting structures compared to broadcast spawners. Extensible papillae like those found in *Gephyrothuria alcocki* may facilitate more directed sperm transfer close to the gonopore of the female and other copulation-based behaviours (O'Loughlin 2001). Some brooders have evolved a combination or hybrid digitate-elongated papilla that may further increase fertilization success (e.g. *Echinopsolus charcoti* and *Echinopsolus splendidus*; O'Loughlin 2001).

While the morphology of male papillae is likely related to the fertilization strategy, female papillae are presumably designed to enhance the release of eggs in broadcast spawning species. Lecithotrophic eggs are typically three to ten times larger in diameter than planktotrophs (Vance 1973; McEdward and Chia 1991; Levitan 1996). The cone-shaped papillae of broadcast-spawning lecithotrophic females may facilitate the expulsion of large eggs by forcing them from a larger space at the base of the papillae through the smaller opening at the tip by muscular contractions. This process is particularly visible in species like *C. frondosa*, *C. miniata*, *Psolus fabricii* and *P. phantapus* that release large "strings" of eggs (reflecting the shape of the gonad tubule) during spawning that could stay intact for several minutes post spawning. The release of

such egg strings, combined with gentle currents generated by the tentacles and positive egg buoyancy could help eggs avoid entrainment to the benthos and benthic predation (Johnson and Shanks 2003; Mercier et al. 2013). This strategy differs from that of planktotrophic species such as *Holothuria* spp. and *Isostichopus* spp. that release most of their eggs in a single, rapid spurt, from a genital papilla that becomes visibly engorged just before spawning. Interestingly, females of brooding species often do not possess papillae at all, since sperm is either transferred close to or reaches the female gonopore by water currents (O'Loughlin 2001). Therefore, the form of genital papillae in sea cucumbers appears to be linked to life history and reproductive needs.

Examination of the genital papillae is an effective way to sex sea cucumbers in species that possess sexual dimorphism throughout their reproductive cycle. In the present study, an experienced investigator could correctly identify males and females 100% of the time. The use of this technique has several distinct advantages. The ability to confirm sex in living animals enables collection of gender-based field/fisheries data, sorting for broodstock or experimentation and sex determination where sacrificing the animal or examining gonad samples are not possible. While sex differences can sometimes be confirmed via a gonad smear, or by examining gametes under the microscope, taking the time to do this is not always practical. In addition, results can be ambiguous when gonads are in the recovery stage (following spawning) as mature gametes have either been released or phagocytized (Hamel et al. 1993). Another challenge with using gonad morphology to determine sex is geographic variability. For instance, the gonad morphology of *C. frondosa* in the St. Lawrence Estuary, Canada is well established and has been used to determine sex in this species quite effectively when gonads are mature (Hamel and Mercier 1996b). However, extensive collections made during the present study revealed that colour in male gonads varies not only throughout the annual reproductive cycle, but also with

geographic location, something that has never been reported in *C. frondosa*. Males from one site had pale orange gonads, whereas males from another site had dark red gonads that could not be distinguished from female gonads. The use of external sexual dimorphism could be applied to any holothuroid species with genital papillae to replace or supplement existing methods of determining the sex by a non-lethal and non-invasive approach.

Acknowledgements

The authors wish to thank Quin-Sea Fisheries Ltd and Memorial University's Field Services Team for their assistance with logistics and animal collections.

Declaration of Interest Statement

This research was supported by the Natural Sciences and Engineering Research Council of Canada (grants to AM) and the Ocean Frontier Institute (grant to AM). The authors have no conflicts of interest to declare. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Tables and Figures

Table 1 Holothuroid species with external dimorphism in the permanent genital papillae of males and females. Male papillae were defined as digitate (multiple protrusions or a cluster of papillae) and/or extensible (elongated or extendable).

Order	Family	Species	Reproductive Mode	Male Papillae Type	Distribution	Source
Dendrochirotida	Cucumariidae	*Colochirus robustus*	Lecithotrophic Pelagic	Digitate	Indo-Pacific	Google Image Search
Dendrochirotida	Cucumariidae	*Cucumaria frondosa*	Lecithotrophic Pelagic	Digitate	N Atlantic	Current study
Dendrochirotida	Cucumariidae	*Cucumaria frondosa japonica*	Lecithotrophic Pelagic	Digitate	Arctic	J.-F. Hamel Pers. Obs.
Dendrochirotida	Cucumariidae	*Cucumaria georgiana*	Lecithotrophic Internal Brooding	Digitate	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Cucumaria japonica*	Lecithotrophic Pelagic	Digitate	N Pacific	Levin and Stepanov (2005)
Dendrochirotida	Cucumariidae	*Cucumaria miniata*	Lecithotrophic Pelagic	Digitate	N Pacific	McEuen (1988)
Dendrochirotida	Cucumariidae	*Cucumaria pseudocurata*	Lecithotrophic External Brooding	Digitate	N Pacific	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Echinopsolus acutus*	Lecithotrophic Internal Brooding	Digitate	Antarctic	O'Loughlin et al. (2009)
Dendrochirotida	Cucumariidae	*Echinopsolus charcoti*	Lecithotrophic Internal Brooding	Digitate	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Echinopsolus charcoti*	Lecithotrophic Internal Brooding	Extensible	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Echinopsolus splendidus*	Lecithotrophic Internal Brooding	Digitate	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Echinopsolus splendidus*	Lecithotrophic Internal Brooding	Extensible	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Parathyonidium incertum*	Lecithotrophic Internal Brooding	Extensible	Antarctic	O'Loughlin (2009)
Dendrochirotida	Cucumariidae	*Pentactella laevigata*	Lecithotrophic Internal Brooding	Extensible	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Pseudocolochirus violaceus*	Lecithotrophic Pelagic	Digitate	Indo-Pacific	Google Image Search
Dendrochirotida	Cucumariidae	*Pseudocolochorus unica*	Lecithotrophic Pelagic	Digitate	Indo-Pacific	Google Image Search
Dendrochirotida	Cucumariidae	*Psolidocnus sacculus*	Lecithotrophic Internal Brooding	Extensible	Antarctic	O'Loughlin (2001)
Dendrochirotida	Cucumariidae	*Trachytyone nina*	Lecithotrophic Pelagic	Digitate	N Atlantic	J.-F. Hamel Pers. Obs.
Dendrochirotida	Psolididae	*Psolus chitonoides*	Lecithotrophic Pelagic	Digitate	N Pacific	McEuen (1988)
Dendrochirotida	Psolididae	*Psolus fabricii*	Lecithotrophic Pelagic	Digitate	N Atlantic	Current study
Dendrochirotida	Psolididae	*Psolus lawrencei*	Lecithotrophic External Brooding	Extensible	S Atlantic	Martinez and Penchaszadeh (2017)
Dendrochirotida	Psolididae	*Psolus phantapus*	Lecithotrophic Pelagic	Digitate	N Atlantic	Current study
Persiculida	Gephyrothuriidae	*Gephyrothuria alcocki*	Lecithotrophic Internal Brooding	Extensible	Global	O'Loughlin (2001)

Table 2 Mean size of permanent male and female genital papillae.

Species	Papillae Length (mm) Male	Papillae Length (mm) Female	Source
Cucumaria anivaensis	5–7	2–4	Levin and Stepanov (2005)
Cucumaria djakonovi	5–7	2–4	Levin and Stepanov (2005)
Cucumaria frondosa	1–4	1–3.5	Current Study
Cucumaria frondosa	5–7	2.5–3	Levin and Stepanov (2005)
Cucumaria georgiana	2	1	O'Loughlin (2001)
Cucumaria japonica	5–7	2.5–3	Levin and Stepanov (2005)
Cucumaria okhotensis	5–7	2–4	Levin and Stepanov (2005)
Cucumaria pseudocurata	1	0*	O'Loughlin (2001)
Echinopsolus charcoti	2	1	O'Loughlin (2001)
Echinopsolus splendidus	2–3	0.5	O'Loughlin (2001)
Gephyrothuria alcocki	2	2	O'Loughlin (2001)
Psolus chitonoides	6	8	McEuen (1988)
Psolus lawrencei	4.3	0*	Martinez and Penchaszadeh (2017)
Overall Mean (± SD)	4.0 (2.0)	2.2 (2.1)	

*female *C. pseudocurata* and *P. lawrencei* do not possess genital papillae.

Table 3 Mean size of genital papillae/clusters of papillae in *Cucumaria frondosa*.

Sex	Papilla/Cluster Length (mm)	Papilla/Cluster Width (mm)	Number of Branches in Cluster
F	2.3	2	0
M	1	2	1
	3	3.5	3
	4	3	4
	2.6	4.3	> 4

Table 4 Frequency distribution of genital papillae branches in males of *Cucumaria frondosa* (n = 20 males).

Number of Papillae Branches in Cluster	Percent of Population (%)
2	25
3	15
4	10
5	10
6	0
7	5
8	5
9	20
10	0
11	0
12	0
13	5
14	0
15	5

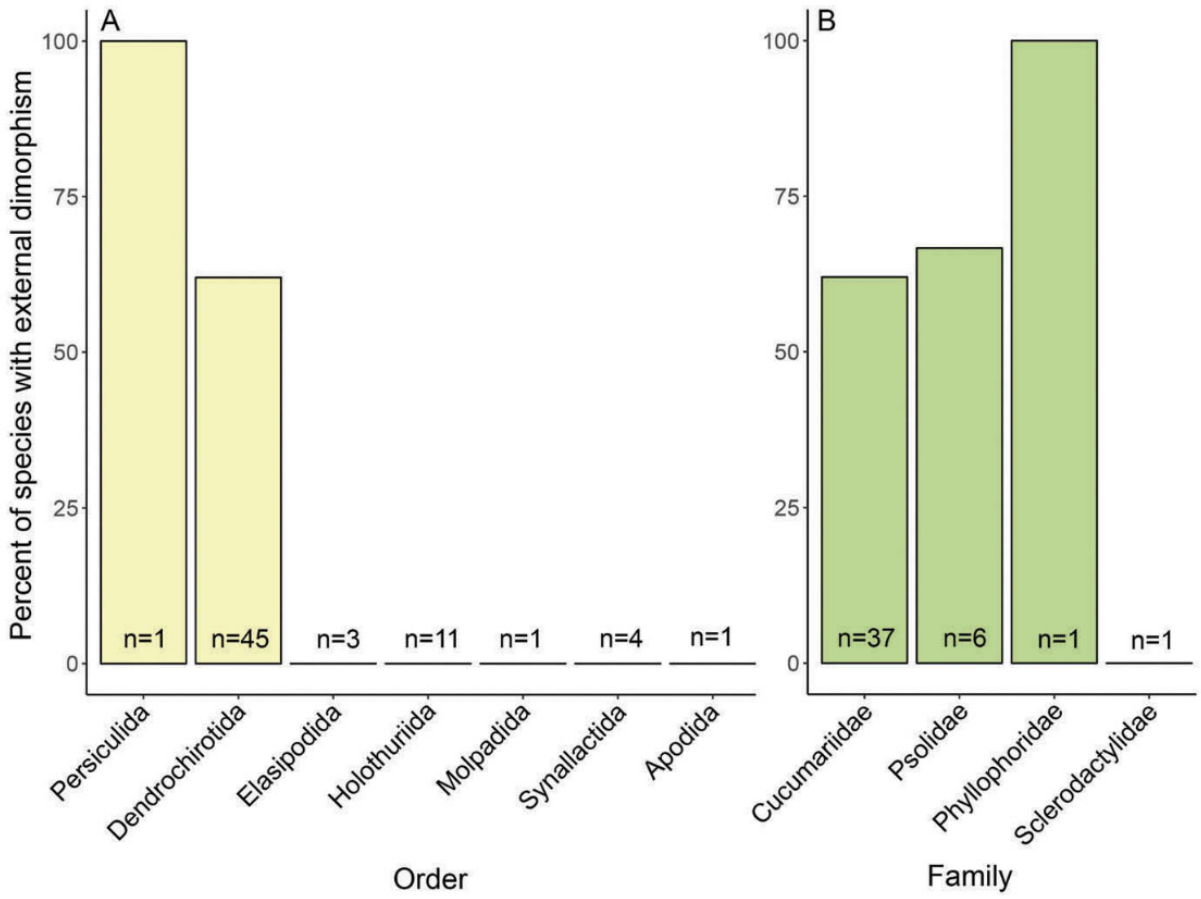

Figure 1 Percent of sea cucumber species that possess external sexual dimorphism in the length and/or shape of permanent genital papillae relative to taxa. Species were considered sexually-dimorphic if macroscopic variation in papillae enabled reliable sexing of individuals *in vivo*. A) Dimorphic species among six orders of sea cucumbers in the dataset. B) Dimorphic species in four families of dendrochirotids in the dataset. The total number (n) of species examined in each taxon is indicated on each bar. This n is unequal because papillae morphology is not equally reported among taxa. Phylogenetic delineations based on Miller et al. 2017. See Table S1 for the full dataset.

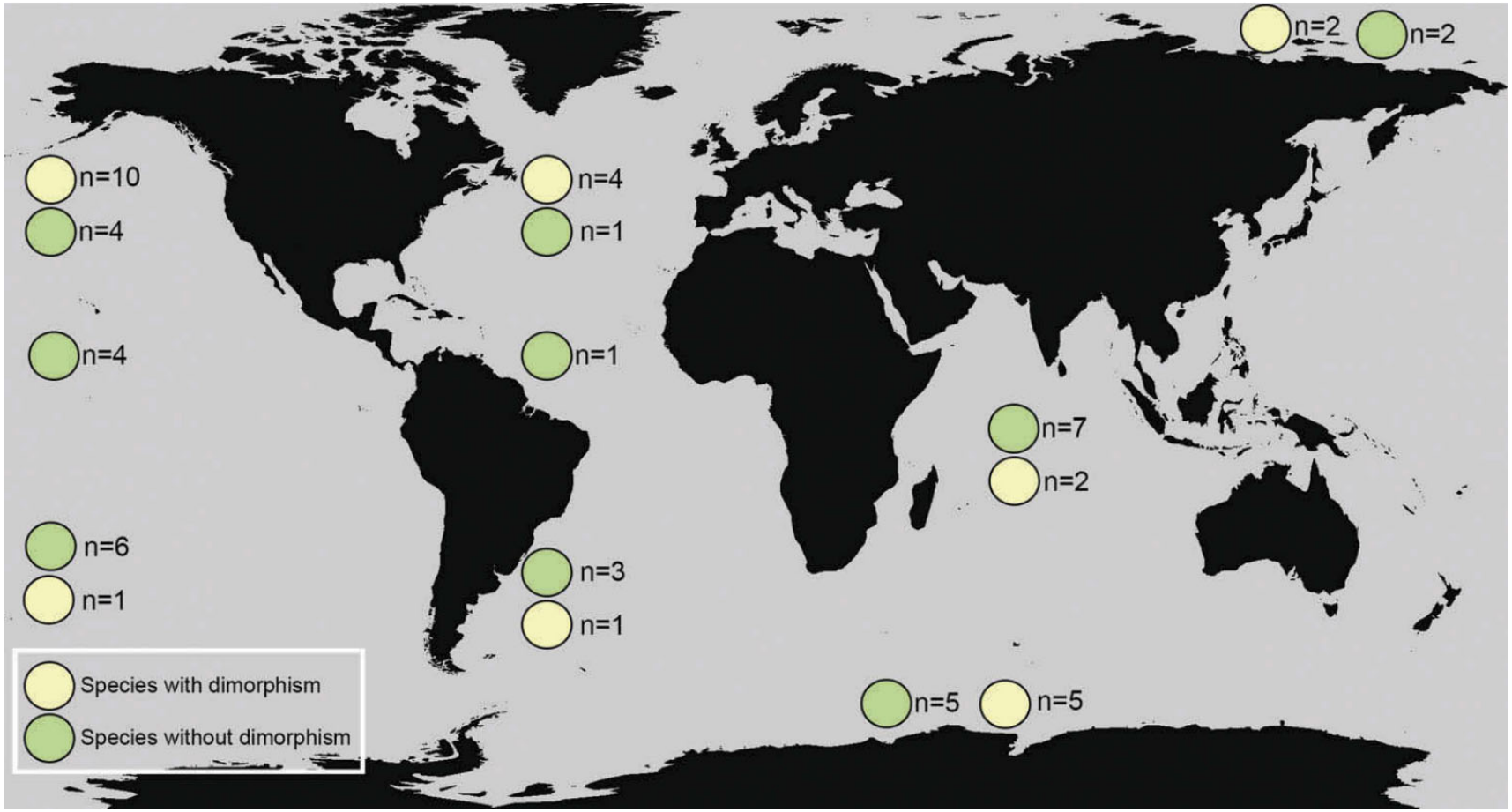

Figure 2 Global distribution of sea cucumber species reported with (yellow) and without (green) external sexual dimorphism. Species were considered sexually dimorphic if macroscopic variation in papillae enabled reliable sexing of individuals *in vivo*.

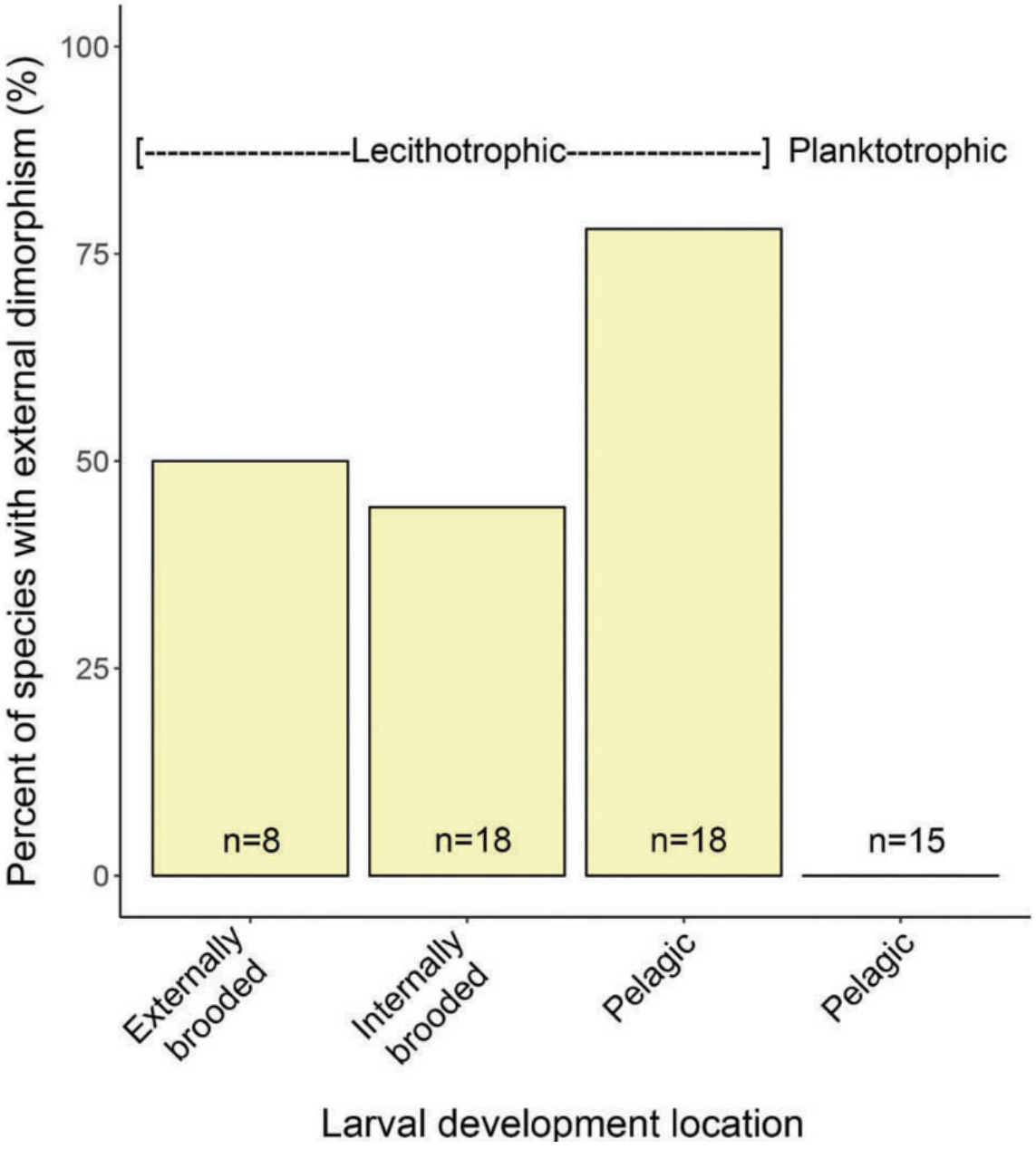

Figure 3 Percent of sea cucumber species that possess external sexual dimorphism in the length and/or shape of the genital papillae relative to larval development location. Species were considered sexually dimorphic if macroscopic variation in papillae enabled reliable sexing of individuals *in vivo*. The total number (n) of species examined in each category is indicated on each bar.

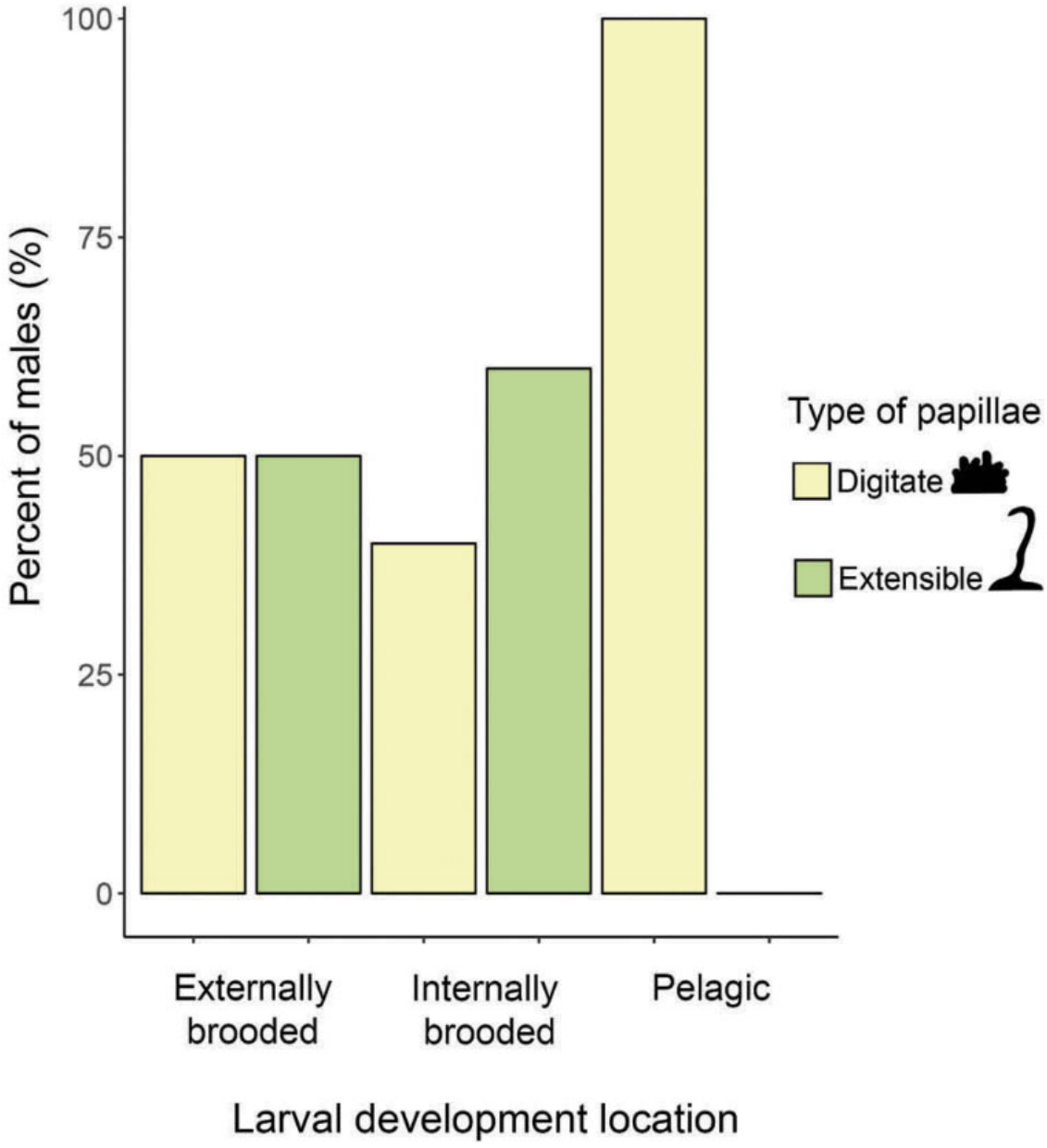

Figure 4 Percent of male sea cucumbers that possess digitate or extensible genital papillae relative to larval development location. *Digitate* papillae were defined by the presence of branching (>1) of the genital papilla. *Extensible* papillae were defined as long papillae with whip-like characteristics. Papillae could be both digitate and extensible but were coded separately for the purpose of comparison. These complex papillae were only found in two species of internal brooders (n=20 species total).

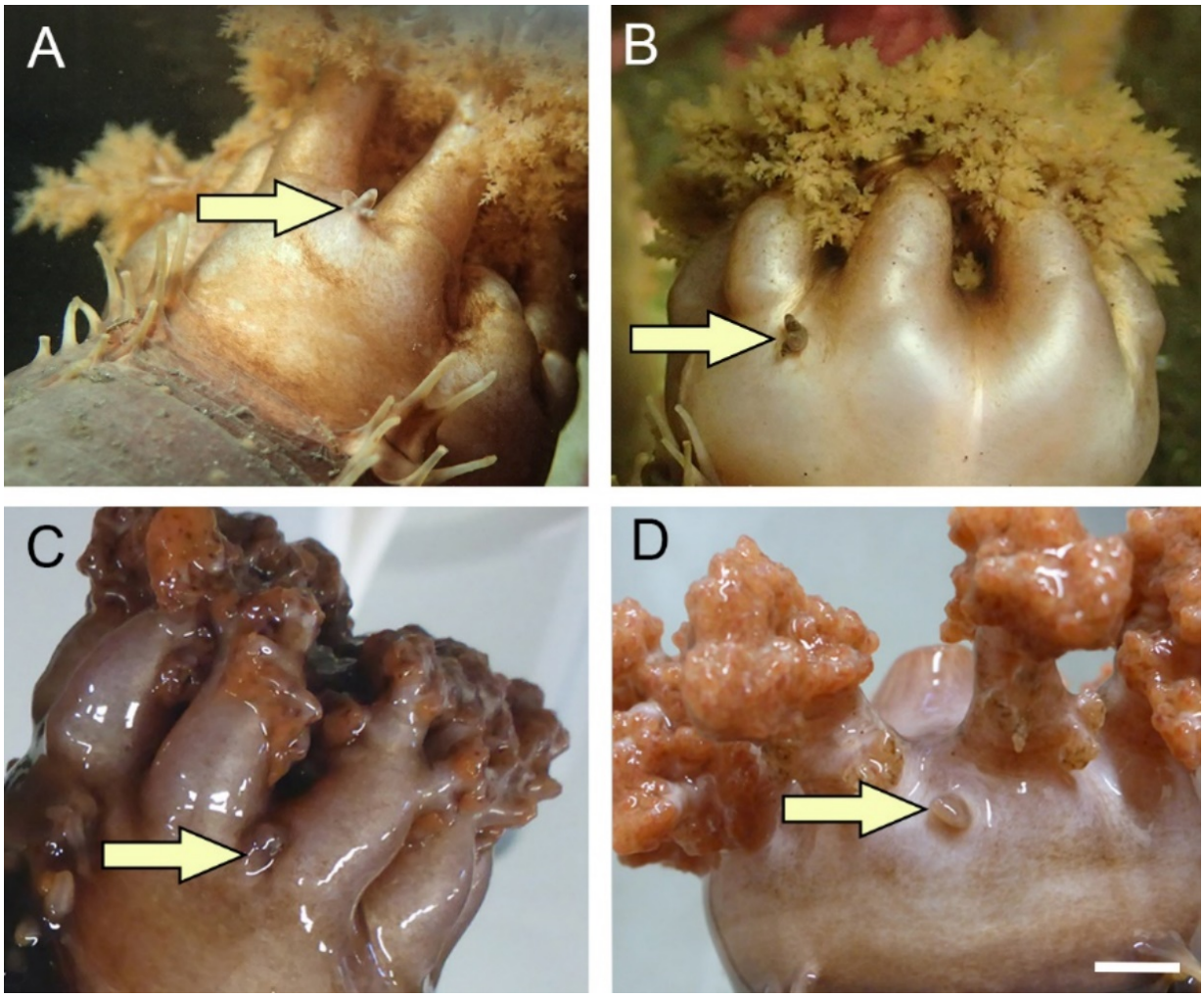

Figure 5 Location of genital papillae in male (A-B) and female (C-D) *Cucumaria frondosa*. Arrows indicate location of papillae. Note the digitate shape of the male papillae (A-B) versus the smooth, non-digitate, cone-shape papillae of the females (C-D). Scale bar indicates 1 cm.

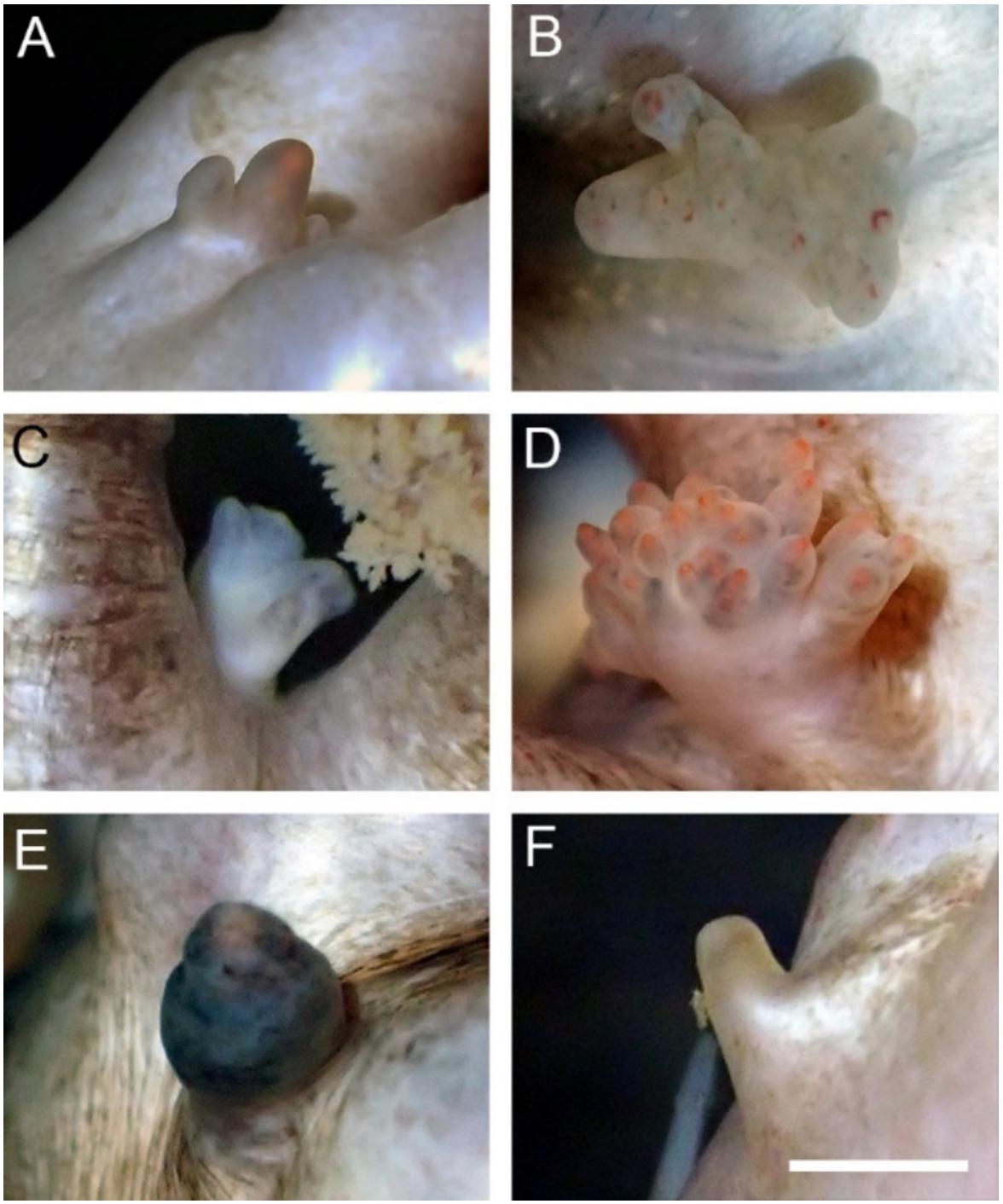

Figure 6 Diversity of genital papillae in male (A-D) and female (E-F) *Cucumaria frondosa*. Panel A shows trifurcated male papillae. Panels B-D show examples of multifurcated male papillae. Panels E-F show smooth, non-digitate, cone-shape female papillae. Scale bar indicates 0.5 cm.

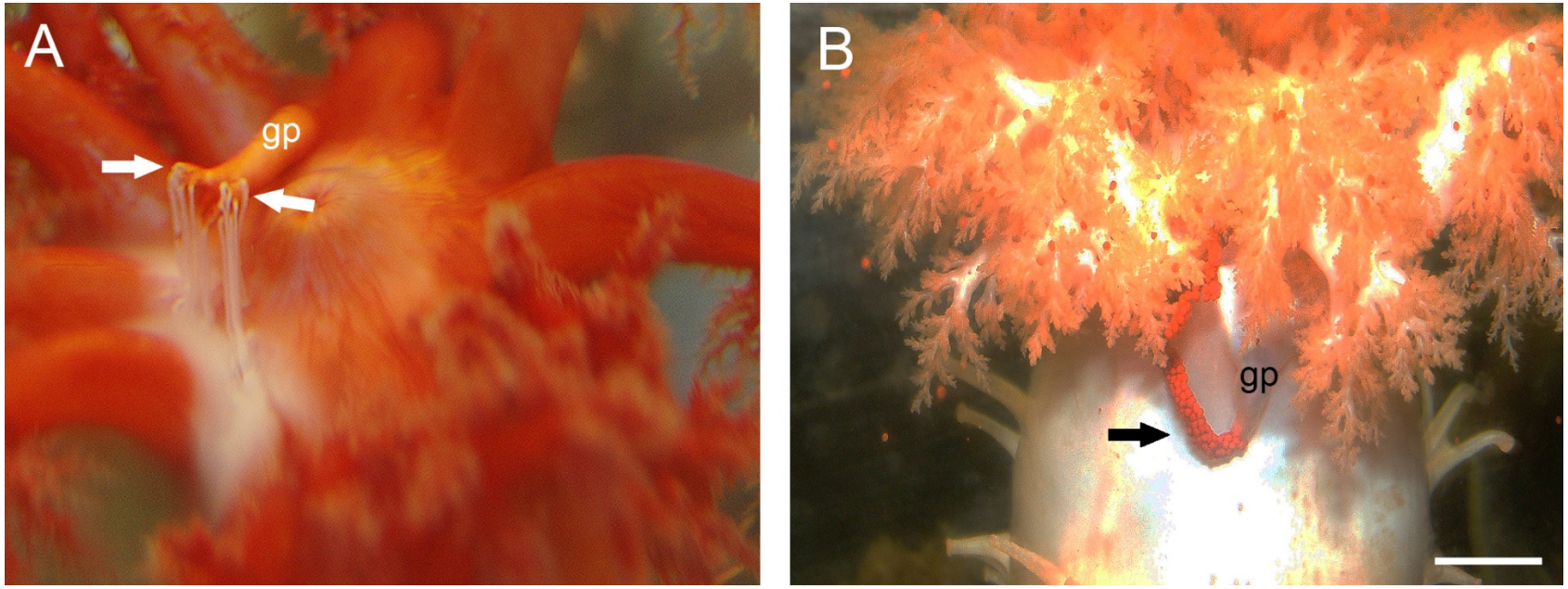

Figure 7 Spawning in sea cucumbers with pelagic lecithotrophic larvae. Male *Psolus fabricii* **(A)** show the typical digitate genital papillae (gp) and multiple streams of sperm emerging from numerous gonopores (white arrow). Female *Cucumaria frondosa* **(B)** release bundled egg "sausages" (black arrow) from a non-digitate genital papilla (gp). Scale bar indicates 2 cm.

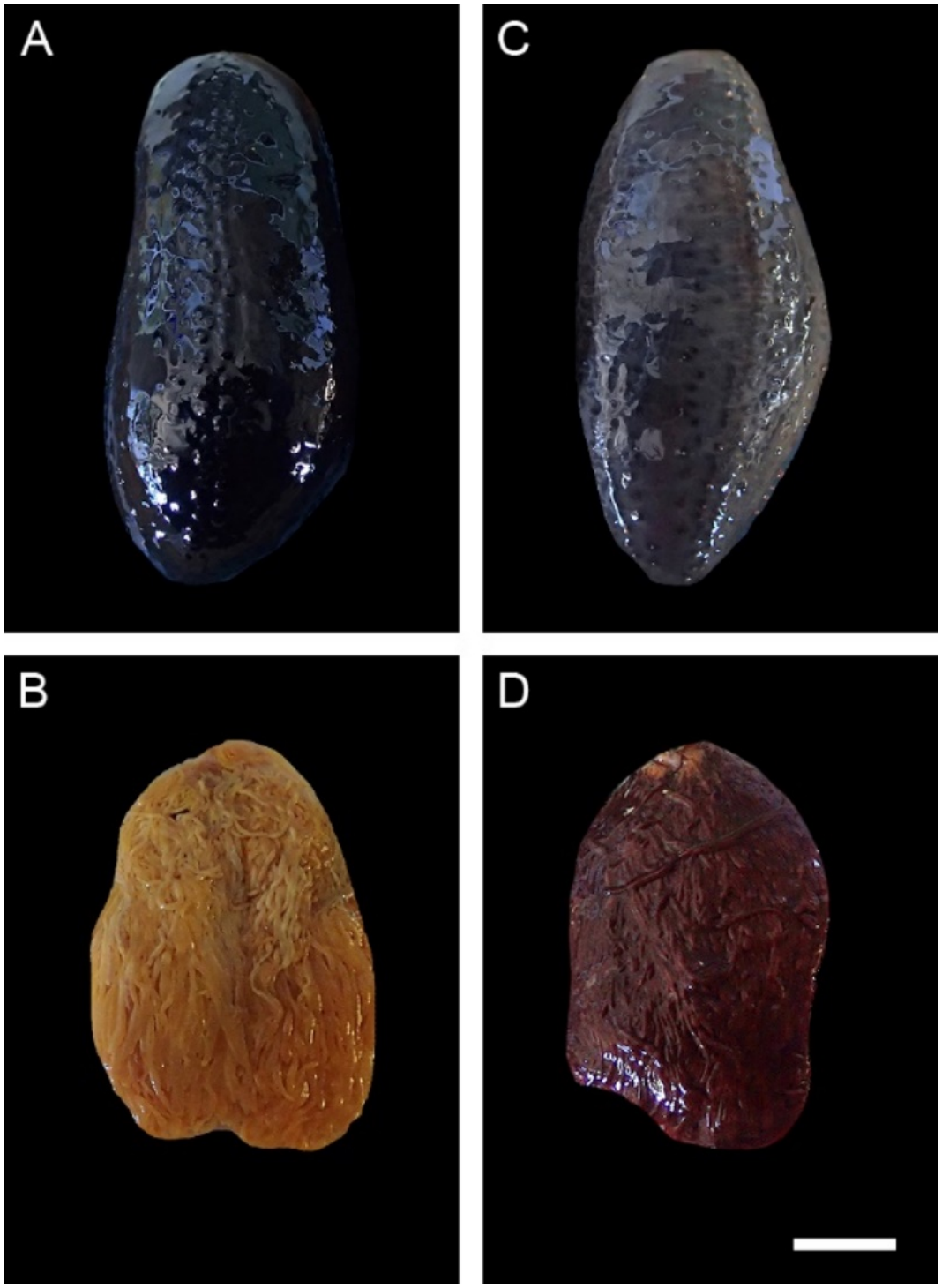

Figure 8 Comparison of the body wall and gonad of two males of *Cucumaria frondosa* originating from two different geographic locations. A-B) Logy Bay, Newfoundland (male 1). **C-D)** Grand Banks, Newfoundland (male 2). Individuals were examined in July, a few months after the natural spawning period of *C. frondosa*. Both males were approximately the same weight (g) and contracted length (mm). Sex was confirmed via the presence of sperm and lack of oocytes in a gonadal smear. Scale bar indicates 3 cm (A-B) and 1 cm (C-D).

Table S1 Full dataset table.

Order	Family	Species	Dimorphism	Development Mode	Male Size (mm)	Female Size (mm)	Distribution	Reference
Apodida	Chiritotidae	*Chiridota laevis*	No	n.d.	n.a.	n.a.	N. Atlantic	Current Study
Apodida	Chiritotidae	*Neotoxodora pacifica*	No***	n.d.	n.a.	n.a.	N. Pacific	Ohshima 1915
Dendrochirotida	Cucumariidae	*Athyonidium chilensis*	No⸎	Lecithotrophic Pelagic	n.a.	n.a.	Pacific	Peters-Didier et al. 2016
Dendrochirotida	Cucumariidae	*Cladodactyla crocea*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	Antarctic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Colochirus robustus*	Yes	Lecithotrophic Pelagic	n.d.	n.d.	Indopacific	Google Image Search
Dendrochirotida	Cucumariidae	*Cucumaria anivaensis*	Yes	n.d.	5-7	2-4	N Pacific	Levin and Stepanov 2005
Dendrochirotida	Cucumariidae	*Cucumaria djakonovi*	Yes	n.d.	5-7	2-4	N Pacific	Levin and Stepanov 2005
Dendrochirotida	Cucumariidae	*Cucumaria fallax*	Yes§	Lecithotrophic Pelagic	n.d.	n.d.	N. Pacific	McEuen 1988
Dendrochirotida	Cucumariidae	*Cucumaria frondosa*	Yes	Lecithotrophic Pelagic	1-4	1-3.5	N Atlantic	Current Study
Dendrochirotida	Cucumariidae	*Cucumaria frondosa*	Yes	Lecithotrophic Pelagic	5-7	2.5-3	N Atlantic	Levin and Stepanov 2005
Dendrochirotida	Cucumariidae	*Cucumaria frondosa japonica*	Yes	Lecithotrophic Pelagic	n.d.	n.d.	Arctic	J.F. Hamel Pers. Obs.
Dendrochirotida	Cucumariidae	*Cucumaria georgiana*	Yes	Lecithotrophic Internal Brooding	2	1	Antarctic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Cucumaria ijimai*	No***	Lecithotrophic Internal Brooding	n.a.	n.a.	N. Pacific	Ohshima 1915

Dendrochirotida	Cucumariidae	*Cucumaria japonica*	Yes	Lecithotrophic Pelagic	5-7	2-4	N Pacific	Levin and Stepanov 2005
Dendrochirotida	Cucumariidae	*Cucumaria lubrica*	Yes§	Lecithotrophic External Brooding	n.d.	n.d.	N. Pacific	McEuen 1988
Dendrochirotida	Cucumariidae	*Cucumaria miniata*	Yes	Lecithotrophic Pelagic	n.d.	n.d.	N Pacific	McEuen 1988
Dendrochirotida	Cucumariidae	*Cucumaria okhotensis*	Yes	n.d.	5-7	2-4	Arctic	Levin and Stepanov 2005
Dendrochirotida	Cucumariidae	*Cucumaria piperata*	Yes§	Lecithotrophic Pelagic	n.d.	n.d.	N. Pacific	McEuen 1988
Dendrochirotida	Cucumariidae	*Cucumaria pseudocurata*	Yes	Lecithotrophic External Brooding	1	0	N Pacific	O'Loughlin 2001, Rutherford 1973
Dendrochirotida	Cucumariidae	*Cucumaria pseudocurata*	No§	Lecithotrophic External Brooding	n.a.	n.a.	N. Pacific	McEuen 1988
Dendrochirotida	Cucumariidae	*Echinopsolus acutus*	Yes	Lecithotrophic Internal Brooding	n.d	n.d	Antarctic	O'Loughlin et al. 2009
Dendrochirotida	Cucumariidae	*Echinopsolus charcoti*	Yes	Lecithotrophic Internal Brooding	2	1	Antarctic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Echinopsolus splendidus*	Yes	Lecithotrophic Internal Brooding	2-3	0.5	Antarctic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Heterocucumis steineni*	No⸎	Lecithotrophic Pelagic	n.a.	n.a.	Antarctic	Gutt et al. 1992
Dendrochirotida	Cucumariidae	*Neoamphicyclus lividus*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	S Pacific	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Neocnus bimarsupiis*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	S Pacific	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Neocnus incubans*	No***	n.d.	n.a.	n.a.	Mediterranean	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Ocnus glacialis*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	N Atlantic	O'Loughlin 2001

Dendrochirotida	Cucumariidae	*Parathyonidium incertum*	Yes	Lecithotrophic Internal Brooding	n.d	n.d	Antarctic	O'Loughlin et al. 2009
Dendrochirotida	Cucumariidae	*Pentactella laevigata*	Yes	Lecithotrophic Internal Brooding	n.d	n.d	Kerguelen	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Pentactella leonina*	No***	n.d.	n.a.	n.a.	S. Atlantic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Pentocnus bursatus*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	S Pacific	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Pseudocnus lamperti*	No***	Lecithotrophic Internal Brooding	n.a.	n.a.	N. Pacific	Ohshima 1915
Dendrochirotida	Cucumariidae	*Pseudocnus lubricus*	No***	Lecithotrophic External Brooding	n.a.	n.a.	N. Pacific	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Pseudocolochirus violaceus*	Yes	Lecithotrophic Pelagic	n.d.	n.d.	S Pacific	Google Image Search
Dendrochirotida	Cucumariidae	*Pseudocolochorus unica*	Yes	Lecithotrophic Pelagic	n.d.	n.d.	Indopacific	Google Image Search
Dendrochirotida	Cucumariidae	*Pseudopsolus macquariensis*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	Antarctic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Psolicrux coatsi*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	Antarctic	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Psolidiella hickmani*	Yes	Lecithotrophic External Brooding	n.d	n.d	New Zealand	O'Loughlin 2000
Dendrochirotida	Cucumariidae	*Psolidiella nigra*	No	Lecithotrophic External Brooding	n.a.	n.a.	New Zealand	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Psolidocnus sacculus*	Yes	Lecithotrophic Internal Brooding	n.d	n.d	New Zealand	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Squamocnus brevidentis*	No	Lecithotrophic External Brooding	n.a.	n.a.	New Zealand	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Squamocnus niveus*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	New Zealand	O'Loughlin 2001
Dendrochirotida	Cucumariidae	*Staurothyone inconspicua*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	S Pacific	O'Loughlin 2001

Dendrochirotida	Phylloporidae	*Pentamera populifera*	Yes§	Lecithotrophic Pelagic	n.a.	n.a.	N. Pacific	O'Loughlin 2001, McEuan 1988
Dendrochirotida	Psolididae	*Echinopsolus acuta*	No***	Lecithotrophic Internal Brooding	n.a.	n.a.	Antarctic	Massin 1992
Dendrochirotida	Psolididae	*Echinopsolus parvipes*	No***	Lecithotrophic Internal Brooding	n.a.	n.a.	Antarctic	Massin 1992
Dendrochirotida	Psolididae	*Psolus chitonoides*	Yes	Lecithotrophic Pelagic	6	8	N Pacific	McEuen 1988
Dendrochirotida	Psolididae	*Psolus dubiosus*	No	Lecithotrophic Internal Brooding	n.a.	n.a.	Antarctic	Gutt et al. 1992
Dendrochirotida	Psolididae	*Psolus fabricii*	Yes	Lecithotrophic Pelagic	n.d	n.d	N Atlantic	Current Study
Dendrochirotida	Psolididae	*Psolus lawrencei*	Yes	Lecithotrophic External Brooding	4.3	0	S Atlantic	Martinez and Penchaszadeh 2017
Dendrochirotida	Psolididae	*Psolus patagonicus*	No	Lecithotrophic External Brooding	n.a.	n.a.	S Atlantic	Martinez and Penchaszadeh 2011
Dendrochirotida	Psolididae	*Psolus phantapus*	Yes	Lecithotrophic Pelagic	n.d	n.d	N Atlantic	Current Study
Dendrochirotida	Sclerodactylidae	*Eupentacta quinquesemita*	No	Lecithotrophic Pelagic	n.a.	n.a.	Pacific	Lambert 1996
Elasipodida	Elpidiidae	*Elpidia glacialis*	No	Planktotrophic or Lecithotrophic^{++}	n.d.	n.d.	Arctic	Théel 1877 and unpublished data
Elasipodida	Elpidiidae	*Kolga hyalina*	No	Planktotrophic or Lecithotrophic^{++}	n.d.	n.d.	Arctic	Rogacheva 2007 and unpublished data
Elasipodida	Laetmogonidae	*Laetmogone maculata*	No	n.d.	n.a.	n.a.	Pacific	Théel 1882
Elasipodida	Laetmogonidae	*Laetmogone wyvillethomsoni*	No***	n.d.	n.a.	n.a.	Indopacific	Théel 1882
Elasipodida	Pelagothuriidae	*Enypniastes eximia*	No***	Lecithotrophic^{++}	n.a.	n.a.	Cosmopolitan	Ohshima 1915

Holothuriida	Holothuriidae	*Actinopyga echinites*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Kohler et al. 2009
Holothuriida	Holothuriidae	*Bohadschia marmorata*	No***	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	O'Loughlin 2001
Holothuriida	Holothuriidae	*Holothuria atra*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Dissanayake and Stefansson 2010
Holothuriida	Holothuriidae	*Holothuria fuscogilva*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Pacific	Ramofafia et al. 2000
Holothuriida	Holothuriidae	*Holothuria grisea*	No‡	Planktotrophic Pelagic	n.a.	n.a.	S Atlantic	Leite-Castro et al. 2016
Holothuriida	Holothuriidae	*Holothuria leucospilota*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Gaudron et al. 2008
Holothuriida	Holothuriidae	*Holothuria mexicana*	No	Planktotrophic Pelagic	n.a.	n.a.	Atlantic	J.-F Hamel personal observation
Holothuriida	Holothuriidae	*Holothuria sanctori*	No‡	Planktotrophic Pelagic	n.a.	n.a.	S Atlantic	Navarro et al. 2012
Holothuriida	Holothuriidae	*Holothuria scabra*	No	Planktotrophic Pelagic	n.a.	n.a.	S Pacific	J.-F Hamel personal observation
Holothuriida	Holothuriidae	*Holothuria spinifera*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Asha and Muthia 2005
Holothuriida	Holothuriidae	*Microthele fuscogliva*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Conand 1981
Holothuriida	Holothuriidae	*Microthele nobilis*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Conand 1981
Molpadida	Molpadidae	*Molpadia intermedia*	No‡	Lecithotrophic Pelagic	n.a.	n.a.	N Pacific	McEuen 1988
Persiculida	Gephyrothuriidae	*Gephyrothuria alcocki*	Yes	Lecithotrophic Internal Brooding	2	2	Global	O'Loughlin 2001
Synallactida	Stichopodidae	*Apostichopus californicus*	No‡	Planktotrophic Pelagic	n.a.	n.a.	N Pacific	Lambert 1996

Synallactida	Stichopodidae	*Apostichopus japonicus*	No‡	Planktotrophic Pelagic	n.a.	n.a.	N Pacific	Slater and Chen 2015
Synallactida	Stichopodidae	*Isostichopus fuscus*	No‡	Planktotrophic Pelagic	n.a.	n.a.	S Pacific	Toral-Granada and Martinez 2007
Synallactida	Stichopodidae	*Stichopus horrens*	No‡	Planktotrophic Pelagic	n.a.	n.a.	Indopacific	Conand 1993

‡ Studies that state the absence of sexual dimorphism in a general capacity but did not explicitly assess the genital papillae. Included in analysis.

*** Studies that state the presence of genital papillae but did not explicitly assess the presence of sexual dimorphism. Not included in analysis.

§ McEuan (1988) commonly discusses the number of gonopores rather than genital papillae. Here we assume that multiple gonopores = multiple papillae because gonopore openings typically occur at the end of each papilla. Included in analysis.

++ Larval development in Elasipodida was never observed so far. Developmental mode is suggested based on mature egg size (i.e. reviewed by Young, 2003).