Exploring the Potential of the Sea cucumber

Cucumaria frondosa as an Aquaculture Species

By

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Abstract

Sea cucumbers are a luxury seafood in China and the increasing demand is driving aquaculture initiatives towards new species worldwide. *Cucumaria frondosa* has been commercially harvested for decades in North America and has also been identified as a potential candidate for aquaculture. So far, captive-breeding methods have focused on tropical/temperate deposit-feeding species with planktotrophic development (small eggs, feeding larvae); however, *C. frondosa* is a cold-water suspension-feeder with lecithotrophic development (large yolky eggs, non-feeding larvae). The aim of this study was to explore the reproductive biology, larval development, and juvenile ecology of *C. frondosa* to identify optimum conditions for culture. In an experimental study, adults kept in ambient environmental conditions showed the best reproductive output and embryo survival relative to those in warmer water, advanced photoperiod, and constant light or dark conditions. An increase in food concentration did not enhance the reproductive output. When methods used to trigger spawning and artificially induce oocyte maturation were investigated, live phytoplankton at 1×10^5 cell ml^{-1} was the most successful technique; moreover, among a handful of chemicals tested, only Dithiothreitol (DTT) at 10^{-1} M induced significantly more oocytes to ovulate than the control (seawater), although eggs remained unfertilizable. These experiments led to the discovery of embryonic fusion and the formation of chimaeras (individuals composed of genetically distinct types of cells). Fusion occurred only among hatched blastulae and occurred in a maximum of 9% of the propagule population, generating individuals up to 5 times larger than singletons. Finally, development and

behaviour of juveniles assessed from settlement to 21 months of age revealed that newly-settled juveniles had low tolerance to light and water flow, fed on deposited material, and preferred rocky substrates and black or red backgrounds. With age, juveniles grew ramified tentacles, shifted to suspension feeding, developed greater tolerance to light and water flow, and showed a preference for coralline algae and red backgrounds. The findings presented here provide important advances to optimize gonad development and egg quality in captive broodstock of *C. frondosa*, to induce or enhance spawning and fertilization, as well as to elucidate the optimal conditions for embryos and juveniles. This work presents a pilot-scale assessment of commercial production in *C. frondosa*, with potential applicability to similar cold-water suspension-feeding species.

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for the subsequent samplings in each treatment. Data shown as mean ± se. Means with different letters are significantly different (p < 0.05); see text for full statistical results. .62

blastulae formed after 15 min. At the end of the experiment, >80% of the blastulae were agglomerated to some degree.

Figure S.4.2: Evidence of chimaerism in unitary animals in the marine environment. Photos of chimaeric sea anemones captured by underwater photographers. A-B) Multi-headed individuals of the genus Urticina photographed off Eastport, Maine, USA. C-D) Bi-headed individuals of the genus Metridium photographed near Whidbey Island, Washington, USA.

Figure 5.1 (previous page): Monthly morphometric measurements of body length, number and length of dorsally oriented tentacles, number of tentacle ramifications, number of ambulacral podia, and tentacle retraction/insertion rate in juveniles of the sea cucumber Cucumaria frondosa from 1 to 21 months old. Data shown as mean ± sd (n = 30).

Figure 5.2 (previous page): Morphological development of juveniles of the sea cucumber *Cucumaria frondosa* from 1 to 21 months old. (A) One month-old juvenile showing primary tentacles (t) and ambulacral podia (ap). (B) Illustration of 3 ramified dorsally-oriented tentacles (dt) used for feeding and 2 non-ramified ventrally oriented tentacles (vt) used for both feeding and attachment. (C) Growth of a new tentacle (nt) occurred between ramified tentacles (t) and a non-ramified and ventrally oriented tentacle (vt). (D) Six-month-old juvenile displaying 7 tentacles (t) and 5 ambulacral podia (ap). Juveniles displayed an erect posture with posterior ambulacral podia (ap) ensuring anchorage. (E) Tentacles of six-month old juveniles are bent in a semicircular movement (arrow) and inserted into the month (mo) one at a time. (F) Six-month-old juveniles with organic particles in the tentacles (t). Ingested food (fo) is visible in the intestinal tract through the semitransparent body wall and juvenile is seen defecating (fc). (G) A yellowish skin pigmentation (pg) appeared around the oral cavity of eight-month-old juveniles. (H) The first dorsal podium is visible on the dorsal side of twelve-month-old juveniles. (I) Dark brownish skin pigmentation (pg) appeared between tentacles of sixteen-month-old juveniles. (J) twenty-one-month-old juvenile displaying 10 ramified tentacles (t) and 9 ambulacral podia (ap). Scale bar presents 0.5 mm.

Figure 5.3 (previous page): Tentacle ramifications in juveniles of the sea cucumber *Cucumaria frondosa*. (A) Unramified primary tentacles are present in one-month-old juveniles. (B) The first ramification of dorsal tentacle appeared when juveniles were two months-old. (C) The second ramification of dorsal tentacle became visible when juveniles were four months old. (D) Dorsal tentacles with 3 ramifications were present in six-month-old juveniles. (E) The development of the fourth ramification on the dorsal tentacles occurred when juveniles reached eight months of age. (F) Twelve-month-old juveniles showed 5 ramifications on dorsal tentacles. (G) Six ramifications were present on dorsal tentacles of sixteen-month-old individuals. (H) Ventrally oriented tentacles (vt) only started branching out when juveniles were twenty-one-month-old. Arrows indicate the number of ramifications in the tentacles. Scale bars present 0.5 mm.

Figure 5.4 (previous page): Scanning electron microscopy of the body wall of 1, 6, and 12-month-old juveniles of *Cucumaria frondosa*. (A) One-month-old juvenile showing

fenestrated ossicles covered with very thin dermal tissue (arrow). (B) Ossicle of one-month-old juveniles are flat, without either knobs or denticles on the inner and outer surface. (C) Small holes and cavities (arrows) are present in ossicles of one-month-old-juveniles. (D) Six-month-old juveniles displaying large fenestrated ossicles covered by a thin layer of dermal tissue (arrow). (E) Sharp knobs and denticles present in the outer edge of the ossicles of six-month-old juveniles (arrows). (F) Ossicles of six-month-old juveniles displayed a solid structure without cavities (arrows). (G) Twelve-month-old juveniles armoured with large ossicle covered by a thick layer of dermal tissue (arrows). (H) Knobs and denticles on the outer surface of the ossicles showed rounded edges (arrows) of twelve-month-old juveniles. (I) Ossicles presented a solid structure without cavities (arrows) of twelve-month-old individuals. Scale bar represents 50 µm.

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Co-authorship Statement

The work described in the present thesis was carried out by Bruno Lainetti Gianasi with supervision and guidance from Annie Mercier, Jean-Francois Hamel, Cyr Couturier, and Iain McGaw. All chapters were written by Bruno Lainetti Gianasi as journal manuscripts with intellectual and editorial input by co-authors as follows:

Authorship for **Chapters 2-5** is Gianasi, B.L; Hamel, J.-F.; Mercier, A.

Chapter 1. General Introduction

1.1 From traditional medicine to aquaculture

Sea cucumbers, also called holothurians or holothuroids, belong to class Holothuroidea of phylum Echinodermata. They have been consumed and used in traditional medicine for centuries; they are considered an important part of many Asian cultures and beliefs whereby their powerful symbolism is based on the promotion of general health and well-being (Fabinyi, 2012; Yang *et al.*, 2015). A profound respect for sea cucumbers has been passed down from generations to generations through many legends and stories that reflect people's beliefs and aspirations.

Ancient Chinese texts assert that sea cucumbers, often referred to as "ginseng of the sea", have been harvested for human consumption since 200 BC (Yang and Bai, 2015). One of the tales mentions that the First Emperor of China sent one of his servants at sea to look for the everlasting elixirs in the three Fairy Mountains (Penglai, Fangzhand, and Yingzhou) located in Bohai Bay, gulf of the Yellow Sea (northeast China); a place where only immortals lived. Everlasting elixirs are considered a type of potion with power that either never runs out or works forever. During his voyage, the boat was hit by a big storm that steered it away to open ocean. After several months lost at sea, the servant ran out of food and landed on a remote island where sea cucumbers were abundant. Despite their unattractive appearance, the cooked sea cucumbers were very tasty and smelled good; so the servant decided to eat them for the rest of his life. When he was almost 90 years old, the servant was still very strong, healthy, and looked quite young for his age. It was then that he finally understood that sea cucumbers were the everlasting elixirs he was looking for several years ago (Yang and Bai, 2015). In another tale, a fisherman saw his parents get

suddenly sick from a very strange disease. To help at home, the young fisherman went fishing day and night; however, one day, he was very tired and fell asleep on the boat. In his dreams, an old man told him that only sea cucumbers could cure his parents from that strange disease. After eating several meals with sea cucumbers, the young fisherman's parents recovered from the disease and named the sea cucumbers "seagods". However, when the Emperor heard about the magical properties of sea cucumbers, he re-named them "ginseng of the sea", because "seagods" could only be referring to immortals (Yang and Bai, 2015).

Over the years, beliefs have been transmitted about the magical powers of the sea cucumber and its benefits to human health. Traditional Chinese medicine asserts that the consumption of sea cucumber promotes general health, increases the essence of life and has curative properties (Cheung and Wu, 2012; Yang *et al.*, 2015). It is believed to relieve asthma, hypertension, rheumatism, impotence, frequent urination, kidney deficiency, tendonitis, arthritis, and pain (Kiew and Don, 2012). Apart from its reputation as an aphrodisiac, sea cucumber contains low levels of fat and high levels of protein when compared to other types of food (Zhong *et al.*, 2007).

In recent years, sea cucumbers have become a prized commodity throughout Asia, typically considered a luxury seafood served at weddings, banquets and corporate events (Fabinyi, 2012; Purcell, 2014). The high demand and market prices for sea cucumbers, driven primarily by the long-standing cultural and social traditions in China, have led to the expansion of sea cucumber fisheries and to the depletion of wild stocks (Anderson *et al.*, 2011; Purcell *et al.*, 2013). At present, over 70 species of holothuroids are commercially exploited and traded (Purcell *et al.*, 2010); however, overfishing has severely impacted

many wild populations throughout the world. The most recent global investigation of the status of sea cucumber fisheries revealed that 20% of the stocks were depleted or collapsed, 38% over-exploited and 14% fully-exploited, with no room for expansion (Purcell *et al.*, 2013). These fisheries are concentrated in the tropical shallow waters of the Indo-Pacific and Asia, and constitute small-scale harvests of high-value species (Purcell *et al.*, 2013). Following an assessment in 2010, seven sea cucumber species were classified as endangered and nine as vulnerable on the IUCN Red List of Threatened Species (Purcell *et al.*, 2014). Moreover, an overall reduction in the average size and weight of harvested sea cucumbers has been observed in many commercially valuable species (Uthicke and Benzie, 2001; Anderson *et al.*, 2011).

Concurrent with the collapse of many natural populations of sea cucumbers in Asia and the Indo-Pacific, aquaculture programs and new fisheries for previously unexploited species blossomed around the globe in an attempt to supply the ever increasing demand for sea cucumber products. By the end of the 1980s, fisheries for sea cucumbers were developing in Chile, Mexico, Egypt, USA and Canada, introducing a number of new species to the global market (Anderson *et al.*, 2011).

1.2 *Cucumaria frondosa* as a candidate for aquaculture

The sea cucumber *Cucumaria frondosa* is widely distribute in cold-waters and may well be the most abundant epibenthic sea cucumber in the North Atlantic (Hamel and Mercier, 2008b). It occurs from the Arctic Ocean to Cape Cod (Massachusetts, USA) as well as in the southern latitudes of Greenland, Iceland, North of the United Kingdom,

Europe and along the Barents Sea on the coast of Russia (Levin and Gudimova, 2000; Hamel and Mercier, 2008b). Several common names are attributed to *C. frondosa* in different location of the globe. In USA and Germany, this species is referred to as the Atlantic sea cucumber and schwarze seegurke, respectively. In Canada, *C. frondosa* is known as the Northern sea cucumber, orange-footed sea cucumber, phenix sea cucumber, and pumpkin sea cucumber (Kluijver and Ingalsuo, 2004). Fisheries for *C. frondosa* first developed in Maine (USA) in the 1980s, later expanding to eastern Canada, Iceland, and western Russia (Hamel and Mercier, 2008b). When it first emerged, landed weights made this species one of the predominant sea cucumbers on the global market (Therkildsen and Petersen, 2006).

The most commonly traded sea cucumber product consists of the body wall (skin), generally including the longitudinal muscle bands, which is dried and sold as beche-de-mer or trepang. The processing of *C. frondosa* follows slightly different protocols than those developed for traditional tropical or temperate species and muscle bands are sometimes fresh frozen and marketed separately from the body wall (Rosalyn, 2015). Dried aquapharyngeal bulbs (labelled 'flowers'), liquid or gel extracts and various supplements can also be found on the market (Hamel and Mercier, 2008a). Although *C. frondosa* is smaller than most other commercial sea cucumbers and possesses a thinner body wall, no papillae, and an irregular shape after processing, market acceptance has nevertheless increased for this species over the past few decades (Therkildsen and Petersen, 2006). Market price for *C. frondosa* (i.e. dried body wall or whole sea cucumber) in Asian markets recently reached approximately US$230 kg^{-1} dried weight (Sze and Conand, 2015).

The use of sea cucumbers for medicinal and cosmetic purposes has gained popularity in recent years. Studies have shown that *C. frondosa* body wall, muscles and intestine are important sources of high-value compounds, which have been explored by nutraceutical and pharmaceutical companies (Haug *et al.*, 2002; Mamelona *et al.*, 2007; Zhong *et al.*, 2007; Janakiram *et al.*, 2010). In particular, studies have shown that the body wall of *C. frondosa* contains high levels of protein and low levels of fat (Zhong *et al.*, 2007) and is an important source of compounds with multiple biological benefits, including anticoagulant, anticancer, antioxidant, anti-inflammatory and antibacterial properties (Haug *et al.*, 2002; Mamelona *et al.*, 2007).

In response to the growing demand for sea cucumber products and the expansion of aquaculture, sound knowledge must be collected to provide a solid base for the development of standard culture protocols. The high marketability of *C. frondosa* for seafood and pharmaceutical/nutraceutical products highlights the importance of this species in the global market and its potential for aquaculture. Moreover, there is an increasing interest to use *C. frondosa* as an extractive species in integrated multi-trophic aquaculture systems to reduce the environmental footprint of salmon and mussel farming (Nelson *et al.*, 2012a; Nelson *et al.*, 2012b). However, *C. frondosa* differs from previously cultivated sea cucumbers in a number of ways: (1) It is a cold-water species; (2) it is a passive suspension-feeder that captures food particles from the water column (Singh *et al.*, 1998); and (3) it produces large yolky eggs that develop into non-feeding (lecithotrophic) larvae (Hamel and Mercier, 1996b, 1998). Although aquaculture of *C. frondosa* remains largely theoretical, this species does have major commercial advantages when compared to other economically important species of sea cucumbers. For instance, as *C. frondosa* is a

suspension-feeder; its diet mostly includes phytoplankton which is perceived to be of better quality than the organic sediment ingested by deposit-feeding sea cucumbers. Moreover, phytoplankton is relatively easy to culture and can be available all year long which puts *C. frondosa* in a favourable position for aquaculture. The second advantage of *C. frondosa* is its large eggs which develop into lecithotrophic (non-feeding) larvae. These larvae do not require feeding until settlement, i.e. ~45 d of development (Hamel and Mercier, 1996a). The production of large eggs with energy-rich yolk reserves generates larger larvae which may have better chances of survival during metamorphosis. In the natural habitat, the density of *C. frondosa* adults can reach up to 50 individuals m^{-2} (Singh *et al.*, 2001), suggesting that this species is able to support high stock densities and might be suitable for intensive aquaculture system. Tools and techniques that might assist holding/handling of *C. frondosa* in aquaculture settings have already been developed, including a way to determine sex in adults based on external sexual dimorphism rather than invasive/lethal approaches (Montgomery *et al.*, 2018, see Appendix), a method to individually mark *C. frondosa* using PIT (passive integrated transponder) tags (Gianasi *et al.*, 2015), and protocols for optimum transport of live individuals from production/fishing sites to processing plants in order to maintain the health and nutritional condition of the sea cucumbers (Gianasi *et al.*, 2016).

1.3 Main objectives and thesis structure

To develop a commercial culture of *C. frondosa*, much deeper knowledge of the specific needs of lecithotrophic, suspension-feeding sea cucumbers must be gathered

(Nelson *et al.*, 2012b). The present work sought to provide significant advances in our understanding of *C. frondosa* and explore aspects of broodstock conditioning, spawning induction, hatchery techniques, embryo development and juvenile morphology and behaviour in captivity.

Sea cucumber aquaculture has relied for many years on the collection of wild adults to obtain gametes and grow juveniles (Jimmy *et al.*, 2012; Liu, 2015). However, with the decline of many natural populations, hatcheries increasingly try to hold their own broodstock either in indoor tanks or outdoor ponds to secure a minimum number of breeders for the next production cycle (Duy, 2012; Purcell *et al.*, 2012). However, in many cases, individuals held in captive conditions showed signs of weight loss, low gonad development, gametogenic asynchrony between sexes, and poor fecundity (Jimmy *et al.*, 2012). In an attempt to identify the main factors mediating the production of gametes in *C. frondosa*, Chapter 2 investigated the influence of water temperature, photoperiod, and food concentration on its gametogenesis, natural spawning, egg quality and embryo survival. Males and females were analyzed for gonad indices, stages of gonad development, and oocyte size frequency distribution at three-time points: pre-trial, just before spawning, and immediately after the spawning period. The number of oocyte released, quality of eggs and survival of embryos were also evaluated.

A consistent supply of healthy gametes had been identified as a bottleneck in many sea cucumber aquaculture worldwide (Purcell *et al.*, 2012). Contrary to sea urchins that can be reliably induced to spawn with injection of potassium chloride (Walker *et al.*, 2007), sea cucumbers are notoriously more difficult (Léonet *et al.*, 2009). Most of the techniques to induce spawning in sea cucumbers were developed for tropical and temperature species and

are either partly or completely ineffective (Battaglene *et al.*, 2002; Léonet *et al.*, 2009). Moreover, oocytes of sea cucumbers are arrested at the meiotic stage and undergo germinal vesicle breakdown (GVBD) and final maturation only during spawning, which makes oocytes freshly collected from the ovaries unfertilizable (Maruyama, 1986). Chapter 3 investigated methods to trigger spawning in adults of *C. frondosa* and to artificially induce GVBD in surgically removed oocytes from mature ovaries. Focal spawning triggers included thermal shock, live phytoplankton, commercial phytoplankton paste, desiccation, sperm from conspecifics, as well as exposure to or injections of solutions of potassium chloride and serotonin. The number of males and females releasing gametes, number and quality of oocytes released, and survival rates of embryos were assessed. Experimental trials were also conducted to test 1-Methyladenine (1-MA), Dithiothreitol (DTT), 2,3-Dimercapto-1-propanol (BAL), and L-cysteine (L-cyst) as artificial inducers of final oocyte maturation in *C. frondosa*.

Embryonic and larval phases are usually the most critical moments in the life history of marine invertebrates where several changes in the morphology and behaviour occur over a short period of time (Lawrence, 1987). Studies examining the adaptation and behaviour of marine larvae can provide valuable insights towards optimizing the transition from larvae to juvenile in aquaculture settings. The investigation of embryonic/larval morphology and developmental tempo in *C. frondosa* led to the discovery of allogeneic fusion whereby two or more embryos fuse, creating a chimaera (organism composed of genetically distinct cells). Chapter 4 explored the formation of chimaeras in embryos of *C. frondosa* and its frequency of occurrence in the propagule population. Histology of normal (non-chimaeric) and chimaeric embryos was also performed to visually assess the

location of external and internal tissues during the fusion process. Moreover, the hypotheses that propagule density and their natural tendency to agglomerate in the water surface could favour the occurrence of chimaeras were tested.

Knowledge of developmental biology and behaviour of post-settlement stages is crucial for producing offspring of good quality and for successful mass rearing of juveniles (Ramofafia *et al.*, 1997). Early life stages of marine organisms have stage-specific environmental requirements. Developments in rearing technology and start-feeding have benefitted significantly from studies of the developmental biology and ecology of sea cucumber juveniles (Yang *et al.*, 2015). There is still a relative scarcity of literature dedicated to the earliest benthic life stages, especially in species that rely on suspension-feeding and/or live in temperate-cold habitats, which has also hindered advances in aquaculture. Chapter 5 investigated morphometric changes occurring on a monthly basis in *C. frondosa*, from newly-settled recruits to juveniles 21 months old. Focal metrics included body length, number and size of tentacles, tentacle ramifications, number of ventral and dorsal ambulacral podia, tentacle insertion rates, ossicles, and skin pigmentation. Experimental trials were also conducted to assess ontogenetic changes in the response of various age groups to different light intensities, substrate types, background colours, current speeds, as well as to briefly describe their daily activity patterns.

Chapters are written in a format compatible with the publication guidelines of scientific journals, which explains the repetition of some of the biological and ecological information. Chapter 6 summarizes the main findings and their contribution to advancing our understanding of the biology and ecology of *C. frondosa* and its requirements pertinent

to the successful establishment of a hatchery. It also presents directions for future research in this area.

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Chapter 2. Environmental Control and Manipulation of Gametogenesis and Spawning: Egg Quality, and Embryo Survival in the Sea Cucumber *Cucumaria frondosa*[1]

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[1]A version of this manuscript is currently being prepared for submission to Aquaculture Research.

2.1 Abstract

The present study aimed to assess how environmental factors influence gametogenesis, spawning, egg quality, and embryo survival in the suspension-feeding holothuroid *Cucumaria frondosa*. Three seawater temperatures (ambient <3°C, 6°C, 12°C), four photoperiods (ambient 8-13-h light, 24-h dark, 24-h light, 4-mo advanced), and two food concentrations (3 and 6 x 10^3 rotifers g^{-1} of sea cucumber d^{-1}) were tested over 120 d. At the onset, males and females showed low gonad indices (GI = 14% and 9%, respectively) and females had small oocytes (main mode ~150 µm in diameter) in the gametogenic growth stage. At pre-spawning, males had mature gonads across all conditions, but differences in the reproductive output of females occurred among treatments. Females in ambient temperature conditions had the highest GI (33%) and ovaries were filled with oocytes (400 µm) typical of the early mature stage, whereas females exposed to 6 and 12°C showed lower GI (24-26%), and smaller oocytes (150 µm), still at the growth stage. In the photoperiod treatments, GI was lowest in females exposed to 24-h light (23%) compared to all other conditions (26-33%). Oocyte size distribution showed that oocytes up to 400 µm were present in females exposed to ambient and advanced photoperiod, whereas females under 24-dark and 24-light exhibited oocytes between 151-350 µm (advanced-growth stage). Females fed high food concentration showed mature ovaries similar to ambient controls. When spawning occurred, females in ambient seawater released 1.2 x 10^3 eggs female^{-1}; whereas spawning did not occur at 6°C or 12°C. In photoperiod trials, females from the ambient group also released the highest number of oocytes, followed by

advanced photoperiod, 24-h dark, and 24-light (12-240 eggs female^{-1}). Females with access to more food released slightly fewer eggs (820 eggs female^{-1}) than ambient controls. In treatments where spawning occurred, sea cucumbers in ambient conditions of temperature, photoperiod and in increased food supply produced the best egg quality and highest survival of embryos to the gastrula stage (17-21% at 20 d post fertilization). In contrast, embryos from constant dark or light and advanced photoperiod treatments died within 10 d post fertilization. Overall, the reproductive output of *C. frondosa* was optimal under ambient/natural environmental conditions, whereas warmer water temperature, and constant light or dark conditions delayed gonad development and prevented spawning, suggesting that enhancement of broodstock productivity in cold-water suspension-feeding sea cucumbers might prove challenging.

2.2 Introduction

The aquaculture of sea cucumber (Echinodermata: Holothuroidea) is a booming industry in Asia (Yang *et al.*, 2015) that is also developing in several other locations worldwide (Purcell *et al.*, 2012). To date, this sector has mostly relied on the collection of wild adults during the breeding season to create broodstock for spawning and subsequent grow-out of juveniles (Jimmy *et al.*, 2012; Liu *et al.*, 2015). However, the decline of the natural stocks has led to a scarcity of wild individuals to serve as broodstock every year (Duy, 2012; Jimmy *et al.*, 2012; Purcell, 2012; Purcell *et al.*, 2012). In an attempt to secure a minimum number of breeders and develop broodstock selection programs, adult sea cucumbers have been held in outdoor tanks and ponds year round under naturally fluctuating environmental conditions (Jimmy *et al.*, 2012). Unfortunately in many cases, sea cucumbers held for an extended period of time in captive conditions have shown signs of weight loss, poor gonad development, low fecundity and gametogenic asynchrony between sexes, as reported for the deposit-feeding species *Holothuria scabra* in Australia (Morgan, 2000), Vietnam (Duy, 2012), and several Pacific islands (Jimmy *et al.*, 2012).

Reproduction in echinoderms is influenced by a combination of endogenous and exogenous factors, which are regulated by changes in the environment (Mercier and Hamel, 2009). The role of environmental parameters on gametogenesis has long been studied in echinoids, or sea urchins, due to the high value of their gonads (i.e. roe) on the seafood market (Walker *et al.*, 2007). Pearse *et al.* (1986) initially demonstrated that the timing of gametogenesis could be shifted by exposing the purple sea urchin *Strongylocentrotus purpuratus* to photoperiod six months out of phase in the laboratory.

Similarly, when the green sea urchin *S. droebachiensis* was held under a photoperiod advanced by four months, gametogenesis started earlier than when individuals were maintained under the natural schedule (Walker and Lesser, 1998). Shpigel *et al.* (2004) observed that periods of darkness (no light) or short daylengths (10L/14D) increased the rates of gametogenesis in another species (*Paracentrotus lividus*); whereas long daylengths (16L/8D) inhibited the production of gametes. In other sea urchins, water temperature appears to have more influence on the production of gametes. When *Pseudocentrotus depressus* was held in captivity under several combinations of photoperiod (24-h dark or dark, 6 months in advance, or ambient) and water temperature (constant 20°C or ambient), gonad maturation was shown to be independent of the photoperiod; however, it occurred several months earlier in individuals kept at 20°C than in individuals held at ambient (12-17°C) temperature (Yamamoto *et al.*, 1988).

In holothuroids, or sea cucumbers, the reproductive cycle has been investigated and correlated to a number of environmental parameters that are acting successively or in combination to modulate gametogenesis and spawning (Mercier and Hamel, 2009). On a broad scale, the main factors involved in gametogenesis (especially in temperate species) include photoperiod (i.e. day length) and water temperature; whereas changes in phytoplankton concentration, light intensity (i.e. time of the day), and lunar cycles have been proposed to act more directly on spawning (Mercier and Hamel, 2009). In eastern Canada, for example, the initiation of gametogenesis of the cold-temperate suspension-feeding sea cucumber *Psolus fabricii* coincided with the annual increase in photoperiod during early winter; whereas spawning occurred during the annual spring bloom (Hamel *et al.*, 1993). At tropical latitudes, the gonad maturation and spawning of several deposit-

feeding sea cucumbers (e.g. *Holothuria scabra*, *H. fuscopunctata*, *H. nobilis, H. fuscogilva, Actinopyga echinites*, *Thelenota ananas*, and *Stichopus variegatus*) coincided with changes in water temperature during warm/cold or dry/wet seasons (Conand, 1981, 1993a, 1993b) and sometimes to precise lunar phases, as demonstrated for *Isostichopus fuscus* (Mercier *et al.*, 2007). More recently, gametogenesis in *H. grisea* was suggested to initiate as phytoplankton concentration starts to increase along the northeastern Brazilian coast during the summer months (Leite-Castro *et al.*, 2016). In captivity, the manipulation of environmental parameters to modify the gametogenic cycle of sea cucumbers has only been reported for the temperate deposit-feeding sea cucumber *Apostichopus japonicus* whereby adults held during the breeding season at a water temperature 6°C above ambient matured two months earlier than control individuals (Liu *et al.*, 2015).

The sea cucumber *Cucumaria frondosa* is widely distributed in temperate and cold waters, occurring from the Arctic Ocean to Cape Cod (Massachusetts, USA) as well as along the coasts of northern Europe and Russia (Hamel and Mercier, 2008). This species has been identified as a potential candidate for aquaculture in North America due to its increasing value as seafood, with market prices reaching US$ 230 kg^{-1} dried (Sze and Conand, 2015), and as a source of pharmaceutical and nutraceutical products (Hamel and Mercier, 2008). Recently, *C. frondosa* has been explored as an extractive species for integrated multi-trophic aquaculture systems (IMTA) in an attempt to reduce the footprint of mussel and salmon farming (Nelson *et al.*, 2012). However, captive breeding of *C. frondosa* poses some unique challenges because it differs from all previously cultivated sea cucumbers in several key ways: (i) it is an arctic/subarctic cold-water species (Hamel

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and Mercier, 2008); (ii) it is a suspension-feeder that primarily ingests phytoplankton (Singh *et al.*, 1998); and (iii) it produces large yolky oocytes that develop into non-feeding (lecithotrophic) larvae (Hamel and Mercier, 1996a). Studies of the reproductive cycle of *C. frondosa* in the field have reported that gametogenesis is initiated in early winter with the first increase in day length, whereas spawning coincides with the annual increase in phytoplankton concentration during spring (Hamel and Mercier, 1995, 1996c). Moreover, experimental trials conducted under laboratory conditions found higher gonad indices and gamete densities in fed than in starved adults of *C. frondosa*, suggesting that abundance and type of food modulate gonad development (Gianasi *et al.*, 2017).

Considering the expansion of sea cucumber aquaculture worldwide to include cold-water species and species with maternally-provisioned larval development, understanding how environmental parameters control their gametogenesis, spawning, egg quality, and embryo survival will be instrumental for broodstock management in captivity. The present study investigated the influence of different water temperatures, photoperiod regimes, and food concentrations on gametogenesis, synchronization of gonad development between sexes, spawning success, quality of eggs, and survival of embryos in *C. frondosa*. Males and females were exposed to various treatments for 120 d. Organ indices, stages of gonad development, total number of gametes produced, and oocyte size frequency distribution were assessed at three time points: pre-trial, pre-spawning and post-spawning. The number of oocytes released, the quality of fertilized oocytes and embryo survival rates were also compared across treatments.

2.3 Material and methods

2.3.1 Sea cucumber collection

Similarly sized sea cucumbers weighing 4.7 ± 1.0 g immersed weight (~310 g wet weight) and measuring 16 ± 2.6 cm contracted body length were hand collected by divers along the Avalon Peninsula, Newfoundland, eastern Canada (47°17'44.6" N: 52°46'8.9" W), at depths between 5 and 10 m. Individuals were held in three 500-L flow-through tanks (~150 individuals in each) with running supply of ambient seawater (40 L h^{-1}) at temperatures of 3 ± 1°C. Photoperiod was adjusted weekly to match natural fluctuations. Plankton and particulate organic matter present in seawater was available as food source during this time. Sea cucumbers were kept in holding tanks for over a month prior to the experimental phase. Only healthy and undamaged individuals displaying normal pigmentation and feeding activity, firm attachment to the substrate, and no skin lesions were selected for the trials.

2.3.2 Experimental design

2.3.2.1 Experimental setup

Each treatment was replicated in three independent tanks, containing 12 sea cucumbers (i.e. 12 individuals x 3 tanks = 36 sea cucumbers per treatment). Tanks (150 L) were supplied with filtered running seawater at a flow rate of 20 L h^{-1}. The filtration system that provided water to experimental tanks comprised sandbed filtration (15 μm), foam fractionation, and ultraviolet filters, which removed essentially all suspended particles from

the water column. Light was provided by a set of white fluorescent bulbs (spectrum of 450-780 nm) to a maximum intensity of 250 lx at the water surface, which covered the probable range of light intensity recorded in the field between 5 and 100 m depth (Kampa, 1970). Tanks were kept at ambient photoperiod and water temperature, except when environmental parameters were manipulated (see description of treatments below). Ambient photoperiod was adjusted weekly to match the natural environment, starting with 8L/16D in the winter and ending with 13L/11D in spring. Ambient water temperature ranged from -1°C to 3°C throughout the experimental period. Air bubbling coming from the centre of the tanks maintained the food in suspension and uniformly mixed in the water column (see diet below). Tanks were randomly distributed in two parallel rows and isolated with black vinyl tarpaulin to avoid bleeding of light among treatments. Physical-chemical parameters of the water and light intensity were monitored daily with a handheld multiparameter probe (YSI 556 MPS) and a light meter (Traceable® 3252). Sea cucumbers were acclimated in the experimental tanks five days prior to the beginning of the trial. An attempt to equally distribute males and females (1:1) in each tank was made based on preliminary description of gonopore morphology, which is visible when tentacles are deployed (Hamel and Mercier, 1996a).

2.3.2.2 Diet and feeding

Although phytoplankton constitutes the main diet of *C. frondosa* in the wild (Hamel and Mercier, 1998), a previous study suggested that phytoplankton might induce spawning in adults held in captivity, even outside the peak of the spawning season (Gianasi *et al.*, 2017). To avoid unwanted spawning of underdeveloped gametes (during early oogenesis)

due to the addition of phytoplankton in the tanks, rotifers (*Brachionus plicatilis*, ~150 µm) were used as an alternative diet for *C. frondosa*. Rotifers were deemed promising as they are very well accepted by *C. frondosa* (based on preliminary tests), can be enriched with commercial diets to add essential nutritive elements (especially fatty acids such as EPA and DHA), and can be reliably produced all year long. In contrast to many phytoplankton, rotifers do not have frustules composed of silica and, therefore, might be entirely digested and absorbed by *C. frondosa*. Rotifers were cultured in a 100-L tank at a stock density of 1 x 10^6 rotifers L^{-1}. The culture was continuously fed with Ori-One® (commercial growing and enrichment diet for rotifers) at a rate of 0.2 g of Ori-One® million-rotifers^{-1} d^{-1}. Rotifers were washed in a collector (mesh size of 40 µm) with seawater for ~30 min to remove food residues, then mixed in 1 L of seawater at the same temperature and added to experimental tanks (see concentration used below).

The amount of rotifers fed to sea cucumbers was chosen to represent the average biomass of phytoplankton in the natural habitat of *C. frondosa* during the experimental period. For this, data on phytoplankton were collected from Station 27, surveyed by the DFO Atlantic Zone Monitoring Program (Pepin *et al.*, 2007) located close to the site where sea cucumbers were collected. In order to standardize the amount of food available to each sea cucumber before and after samplings, the concentration of rotifers was determined based on a ratio of rotifers to average immersed weight of sea cucumbers in each tank. In this context, sea cucumbers were fed once a day (at 10:00) with a concentration of 3 x 10^3 rotifers g^{-1} of sea cucumbers d^{-1}, except when otherwise mentioned. During feeding times, the water flow was interrupted for 3 h to ensure all sea cucumbers had enough time to react to and ingest the food (Gianasi *et al.*, 2015).

2.3.2.3 Experimental conditions

Sea cucumbers were exposed to different treatments of water temperature, photoperiod, and food concentration for a total of 120 d during a period that included the natural gametogenic and spawning time of *C. frondosa* in eastern Canada (Mercier and Hamel, 2010). All treatments were compared to a control group which was exposed to ambient conditions that fluctuated over the study period: seawater temperature (-1 to 3°C), photoperiod (8 to 13 h of light), and food availability (3 x 10^3 rotifers g^{-1} of sea cucumbers d^{-1}).

For the temperature treatments, sea cucumbers were exposed to ambient photoperiod (adjusted weekly) and kept in seawater at either 6 ± 1°C or at 12 ± 1°C. Temperatures were chosen based on the fact that *C. frondosa* displays high feeding activity at ~7°C when food is abundant (Gianasi *et al.*, 2017), and because 12°C is close to the upper end of the temperature range for this species in Newfoundland (So *et al.*, 2010). Individuals were initially placed in their experimental tanks while seawater was at the same temperature as their holding tanks (≤ 3°C). Acclimation was conducted by gradually increasing the water temperature in header tanks at a rate of 1°C h^{-1} until it reached the desired temperature in the tanks; *C. frondosa* is exposed to such temperature variations in its natural habitat, especially during changing tides at different times of the year (Hamel and Mercier, 1996a).

Photoperiod treatments were conducted in ambient seawater temperature and included: 24-h dark, 24-h light, and photoperiod advanced by 4 months relative to ambient (adv photo). The latter treatment was a preliminary experiment based on sea urchin research (Walker and Lesser, 1998), since the sensitivity of *C. frondosa* to the photoperiod cue is

currently unknown. Experimental tanks in treatment 24-h dark had black walls and fiberglass covers, which did not allow the penetration of surrounding light. Tanks in advanced photoperiod started on a photoperiod of 13L/11D (spring) and ended with 15L/9D (summer).

Another group of sea cucumbers (high food) was fed twice the quantity of rotifers as the other treatments, under ambient conditions of seawater temperature and photoperiod. This concentration (6 x 10^3 rotifers g^{-1} of sea cucumbers d^{-1}) was chosen to mirror the biomass during natural spring phytoplankton bloom, which usually occurs five months after *C. frondosa* starts synthetizing gametes.

2.3.3 Monitoring and data collection

2.3.3.1 Assessment of health condition and reproductive output

Deployment/retraction of the tentacles and feeding activity (i.e. movement of the tentacles towards the mouth) were determined in each tank before and after the addition of the food. The number of individuals reacting to the rotifers by extending their tentacles and inserting them into the mouth was documented daily 1 h post feeding.

In order to assess health condition and gonad maturation of sea cucumbers, an initial sampling was conducted prior to the start of the experiment (pre-trial). A second sampling (pre-spawning) was performed four days before the estimated spawning of *C. frondosa* in Newfoundland, generally corresponding to the first spring full moon (Mercier and Hamel, 2010). The third sampling was conducted seven days after the spawning time (post-spawning).

For the first pre-trial sampling, 12 males and 12 females were haphazardly selected from the holding tanks. In each subsequent sampling, 4 sea cucumbers were haphazardly selected from each experimental tank (4 individuals x 3 tanks per treatment = 12 sea cucumbers per treatment) and males and females were analyzed separately. Their general health condition and gonad maturation were assessed through organ indices, gonad phenotype, diameter of the gonad tubules, and oocyte size frequency distribution.

Organ indices were calculated as the wet weight of the organ, such as gonad, muscle bands, intestine, and respiratory tree, divided by the wet weight of the body wall (after removing the aquapharyngeal bulb and the longitudinal and circular muscle bands). All organs were blotted on paper towel to remove excess water prior to weighing on a digital scale (Sartorius TE313S).

The oocyte size frequency distributions were obtained by weighing a 2-cm subsample of gonad tubule from each individual (4 individuals x 3 tanks = 12 sea cucumbers per treatment). Oocytes or spermatozoa were extracted by gently pressing a glass rod over the tubule toward the opening. Gametes collected from gonad subsamples were preserved in 10% ethanol and kept refrigerated at 4°C until further analysis. Oocytes were measured at their longest axis (Feret diameter) under a stereo-microscope (Leica M205FA) and counted. The concentration of spermatozoa in the male gonad was determined by collecting 10 µl of well-mixed sperm solution and placing it in a hemocytometer to perform counts under the same microscope. Dilution factors were applied to estimate the total amount of spermatozoa in the testis subsample.

Gonad maturation has been associated with changes in gonad colour and size in *C. frondosa*. In order to better discriminate gametogenic stages, 30 gonad tubules of

C. frondosa were haphazardly selected from each gonad and their diameter measured (at mid tubule length) with a digital caliper (Traceable® 12777-830). The large size of the mature oocytes in *C. frondosa* (~550 µm) combined with frequent measurements of gonad tubules, and diameter of oocytes before and after spawning, allowed the classification of gametogenic stages of females into: growth, advanced growth, mature, partially spent, and spent (Table 2.1). Finally, distribution of oocyte diameters (number of oocytes per size class from 100 to 800 µm, using 50-µm bins) was conducted in order to visualize the size range of oocytes in each experimental group before and after spawning.

2.3.3.2 Spawning, egg quality and embryo survival

Experimental tanks were monitored daily for evidence of natural spawning. When spawning occurred, the time of spawning and the synchronicity among individuals and replicate treatment tanks were recorded. Eggs were gently skimmed from the surface of the experimental tank into a glass breaker and incubated in 4-L perforated plastic containers covered with 300 µm mesh. Egg samples from the same treatment (1 sample per replicate tank = 3 samples per treatment) were placed inside 150-L flow-through tanks with the same water temperature and light conditions as the parental tanks. The number of eggs released in each tank was estimated from aliquots, and fertilization was confirmed under a microscope (Leica M205FA) based on elevation of the fertilization envelope and/or cleavage.

The quality of eggs and embryos from each treatment was monitored until the gastrula stage (20 days post fertilization). Quality criteria for eggs included buoyancy,

colouration, and fertilization; whereas cleavage pattern, morphology and swimming patterns were used to evaluate quality of embryos, as described in Table 2.2.

A sample of 50 eggs/embryos was collected from each culture three times daily for a total of 20 days post fertilization (i.e. until the gastrula stage); propagules were measured and assessed for quality as per Table 2.2. A new developmental stage was scored when 50-60% of the eggs/embryos had reached it. Finally, survival rates of eggs/embryos were recorded every 5 days.

2.3.4 Data analysis

Data on sea cucumber feeding, gonad index, gonad phenotype, total number of gametes, quality of fertilized eggs, blastulae and gastrulae, and embryonic survival rate were tested for normality and equal variance using Kolmogorov-Smirnov and Levene's tests ($\alpha = 0.05$), respectively. Analysis of variance (ANOVA) was conducted when assumptions were met; otherwise data transformations were attempted, failing which a non-parametric test was adopted.

The number of sea cucumbers with deployed tentacles and displaying feeding activity violated the assumptions for parametric statistics even after transformations. For this reason, Friedman repeated measures analysis of variance on ranks followed by multiple comparison Tukey tests were used to compare the number of deployed/feeding sea cucumbers among treatments.

Two-way ANOVA followed by Tukey multiple comparison tests were used to compare organ indices (gonad, muscle, intestine, and respiratory tree). Three-way ANOVA followed by Holm-Sidak multiple comparison tests were used to compare gametogenic

stages (growth, advanced growth, mature, partially spent, and spent) among treatments over time (in pre-trial, pre-spawning and post-spawning samplings). When spawning occurred, the total number of eggs released violated the assumptions for parametric statistics even after transformations; therefore, one-way ANOVA on ranks followed by Tukey tests was used compared the number of eggs spawned in each treatment. The quality of fertilized eggs (0 d post fertilization), blastulae (10 d post fertilization) and gastrulae (20 d post fertilization) was compared among treatments using one-way repeated measures ANOVA followed by Holm-Sidak multiple comparison tests. Finally, survival rates of gastrulae (20 d post fertilization) was assessed among treatments with Logrank survival analysis, using Kalpan-Meier estimator followed by Holm-Sidak multiple comparison tests. Male and female sea cucumbers were analyzed separately. Data in the text are expressed as mean ± se. Statistical analysis were performed with SigmaPlot® using $\alpha = 0.05$.

2.4 Results

2.4.1 Health condition, reproductive output, and spawning

The proportion of sea cucumbers displaying feeding activity varied under different environmental conditions (Fig. 2.1). In the seawater temperature treatments, the proportions of individuals feeding in the control (ambient temperature) and at 6°C were similar (62.4 ± 7.2% and 66.8 ± 8.5%, respectively, Fig. 2.1a), and both significantly higher than at 12°C (15.7 ± 2.2%; $\chi^2 = 7.6$, df = 2, p = 0.011). Similarly, in the photoperiod trials, the highest proportion of sea cucumbers feeding was in the control group (62.4 ± 7.2%; χ^2 = 8.4, df = 3, p = 0.002; Fig. 2.1b); whereas in 24-h dark, 24-h light, and advanced

photoperiod, only $37.7 \pm 6.3\%$, $46.6 \pm 4.2\%$, and $42.9 \pm 8.3\%$, respectively, were feeding, without any significant differences among treatments (Fig. 2.1). The increased food treatment (high food) did not yield an increase in feeding activity (Fig. 2.1c); instead it showed similar proportions of feeding individuals as the control group ($71.1 \pm 9.4\%$ and $62.4 \pm 7.2\%$, respectively).

When sea cucumbers were assessed for organ indices, only the gonad index (GI) showed an increase toward the spawning period for the species (Fig. 2.2); whereas muscle, intestine, and respiratory tree indices remained unchanged throughout the study (Supplementary material Figs. S.2.1, S.2.2, and S.2.3), and thus will not be considered further. At the onset of the experiment (time 0), female sea cucumbers had a GI of $14 \pm 3.2\%$ (Fig. 2.2) and gonad tubules measuring 2.2 ± 0.5 mm in diameter. Some reddish vitellogenic oocytes and smaller yellowish oocytes were present in the lumen closer to the germinal epithelium, which overall imparted a light reddish colour to the whole gonad (Supplementary material, Fig. S.2.4). Oocyte size frequency distribution showed the predominance of small oocytes with a mode around 150 µm in diameter (Fig. 2.3). Ovaries were all scored to be in the growth stage of oogenesis (Fig. 2.4; Table 2.1). Males had a GI of $9 \pm 4.1\%$ at the onset of the experiment (Fig. 2.2) and gonad tubules measuring 1.6 ± 0.3 mm in diameter. Some spermatozoa were visible in the lumen, which gave a uniform dark peach colour to the gonad (Supplementary material, Fig. S.2.4). Testes were also scored to be at the growth stage of development (Fig. 2.4; Table 2.1).

When the spawning time approached (pre-spawning samples; ~100 d of exposure), ovaries changed colour, tubules became larger, the GI and the size of oocytes increased, and significant differences in the latter two metrics occurred across the treatments (Figs.

2.2 and 2.3). In temperature trials, control females exhibited the highest GI ($33.2 \pm 3.6\%$; Fig. 2.2) among all treatments ($F_{3,36} = 7.5$, $p = 0.001$). The diameter of the gonad tubules increased to 2.8 ± 0.4 mm and large reddish oocytes appeared (Supplementary material Fig. S.2.4) with a size mode centred around 400 µm (Fig. 2.3), which classified these ovaries in the mature gametogenic stage (Fig 2.4; Table 2.1). Despite females exposed to 6 and 12°C showed an increase in GI ($24.3 \pm 3.8\%$ and $26.4 \pm 5.1\%$, respectively; Fig. 2.2) compared to time 0 (onset), the diameter of gonad tubules (~2.2 mm) and the mode of the oocyte size distribution (~150 µm) remained unchanged, i.e. at the growth gametogenic stage (Fig 2.4; Table 2.1). Differences in the reproductive output were also detected in pre-spawning females from the photoperiod treatments. The GI from the control ($33.2 \pm 3.6\%$), 24-h dark ($26.6 \pm 3.9\%$) and advanced photoperiod ($31.4 \pm 3.1\%$) were similar; however, they were significantly higher than for females under 24-h light ($23.5 \pm 3.3\%$; $F_{4,48} = 13.8$, $p = 0.003$; Fig. 2.2). Greater differences were measured in gonad tubule diameters and oocyte size distributions among treatments. Both control and advanced photoperiod treatments showed gonad tubules of 2.8 ± 0.4 mm in diameter filled with large reddish oocytes with a mode around 400 µm (Fig. 2.3) scoring them as gametogenically mature ovaries (Fig. 2.4). However, females exposed to 24-h dark and 24-h light presented tubules measuring ~2.4 mm in diameter filled with a mix of large and small oocytes, with a mode around 200 µm (Fig. 2.3), which characterized them at the advanced-growth gametogenic stage (Fig. 2.4). Females fed with a high concentration of food (high food) had similar GIs ($34.3 \pm 2.7\%$; Fig. 2.2) and gonad tubules (~2.8 mm diameter) filled with large oocytes (mode ~400 µm diameter) than females in control conditions, i.e. classified as mature gametogenically (Fig. 2.4). Males at pre-spawning, on the other hand, exhibited similar GIs (30-34%; Fig. 2.2)

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and tubules (2.6 ± 0.4 mm diameter) filled with spermatozoa, giving a uniform light peach colour to the gonads (Supplementary material Fig. S.2.4; Table 2.1), indicating they were mature across treatments (Fig. 2.4).

Sea cucumbers began to spawn in the morning (~7:00 h) of the first spring full moon (March). Males started to spawn first, followed by females ~30 min later. Spawning occurred simultaneously in all three tanks of the control and high-food treatments; however, spawning was only detected in two tanks of the advanced-photoperiod, and only in one tank each of 24-h dark and 24-h light treatments (Table 2.3). The total number of eggs released in the control group (1,250.4 ± 82.6 eggs female^{-1}) was significantly higher than in all other treatments (H = 14.8, df = 6, p = 0.013; Table 2.3), followed by high-food treatment with 820.6 ± 76.3 eggs female^{-1} (Table 2.3). The advanced-photoperiod treatment yielded intermediate numbers of eggs released (240.3 ± 92.1 eggs female^{-1}), while the lowest number of eggs released was in the 24-h dark (12.5 ± 10.2 eggs female^{-1}) and 24-h light (89.4 ± 82.3 eggs female^{-1}) treatments (Table 2.3). Sea cucumbers exposed to the water-temperature treatments (6 and 12°C) did not spawn (Table 2.3).

After spawning had ceased and eggs had been collected, sea cucumbers were dissected to determine the proportion of individuals that had spawned and their gonad stages post-spawning (~120 d of exposure). For both water temperature and photoperiod treatments, the largest drops in GIs were detected in females and males from the control group (down to 15.1 ± 7.5% and 17.6 ± 6.4%, respectively) (Fig. 2.2); whereas females and males at 6°C (23.5 ± 2.3% and 29.6 ± 4.2%, respectively) and 12°C (31.4 ± 7% and 29.2 ± 5.2%), and in 24-h dark (26.6 ± 5.6% and 28.5 ± 6.7%), 24-h light (26.8 ± 3.6% and 27.7 ± 4.5%) and advanced photoperiod (24.3 ± 7.1% and 22.5 ± 6.5%, respectively) treatments

had GIs that were unchanged from the pre-spawning sampling (Fig. 2.2). In the food supply treatments, however, females and males from both control ($15.6 \pm 7.4\%$ and $17.2 \pm 6.7\%$, respectively) and high-food ($14.1 \pm 6.4\%$ and $13.3 \pm 5.8\%$, respectively) treatments exhibited drops in their GIs by almost half compared to pre-spawning (Fig. 2.2).

The assessment of gametogenic stages revealed that, in temperature treatments only females and males from the control group showed spent ($25.2 \pm 11.1\%$ and $25.2 \pm 10.4\%$, respectively) and partly-spent gonad stages ($33.4 \pm 13.5\%$ and $42.5 \pm 16.2\%$, respectively; Fig. 2.4). Moreover, the oocyte size mode of un-spawned oocytes remaining in tubules post-spawning decreased to around 250 µm in diameter (Fig. 2.3). Females exposed to 6 and 12°C (which had not spawned) showed ovaries at the mature stage with large oocytes and a mode around 400 µm (Fig. 2.3); males exposed to 6 and 12°C were still at the mature gametogenic stage, comparable to the pre-spawning samples (Fig. 2.4). Spent ovaries were characterized by 90-100% of tubules showing a yellowish colour; gonad tubule diameters reduced to 1.3 ± 0.2 mm, with few remaining oocytes in the lumen (Supplementary material Fig. S.2.4; Table 2.1). Spent testes showed 90-100% of yellowish tubules; gonad tubule diameters reduced to 1.1 ± 0.3 mm with no visual evidence of remaining spermatozoa in the lumen (Supplementary material Fig. S.2.4; Table 2.1). Partially-spent ovaries showed 20-50% of tubules with a yellowish colour and only a few unspawned oocytes in the lumen; whereas the remaining tubules were still filled with large reddish oocytes. Partially-spent testes exhibited 20-50% yellowish tubules with no visual evidence of spermatozoa in the tubules; whereas the remaining tubules were still filled with sperm and displayed the typical light peach colour of gametogenically mature tubules (Supplementary material Fig. S.2.4; Table 2.1). In the photoperiod treatment, females and males from the control group were

also the only ones to exhibit ovaries and testes in the spent stage ($25.2 \pm 11.1\%$ and $25.2 \pm 10.4\%$, respectively; Fig. 2.4). The highest proportions of partly-spent ovaries and testes were found in the control ($33.4 \pm 13.5\%$ and $42.5 \pm 16.2\%$, respectively), followed by the advanced photoperiod treatment ($25.2 \pm 9.1\%$ and $16.2 \pm 5.3\%$); whereas the lowest proportions were found in the 24-h dark and 24-h light treatments ($8.1 \pm 3.1\%$ and $8.1 \pm 4.1\%$; Fig. 2.4). Ovaries from the control and advanced-photoperiod treatment experienced a decrease in the oocyte size mode to around 250 µm in diameter post spawning; whereas ovaries of 24-h dark and light treatments remained with similar modes as pre-spawning (Fig. 2.3). In the food supply trial, the proportions of ovaries and testes from the high-food treatment at mature ($42.5 \pm 6.2\%$ and $33.4 \pm 13.5\%$, respectively), partly-spent ($33.4 \pm 13.5\%$ and $42.5 \pm 16.2\%$), and spent ($25.2 \pm 13.1\%$ and $25.2 \pm 12.1\%$, respectively) stages were similar to the control (Fig. 2.4) with an overall drop in the oocyte size mode to around 250 µm post spawning (Fig. 2.3).

2.4.2 Egg/embryo quality and survival

Among treatments where spawning occurred, sea cucumbers in control conditions showed the healthiest eggs ($F_{3,72} = 15.6$, $p = 0.001$), blastulae ($F_{3,66} = 13.4$, $p = 0.001$), and gastrulae ($F_{3,52} = 8.1$, $p < 0.001$) as well as the highest survival rates of gastrulae ($\chi^2 = 9.8$, $df = 3$, $p < 0.001$; 20 d post fertilization; Fig. 2.5a). Eggs and early embryos scored as normal displayed positive buoyancy, round shape, dark reddish colour, clear fertilization envelope (Fig. 2.6a; Table 2.2), and normal blastomere cleavages (Figs. 2.6b and 2.6c). Eggs and embryos undergoing abnormal development exhibited negative buoyancy, pale colour (Fig. 2.6f), lipid droplets in the interstitial space between the plasma membrane and

the fertilization envelope (Fig. 2.6g), recess or depression on the surface of blastomeres (Figs. 2.6h and 2.6i), and abnormal and incomplete cleavages (Fig. 2.6j; Table 2.2). Normal blastulae displayed a dark reddish colour, round shape covered in cilia and spinning (Fig. 2.6d). Abnormal blastulae exhibited pale colour, lipid droplets in the interstitial space (Fig. 2.6k), degraded cytoplasm (Fig. 2.6l), and no cilia. In the photoperiod treatments, the proportion of eggs scored as normal in the control was $92.5 \pm 5.3\%$ (0 d post fertilization; pie charts Fig. 2.5a), followed by 24-h dark ($86.3 \pm 6.3\%$) and advanced-photoperiod ($77.4 \pm 5.6\%$) treatments. The lowest proportion of normal eggs was found in the 24-h light treatment ($60.2 \pm 7.7\%$; Fig. 2.5a). Survival of eggs and early embryos (0 d post fertilization) was 100% for all treatments (Fig. 2.5a). The greatest proportion of blastulae undergoing normal development was 100% in the control (10 d post fertilization; pie charts Fig. 2.5a), followed by 24-h dark ($86.1 \pm 7.4\%$) and 24-h light ($62.7 \pm 5.9\%$); whereas the lowest proportion of normally-developing blastulae was observed in the advanced-photoperiod ($43.3 \pm 8.4\%$; Fig. 2.5a). Survival rates of blastulae (10 d post fertilization) were highest in the control ($33.2 \pm 6.1\%$), followed by advanced-photoperiod ($21.5 \pm 4.4\%$), 24-h dark ($18.4 \pm 6.5\%$), and 24-h light ($12.7 \pm 8.2\%$) treatments (Fig. 2.5a). Only embryos from the control survived to the gastrula stage ($21.2 \pm 3.8\%$ at 20 d post fertilization; Fig. 2.5a) and all of them exhibited normal development (Fig. 2.5a). Normal gastrulae displayed a reddish colour, a bean-shaped form; they were covered with cilia and spinning (Fig. 2.6e; Table 2.2).

When comparing the control with high-food treatments, no difference was detected in the quality of eggs (0 d post fertilization, pie charts Fig. 2.5b), blastulae (10 d post fertilization), and gastrulae (20 d post fertilization), or in the survival rates of gastrulae (20

d post fertilization; Fig. 2.5b). The proportions of normal eggs in control and high-food treatments were $92.5 \pm 5.3\%$ and $94.6 \pm 6.1\%$, respectively (Fig. 2.5b; Table 2.2) and survival rates were 100% for both treatments at 0 d post fertilization (Fig. 2.5b). The proportion of blastula (10 d post fertilization) and gastrula (20 d post fertilization) embryos undergoing normal development was 100% for both treatments (Fig. 2.5b). Survival rates of gastrula embryos (20 d post fertilization) were $21.5 \pm 3.8\%$ and $17.3 \pm 4.5\%$ in the control and high-food treatments, respectively (Fig. 2.5b).

2.5 Discussion

The manipulation of photoperiod and water temperature over 120 d markedly influenced the reproductive output and spawning of *Cucumaria frondosa* in captivity. Sea cucumbers exposed to ambient temperature (varying from -1 to 3°C in the study region) and photoperiod (8 to 13 h of light) exhibited the highest gonad indices and yielded the greatest production of large mature oocytes among treatments, irrespective of food supply. In addition, their spawning was synchronized across tanks and produced the greatest abundance of healthy normal oocytes, which resulted in the highest embryo survival rates.

While these results were somewhat unexpected, it is perhaps not surprising that *C. frondosa* exposed to environmental conditions similar to those encountered in their natural habitat exhibited the highest reproductive output, synchronized spawning, and the best quality and survival of offspring. This very abundant temperate/polar species (Hamel and Mercier, 2008) seems to reach optimum reproductive success in specific conditions of seawater temperature and photoperiod that are found naturally in winter and spring across

its distribution range. Despite it was shown in the polar and temperate sea urchin *S. droebachiensis* that warmer water temperature and increased food availability favoured its reproductive output (Garrido and Barber, 2001), no such improvement was noticed in *C. frondosa* after raising temperature and food supply. Field studies have suggested that *C. frondosa* undergoes peak gametogenesis during early winter when water temperature is ~0°C and photoperiod (i.e. daylength) starts to increase from its minimum (Hamel and Mercier, 1995, 1996c). The low water temperature combined with an increasing photoperiod may act synergistically to regulate and trigger endogenous factors (e.g. hormones) associated with oogenesis and/or vitellogenesis. Modifying the environmental conditions (as in the present study) could have disrupted the normal gametogenic development and the quality of the oocyte produced. In support of this, specific environmental conditions in the sea urchin *S. purpuratus* were demonstrated to induce the synthesis of glycoproteins, derived from the expression of a single vitellogenin gene, which become the major constituent of oocyte yolk (Shyu *et al.*, 1986) and potentially contribute to the quality of oocyte and embryos. If some environmental factors disrupt similar genes in *C. frondosa*, it could explain why under some environmental parameters tested here (outside of ambient-natural conditions), the growth of gametes did not progress or was altered to some degree, mainly during vitellogenesis. Although little is known about how echinoderms translate environmental factors into seasonal cycles of reproduction and hormone regulation (Mercier and Hamel, 2009), the combination of natural conditions of water temperature and photoperiod with endogenous factors that regulate the reproductive physiology of *C. frondosa* presumably optimized the synthesis of gametes, contributing to the overall abundance and quality of gametes. Why the increased temperature within the

natural tolerance of the species neither promoted gonad growth nor increased the number and quality of gametes produced by *C. frondosa* remains puzzling. The lecithotrophic (non-feeding) larvae of *C. frondosa* rely on maternally-provisioned yolky oocytes (Hamel and Mercier, 1996a), as do newly-settled juveniles during the first weeks post settlement (Gianasi *et al.*, 2018), in a manner that is very different from the more commonly studied planktotrophic species (e.g. *S. droebachiensis* and *S. purpuratus*).

The exposure of *C. frondosa* adults to ambient environmental conditions also favoured spawning synchronization and the quantity of gametes produced in captivity, compared to treatments where environmental parameters were manipulated. In the present study, spawning of *C. frondosa* occurred around the first full moon of spring, similar to previous reports of spawning either in the field or in mesocosms (Mercier and Hamel, 2010). Ambient water temperature and photoperiod likely optimized the perception of the spawning cue(s) and allowed the coordination of spawning among individuals; moreover, it allowed the timing of spawning to be relatively predictable, which facilitated the collection of eggs.

In many marine organisms, nutrition has a direct impact on gametogenic steps, particularly during vitellogenesis (Thompson, 1983). However, an increase in the quantity of food provided to *C. frondosa* throughout the experimental period did not enhance its reproductive output. Individuals that were fed twice the amount of food provided to the control group produced similar numbers of healthy oocytes and yielded equally high embryo survival rates. This result suggests that an increase in food availability during the gametogenic period is unlikely to increase the production of gametes in captivity, at least with the type of food used during the present study. The control (ambient) food

concentration used here reflected the average plankton biomass typical in the natural habitat of *C. frondosa* during winter and spring months, when gametogenesis and spawning occur. Therefore, food was assumed to be abundant enough for maintenance of metabolism and production of gametes. This was confirmed by measurements of body indices, particularly muscle indices, which remained unchanged over the 120-d period. If food had been a limiting factor, muscle indices would have dropped considerably during gametogenesis due to a transfer of nutrients from the muscle bands to the gonads for the production of yolky oocytes, as observed in starved individuals of *C. frondosa* during their gametogenic period (Hamel and Mercier, 1996b; Gianasi *et al.*, 2017).

The conditioning of *C. frondosa* in warmer water temperatures (6 or 12°C) or constant photoperiods (24-h dark or light) delayed gonad development compared to individuals under ambient conditions. At pre-spawning, females exposed to 6 and 12°C showed ovaries at the growth gametogenic stage; whereas females exposed to constant dark or light exhibited advanced-growth stage at the same time point. Exposure to warmer water temperature and different photoperiod regimes might have caused some stress in *C. frondosa*, disrupting gamete production. A similar response was reported when the sea urchin *S. droebachiensis* was kept in captivity under shifted environmental conditions (4 months out-of-phase photoperiod, under ambient water temperature); primary oocytes were about half the diameter of normal oocytes produced under ambient photoperiod and water temperature (Walker and Lesser, 1998). In contrast, *C. frondosa* exposed to 4-mo advanced photoperiod showed mature gonads at pre-spawning, similar to the control group, suggesting that the rates of gametogenesis were comparable between these two groups and that *C. frondosa* is able to retain its maturation tempo, at least for a short period, under

spring to summer photoperiod. Contrarily to *S. droebachiensis* (Walker and Lesser, 1998) where 4-mo advanced photoperiod was enough to reprogram gametogenesis to a new phase, a longer exposure duration (>120 d) to different water temperatures and photoperiods might be needed to completely shift the reproductive tempo of *C. frondosa* and potentially achieve out-of-phase gametogenesis and spawning in captivity.

No spawning occurred when *C. frondosa* was kept in water temperatures of 6 or 12°C. Although well within the tolerance threshold for this species, the warmer water temperature apparently cancelled the environmental stimulus that triggers natural spawning. The timing of spawning in marine organisms is commonly proposed to maximize offspring survival. Sea cucumber larval stages are known to be sensitive to changes in environmental factors such as temperature (Asha and Muthiah, 2005); their exposure to suboptimal conditions could have dire consequences on survival. Despite not being the optimal conditions, previous study induced spawning in *C. frondosa* kept at 6°C when phytoplankton was used as food (Gianasi *et al.*, 2017). Phytoplankton has been shown to be a powerful trigger of spawning in *C. frondosa* capable of inducing the release of mature and non-mature oocytes (Gianasi *et al.*, 2017). These results suggest that a warmer water temperature may disturb the reproductive physiology of *C. frondosa* preventing it from spawning naturally without an artificial trigger. It is also important to mention that the environmental conditions tested here are representative of Newfoundland (eastern Canada), and because of the broad distribution of *C. frondosa* in the North Hemisphere, other populations might experience slightly different ranges of ambient seawater temperature, photoperiod, and food concentration.

2.6 Conclusion

Understanding how environmental parameters influence the condition of sea cucumber broodstock in captivity is fundamental to ensure production of quality eggs and juveniles. The present study investigated for the first time how water temperature, photoperiod, and food concentration modulate gametogenesis, spawning and egg quality in the suspension-feeding cold-water sea cucumber *Cucumaria frondosa*. Individuals kept in ambient water temperature, photoperiod and food supply exhibited the highest reproductive output in combination with coordinated spawning and high embryo survival rates. An increase in food availability (relative to ambient) did not directly enhance the production of gametes, probably because the amount that was naturally available was enough to sustain nutritional requirements during gametogenesis. These findings suggest that manipulation of water temperature, photoperiod, and food concentration is not advantageous for conditioning of *C. frondosa* broodstock over 4 months. Further study will be needed to evaluate the impact of a longer exposure to shifted environmental conditions to achieve production of gametes out of the natural breeding season.

2.7 Acknowledgments

dissections, gamete collection and counts.

2.8 References

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2.9 Tables

Table 2.1: Classification of gametogenic stages in females and males of *Cucumaria frondosa* based on gonad phenotype. See Fig. S.2.4 (Supplementary material) for illustrations of the gametogenic stages.

Gonad	Stage	Description
Ovaries	Growth	Tubules (2.2 ± 0.5 mm diameter) filled with reddish vitellogenic oocytes and small yellowish oocytes. Main mode of oocyte size distribution <150 µm. Oocytes in different stages of development in lumen provide an overall light reddish colouration to entire gonad.
	Advanced growth	Tubules (2.4 ± 0.2 mm diameter) filled mostly with reddish oocytes. The mode of size distribution between 151 to 350 µm.
	Mature	Tubules (2.8 ± 0.4 mm diameter) filled with large vitellogenic oocytes; main mode of size distribution > 351 µm. Small yellowish oocytes still present, but less abundant. Predominance of large vitellogenic oocytes gives dark reddish colouration to entire gonad.
	Partially spent	Majority of tubules present dark reddish colour filled with oocytes. However, 20-50% of tubules yellowish with few dispersed oocytes in lumen, indicating partial spawning.
	Spent	90-100% of tubules yellowish and only few dispersed reddish oocytes in lumen. Average diameter of tubules 1.3 ± 0.2 mm.
Testes	Growth	Tubules with diameter of 1.6 ± 0.3 mm have some spermatozoa in lumen and uniform dark peach colour.
	Advanced growth	Tubules (1.8 ± 0.4 mm diameter) filled with spermatozoa and presenting uniform light peach colour.
	Mature	Tubules (2.6 ± 0.4 mm in diameter) filled with spermatozoa, and uniform light peach colour.
	Partially spent	Some tubules still filled with spermatozoa (forming pockets). However, 20-50% of them have no evidence of spermatozoa and are yellowish in colour.
	Spent	90-100% of tubules yellowish colour and do not present visual evidence of spermatozoa. Average diameter of tubules is 1.1 ± 0.3 µm.

Table 2.2: Description of egg and embryo quality in the sea cucumber *Cucumaria frondosa*.

Propagule	Quality	Description
Eggs (fertilized oocytes) and early embryos	Normal	Dark reddish colour, surrounded by clear fertilization envelope (Fig. 2.6a), positively buoyant. When cleavage started, blastomeres were tight together, dividing completely and uniformly (Fig. 2.6b and 2.6c).
	Abnormal	Unusual pale colour (yellowish-whitish; Figs. 2.6f and 2.6l), interstitial lipid droplets between fertilization envelope and plasma membrane (Fig. 2.6g), recessed surface (Fig. 2.6h), negatively buoyant. Early embryos exhibit asymmetric arrangement of blastomeres or incomplete cleavage (Fig. 2.6j). Blastomeres may show unusual recessed surface (Fig. 2.6i).
Blastula embryos	Normal	Uniform dark reddish colour, covered in cilia and spinning (Fig. 2.6d).
	Abnormal	Irregular shape (Fig. 2.6k), sparse yellowish lipid droplets between fertilization envelop and plasma membrane (Fig. 2.6k).
Gastrula embryos	Normal	Bean-shaped, uniform dark reddish colour, ciliated and spinning (Fig. 2.6e).
	Abnormal	Irregular shape, recessed surface, cilia hardly apparent, and embryos not spinning.

Table 2.3: Overview of the number of tanks in which spawning of *Cucumaria frondosa* occurred, also showing the mean number of eggs (± se) released per female in each treatment.

Treatments	Total # of tanks	# of tanks where spawning occurred	# of eggs released female^{-1*}
6°C	3	0	0 [a]
12°C	3	0	0 [a]
24-h dark	3	1	12.5 ± 10.2 [b]
24-h light	3	1	89.4 ± 82.3 [b]
Advanced photoperiod	3	2	240.3 ± 92.1 [c]
High food	3	3	820.6 ± 76.3 [d]
Control (ambient)	3	3	1,250.4 ± 82.6 [e]

[*] Means with different letters are significantly different (ANOVA on ranks, $p < 0.05$).

2.10 Figures
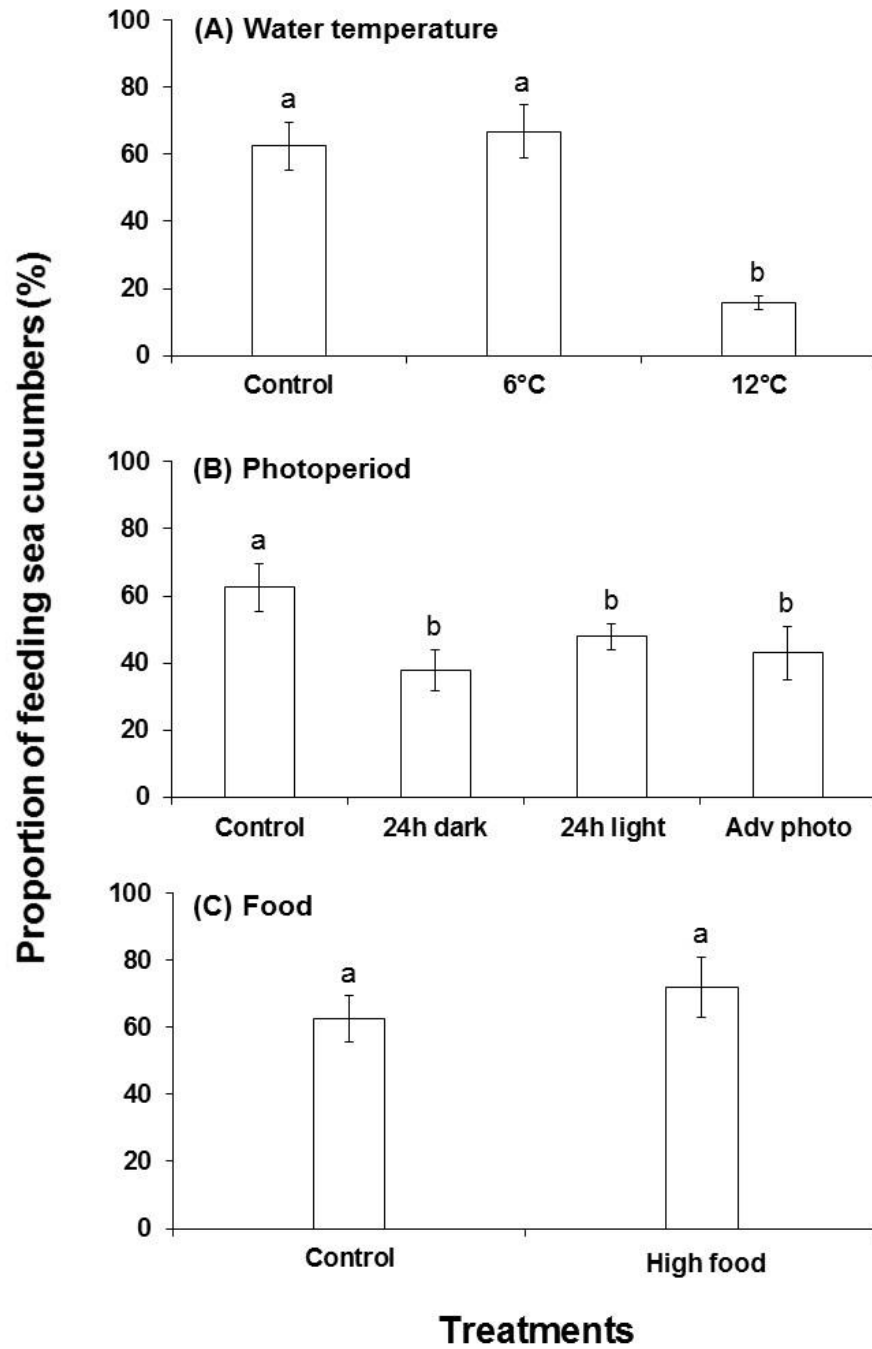
Figure 2.1 (previous page): Proportion of individuals displaying feeding activity throughout the experimental period (120 d) in *Cucumaria frondosa*. Individuals were exposed to different environmental conditions of (A) water temperatures (6 or 12°C), (B) photoperiods (24-h dark, 24-h light, Adv photo = 4-mo advanced photoperiod of 13-15 h light) and (C) twice the ambient food concentration (high food). They were compared to control individuals exposed to ambient water temperature (-1 to 3°C), photoperiod (8-13 h light), and food concentration. Data shown as mean ± se (n = 3). Means with different letters are significantly different (p < 0.05); see text for full statistical results.
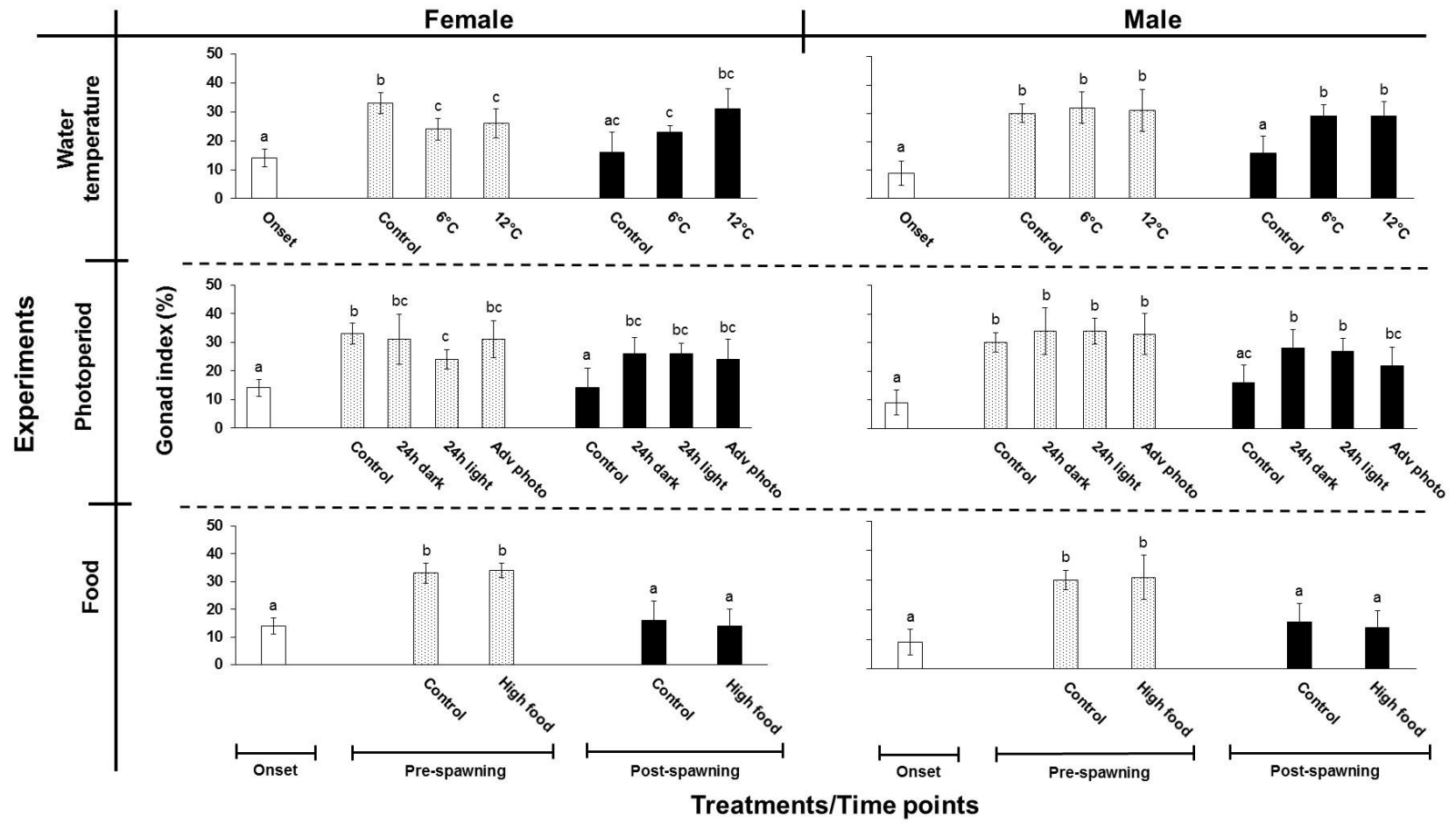
Figure 2.2 (previous page): Gonad index (GI) in females and males of *Cucumaria frondosa* at the onset of the experiment, just before spawning time (pre-spawning) and after the estimated spawning time (post-pawning). Individuals were exposed to different environmental conditions of water temperatures (6 or 12°C), photoperiods (24-h dark, 24-h light, Adv photo = 4-mo advanced photoperiod of 13-15 h light) and twice the ambient food concentration (high food). They were compared to control individuals exposed to ambient water temperature (-1 to 3°C), photoperiod (8-13 h light), and food concentration. A total of 12 females and 12 males were analyzed at the onset sampling and 6 females and 6 males from the subsequent samplings in each treatment. Data shown as mean ± se. Means with different letters are significantly different ($p < 0.05$); see text for full statistical results.
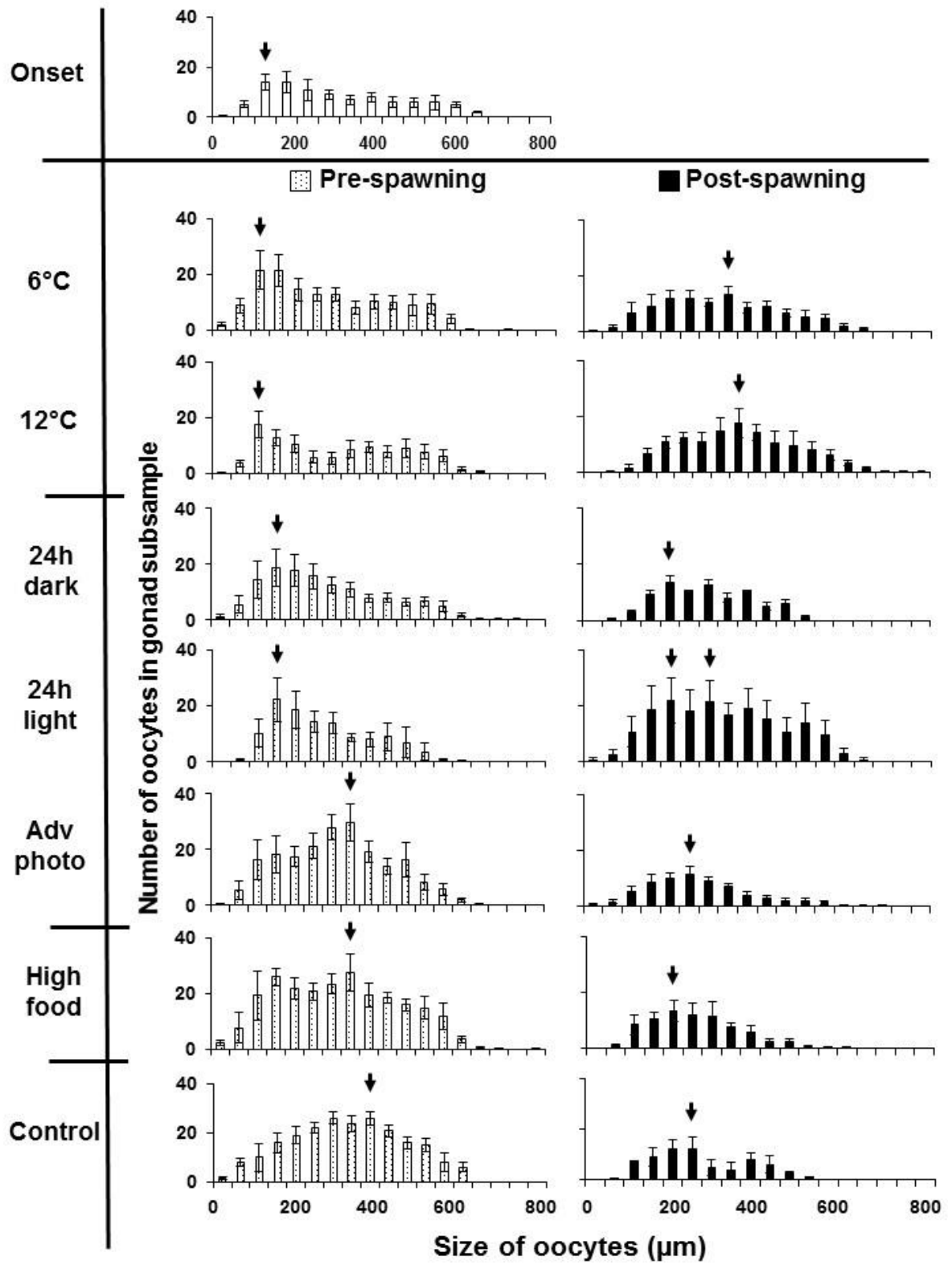
Figure 2.3 (previous page): Oocyte size structure from a segment of the gonad tubule (2 cm long) of *Cucumaria frondosa* at the onset of the experimental period, just before the spawning time (pre-spawning) and after the estimated spawning time (post-pawning). Individuals were exposed to different environmental conditions of water temperatures (6 or 12°C), photoperiods (24-h dark, 24-h light, Adv photo = 4-mo advanced photoperiod of 13-15 h light) and twice the ambient food concentration (high food). They were compared to control individuals exposed to ambient water temperature (-1 to 3°C), photoperiod (8-13 h light), and food concentration. Arrows above the graphs indicate the mode of the oocyte size distribution. A total of 12 females and 12 males were analyzed in the pre-trial sampling and 6 females and 6 males for the subsequent samplings in each treatment. Data shown as mean ± se.
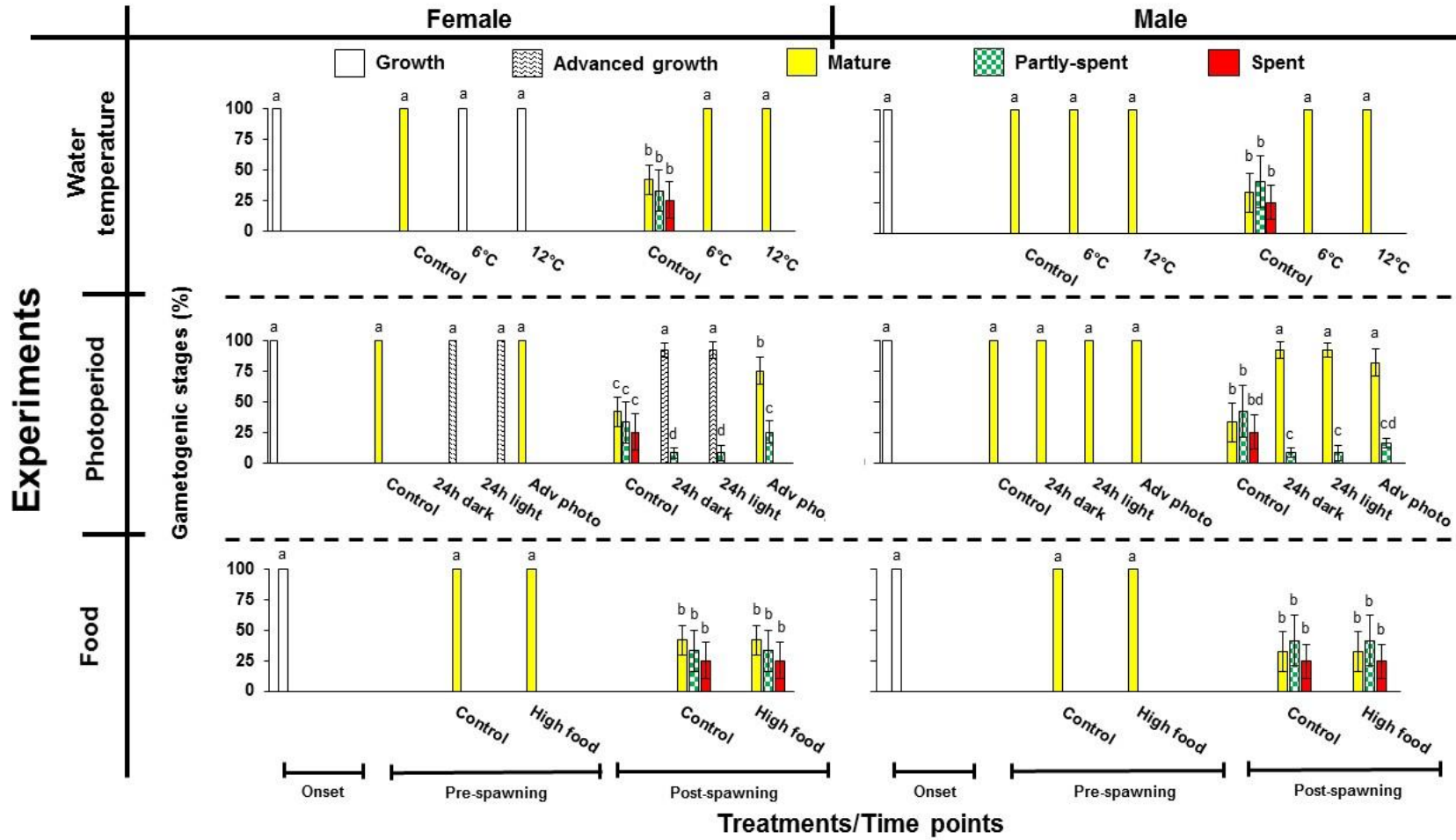
Figure 2.4 (previous page): Proportion of gonad phenotypes at different sampling times in *Cucumaria frondosa*. Individuals were exposed to different environmental conditions of water temperatures (6 or 12°C), photoperiods (24-h dark, 24-h light, Adv photo = 4-mo advanced photoperiod of 13-15 h light) and twice the ambient food concentration (high food). They were compared to control individuals exposed to ambient water temperature (-1 to 3°C), photoperiod (8-13 h light), and food concentration. See Table 2.1 for description of gametogenic stages and Fig. S.2.4 (Supplementary material) for illustrations. A total of 12 females and 12 males were analyzed in the pre-trial sampling and 6 females and 6 males for the subsequent samplings in each treatment. Data shown as mean ± se. Means with different letters are significantly different ($p < 0.05$); see text for full statistical results.
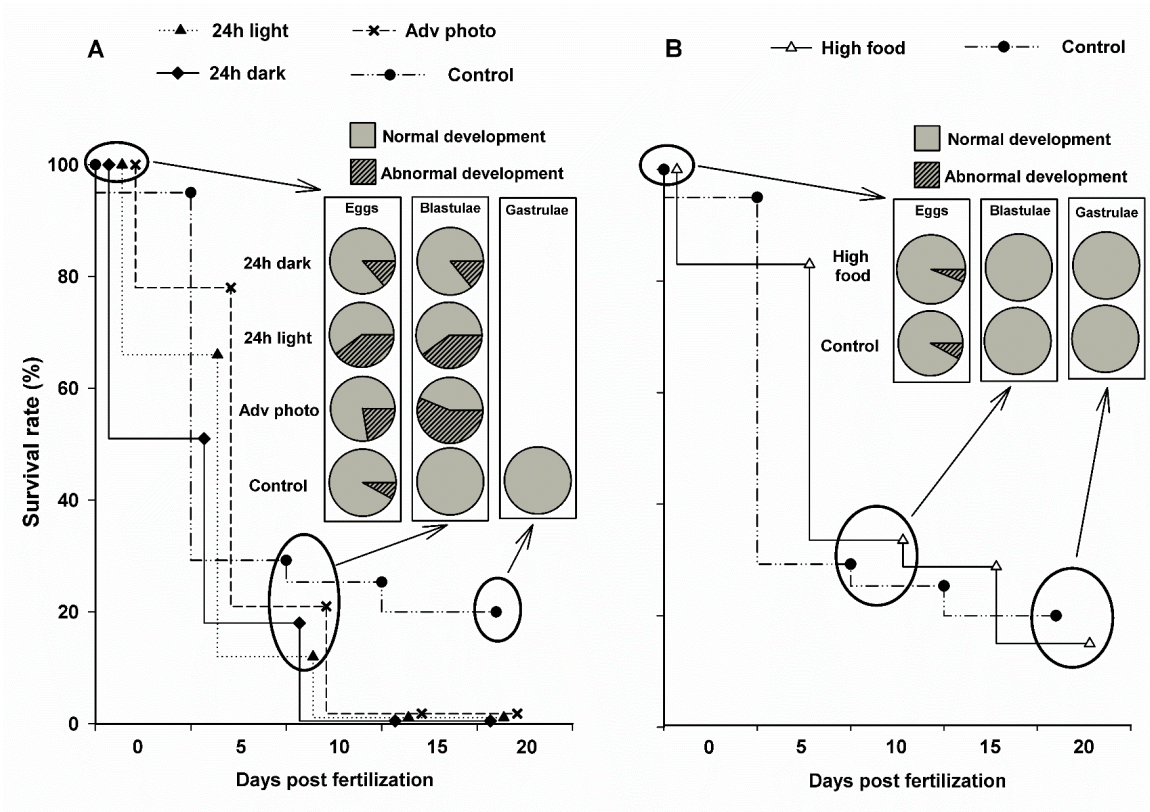
Figure 2.5: Survival rates and quality (pie charts) of fertilized oocytes (eggs), blastulae and gastrulae of *Cucumaria frondosa* produced by adults exposed to different (A) photoperiods (24-h dark, 24-h light, Adv photo = 4-mo advanced photoperiod of 13-15 h light) and (B) twice the ambient food concentration (high food). They were compared to control individuals exposed to ambient water temperature (-1 to 3°C), photoperiod (8-13 h light), and food concentration. No spawning occurred in water temperature 6-12°C.
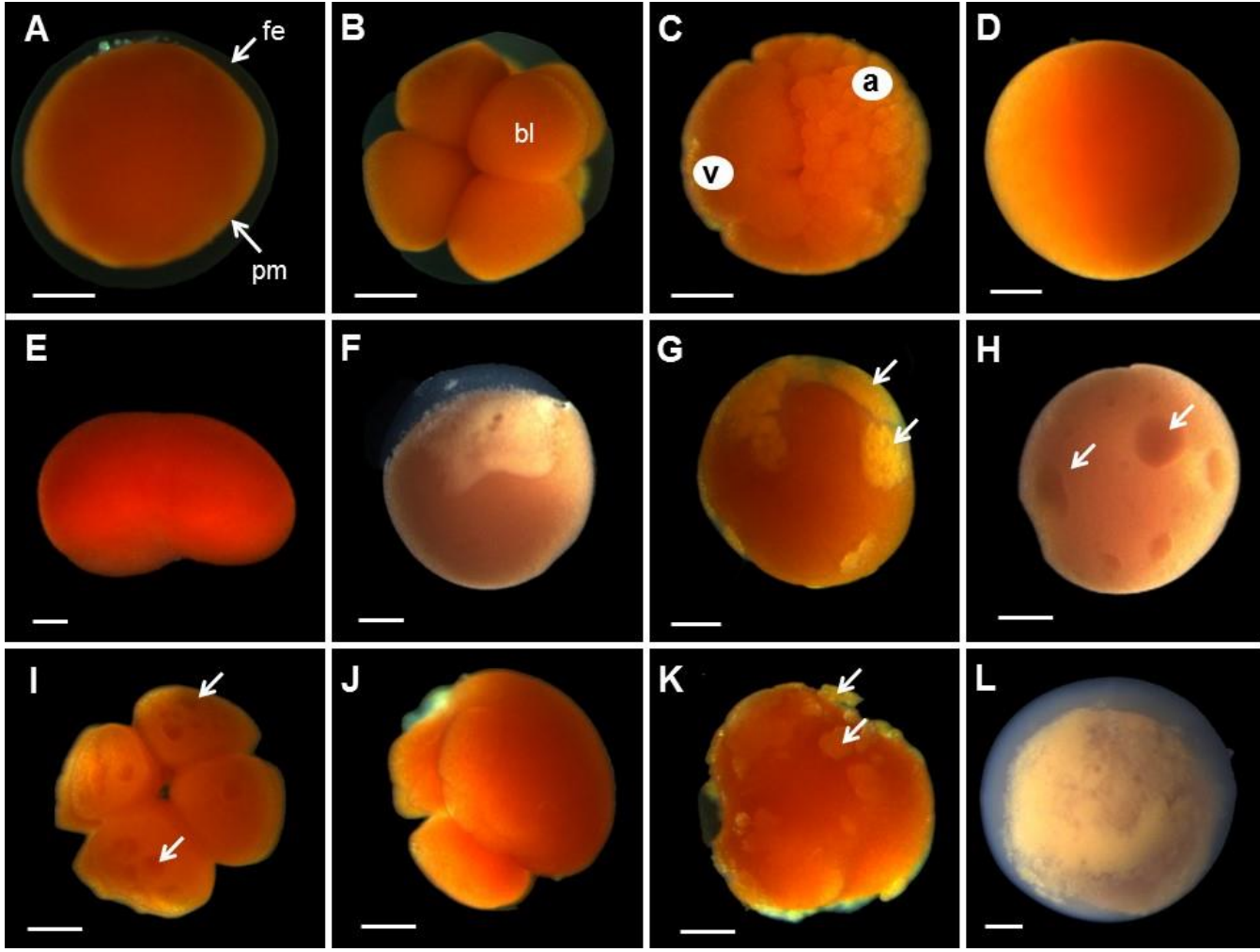
Figure 2.6 (previous page): Normal and abnormal development of eggs and embryos of *Cucumaria frondosa*. (A) Normal fertilized egg (0-1 d post fertilization) showing non-damaged clear fertilization envelop (fe) and plasma membrane (pm). (B) Normal 8-cell stage embryo (3-4 d post fertilization) with symmetric cleavage of blastomeres (bl). (C) Normal divisions of blastomeres in the vegetal (v) and animal (a) poles. (D) Normal blastula embryo (10 d post fertilization) displaying reddish coloration and round-shaped form. (E) Normal gastrula embryo (20 d post fertilization) exhibiting reddish colouration and bean-shaped form. (F) Abnormal egg displaying yellowish-whitish coloration. (G) Abnormal egg with yellowish interstitial lipid droplets (arrows) between plasma membrane and fertilization envelope. (H) Abnormal egg with recess on the surface (arrows). (I) Abnormal 8-cell embryo with recesses (arrows) on the surface of blastomeres. (J) Abnormal eggs showing irregular or incomplete cleavage of blastomeres. (K). Abnormal blastula embryo with lipid droplets (arrows) between plasma membrane and fertilization envelop. (L) Wasting blastulae showing pale coloration. Scale bars represent 100 µm.

2.11 Supplementary Material
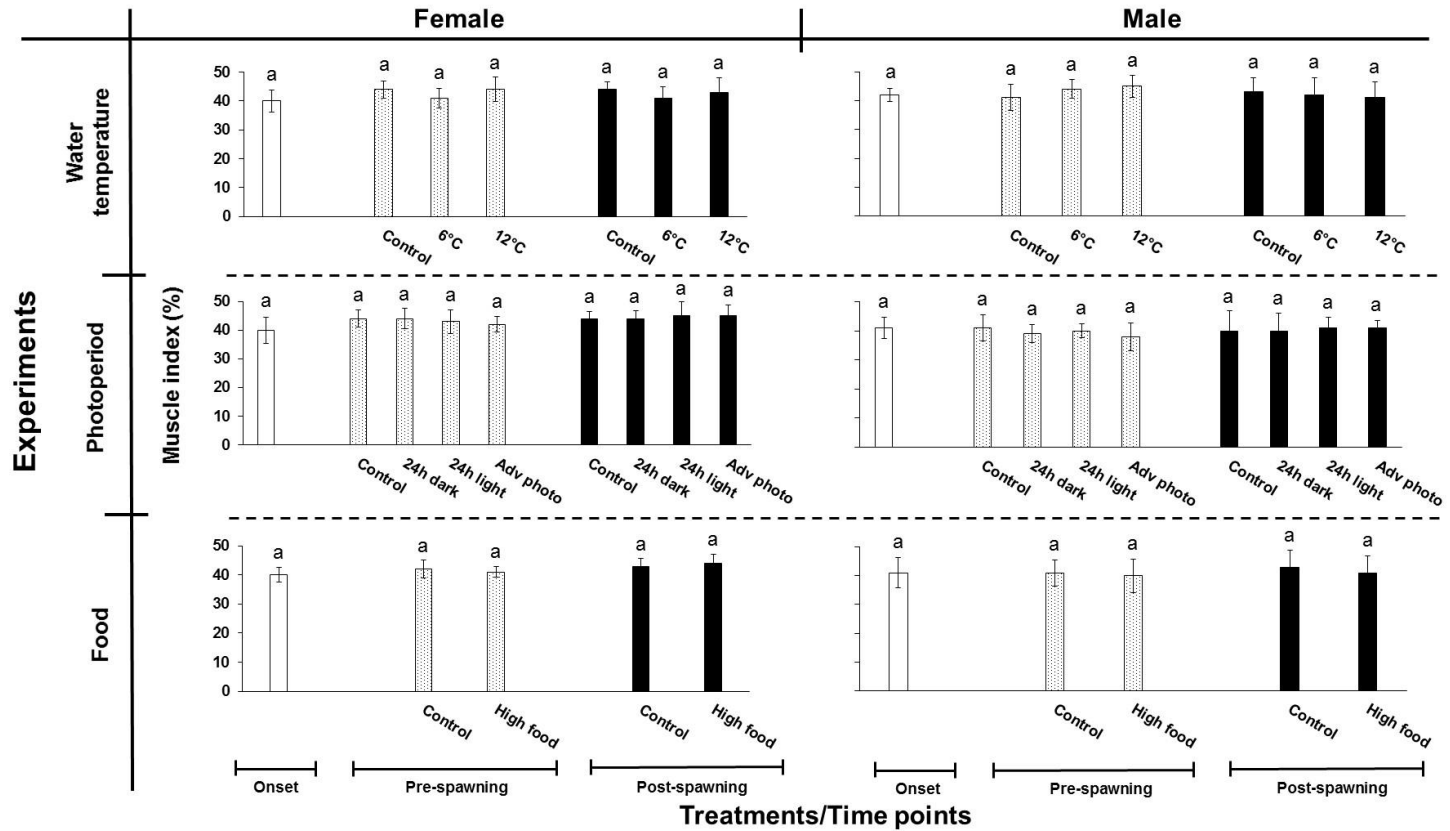
Figure S.2.1: Comparison of muscle indices in females and males of the sea cucumber *Cucumaria frondosa* among treatments at three time points: onset of the experiment, pre-spawning, and post-spawning.
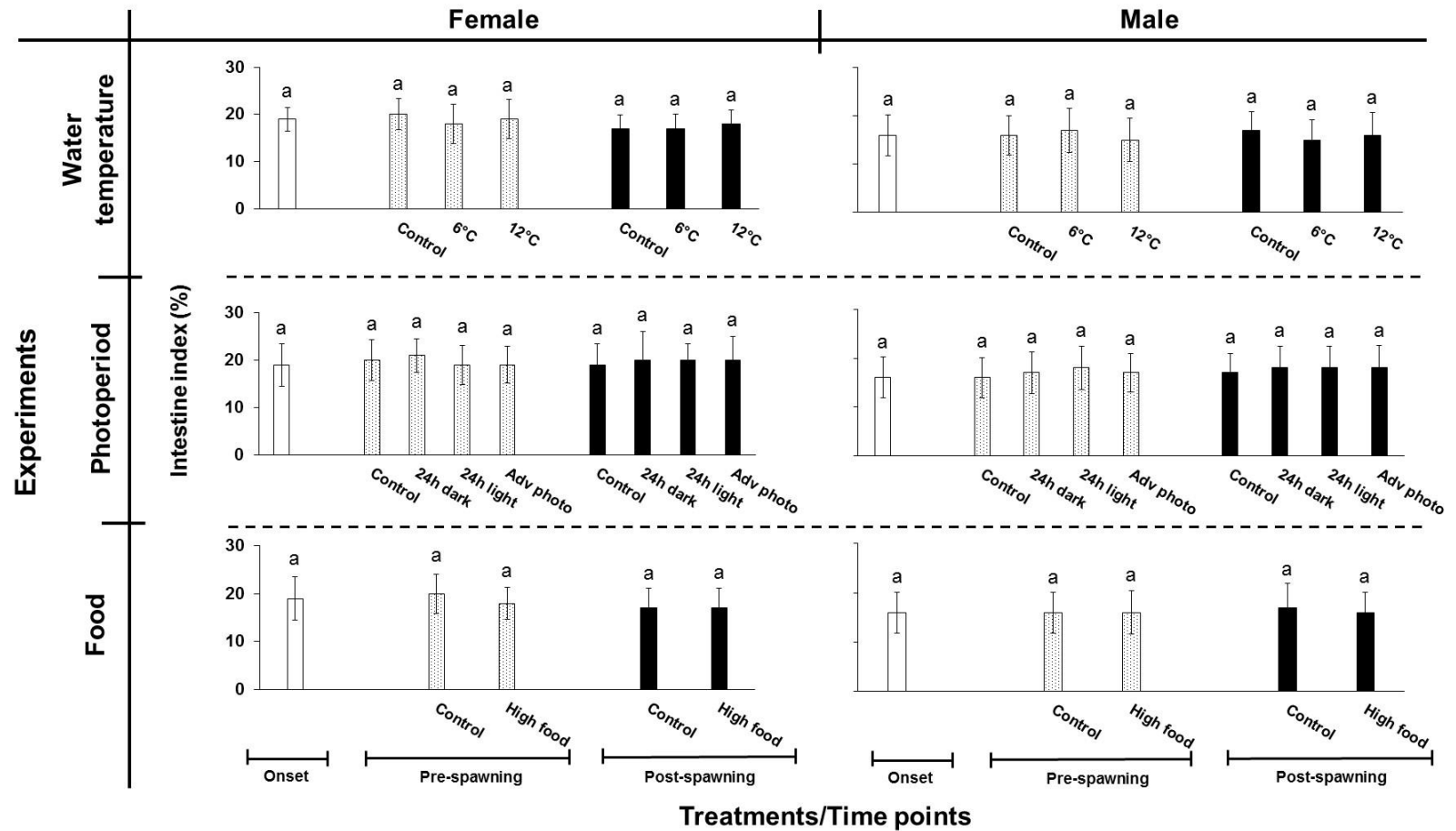
Figure S.2.2: Comparison of intestine indices in females and males of the sea cucumber *Cucumaria frondosa* among treatments at three time points: onset of the experiment, pre-spawning, and post-spawning.
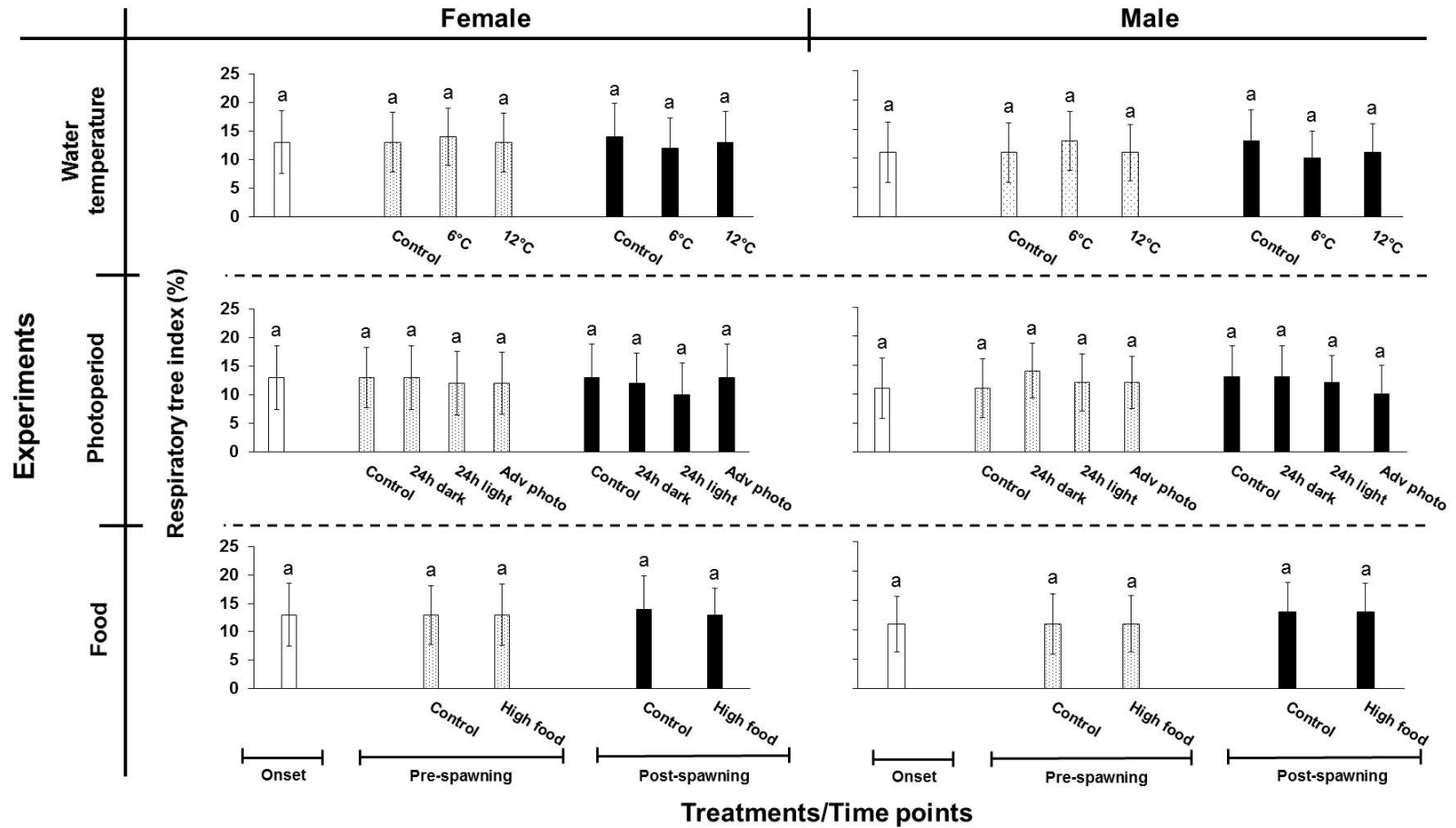
Figure S.2.3: Comparison of respiratory tree indices in females and males of the sea cucumber *Cucumaria frondosa* among treatments at three time points: onset of the experiment, pre-spawning, and post-spawning.
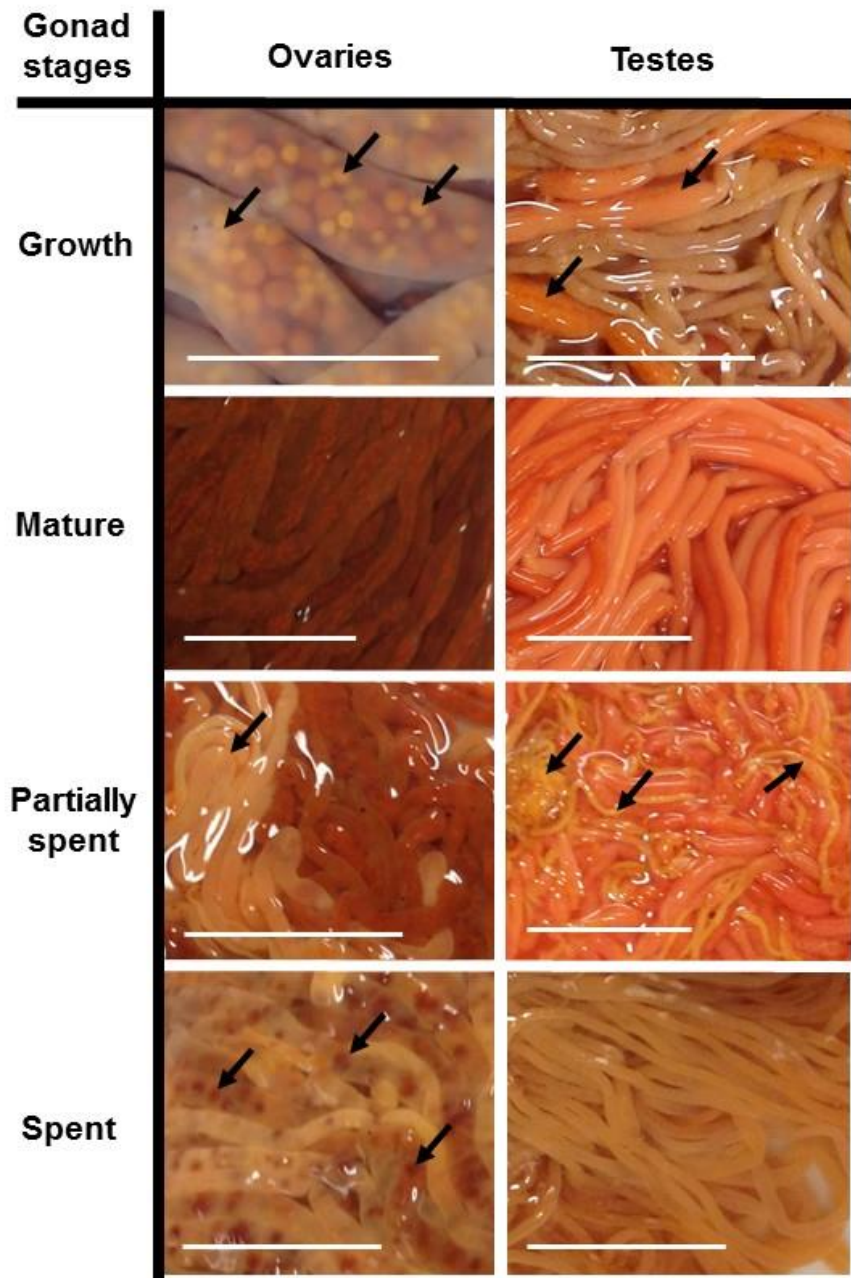
Figure S.2.4: Illustrations of the dominant gametogenic stages in females and males of *Cucumaria frondosa* based on assessment of gonad phenotype. See Table 2.1 for description of each stage. Scale bars represents 1 cm.

Chapter 3. Triggers of Spawning and Oocyte Maturation in the Commercial Sea Cucumber *Cucumaria frondosa*[2]

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3.1 Abstract

The present study investigated methods to trigger spawning and artificially induce oocyte maturation in the commercial cold-water suspension-feeding sea cucumber *Cucumaria frondosa*. Spawning occurred after exposure to live phytoplankton, commercial phytoplankton paste, and conspecific sperm. Live phytoplankton at 1 x 10^5 cells ml^{-1} induced the highest proportion of females to spawn (mean of 56%); promoted the greatest oocyte release (mean of 19,200 oocytes female^{-1}), best quality of eggs and highest survival of embryos (65%; 20 d post fertilization). More concentrated live phytoplankton (1 x 10^6 cells ml^{-1}) triggered 43% of the females to release ~16,100 oocytes female^{-1} and survival rates 20 d post fertilization were lower (50%). Both phytoplankton paste treatments (1 x 10^5 and 1 x 10^6 cells ml^{-1}) yielded intermediate results in terms of percent spawning females (26%), abundance of oocytes released (11,000 and 7,300 oocytes female^{-1}, respectively), quality of eggs, and survival of embryos (44 and 50%, respectively). Sperm from conspecific (2 and 10 x 10^6 spermatozoa ml^{-1}) induced the lowest proportion of females to spawn (16%) with release of 3,000 and 1,700 oocytes female^{-1}, respectively; furthermore, all embryos died within 10 d post fertilization. Results for males were slightly different: spawning was more frequent (63%) in the sperm treatment at 2 x 10^6 spermatozoa ml^{-1} followed by live phytoplankton at 1 x 10^6 and 1 x 10^5 cells ml^{-1} (43%), and phytoplankton paste at 1 x 10^5 cells ml^{-1} (46%); the lowest spawning success was observed for phytoplankton paste at 1 x 10^6 cells ml^{-1} and conspecific sperm at 10 x 10^6 spermatozoa ml^{-1} (26%). Thermal shock, desiccation, potassium chloride (injection or bath), and serotonin (injection) did not induce spawning in *C. frondosa*. When oocytes isolated from

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mature ovaries were exposed to three different concentrations of 1-Methyladenine (1-MA), Dithiothreitol (DTT), 2,3-Dimercapto-1-propanol (BAL), and L-cysteine (L-cyst), DTT at 10^{-1} M induced the highest proportion of oocytes (48%) to shed follicle cells (i.e. ovulate); whereas other treatments induced only 2-6% of ovulation, similar to the control (seawater). All oocytes that shed follicle cells remained unfertilizable. Overall, live phytoplankton emerged as the most suitable spawning trigger for *C. frondosa*, a Dendrochirotida that produces maternally-provisioned oocytes. In the context of expanding sea cucumber aquaculture, this finding provides a tool to maximize the availability of gametes in this cold-water species, with possible applicability to similar commercial dendrochirotids.

3.2 Introduction

Sea cucumbers have been commercially harvested as seafood and as a source of nutraceutical and pharmaceutical products for centuries (Purcell, 2014; Xia and Wang, 2015). The high demand and market price commanded by sea cucumbers have led to the intensification of the fisheries and, consequently, to the overexploitation of many stocks around the world (Purcell *et al.*, 2014), encouraging the emergence of new fisheries for previously unexploited species (Hamel and Mercier, 2008a) and the development of aquaculture programs in numerous countries (Purcell *et al.*, 2012; Yang *et al.*, 2015).

One of the primary objectives of aquaculture is to develop reliable ways of getting a consistent supply of healthy gametes. However, contrary to sea urchins that can reliably be induced to spawn through injection of potassium chloride (KCl) in the coelomic cavity (Walker *et al.*, 2007), sea cucumbers are notoriously less predictable, whereby most spawning techniques are either partly or completely ineffective (Battaglene *et al.*, 2002; Léonet *et al.*, 2009). Moreover, oocytes of sea cucumbers are arrested at the first meiotic stage and undergo ovulation, germinal vesicle breakdown (GVBD), and final maturation only during spawning; therefore, they are typically not fertilizable *in vitro* (Maruyama, 1986). In an attempt to optimize the supply of recruits, sea cucumber hatcheries are always looking for improved techniques to induce oocyte maturation or oocyte release, derived from current knowledge of natural reproductive triggers.

The direct observation of natural spawning in the field is rare and the identification of environmental factors that trigger gamete release is a difficult task, mainly because many of these factors act synergistically in the natural environment (Mercier and Hamel, 2009).

At the broadest scale, some of these triggers were highlighted based on reproductive studies where stages of gametogenesis were correlated to environmental factors. Changes in phytoplankton concentration, water temperature, light intensity (i.e. time of the day), and lunar cycles have been proposed to be the main cues involved with spawning in sea cucumbers (Mercier and Hamel, 2009). Species that have been investigated in that regard include the temperate sea cucumbers *Parastichopus californicus* in British Columbia (Canada), *Psolus chitonoides*, *Psolidium bullatum*, *Cucumaria miniata*, *C. piperata*, *C. fallax*, *Eupentacta quinquesemita*, and *Pentamera populifera* in San Juan Archipelago (Washington, USA), and *P. fabricii* in the Gulf of St. Lawrence (eastern Canada). All of them showed a marked reproductive seasonality coinciding with the annual phytoplankton bloom (Cameron and Fankboner, 1986; McEuen, 1988; Hamel *et al.*, 1993), days of bright sunshine, and/or with the full or new moon (McEuen, 1988) throughout spring and summer months. Other examples exist at tropical latitudes, including the sea cucumbers *Holothuria scabra*, *H. fuscogilva*, and *Isostichopus fuscus*, which exhibit two or more annual spawning events associated with changes in water temperature during warm/cold or dry/wet seasons (Conand, 1981), and with lunar periodicity (Babcock *et al.*, 1992; Mercier *et al.*, 2007). This body of literature outlines key environmental correlations and possible spawning cues, which are valuable but not directly applicable in a captive-breeding context.

In laboratory settings, the identification of parameters (either natural or artificial) that can trigger spawning have been attempted in a limited number of sea cucumber species belonging to the order Holothuriida (formerly Aspidochirotida). Apart from spontaneous spawning following the stress of capture and handling (Battaglene *et al.*, 2002), the use of

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thermal shock (i.e. abrupt change in water temperature), alone or combined with other stressors, is the most common spawning technique in tropical species (James *et al.*, 1994; Battaglene *et al.*, 2002). Thermal shocks (3-5°C above ambient) have been reported to induce spawning in 100% (Morgan, 2000) and 35% (Battaglene *et al.*, 2002) of *H. scabra*, 60% of *Actinopyga mauritiana* (Battaglene *et al.*, 2002), 40% of *Australostichopus mollis* (Morgan, 2009), and 50-80% of *A. japonicus* (Liu *et al.*, 2015). Desiccation (i.e. exposure to air) of sea cucumbers can also trigger spawning in *H. scabra* (James *et al.*, 1994), *H. atra* (Laxminarayana, 2005), and *A. japonicus* (Liu *et al.*, 2015). Addition of dried algae (*Schizochytrium* sp.) in tanks with *H. fuscogilva* induced spawning in 10-35% of individuals (Ramofafia *et al.*, 2000; Battaglene *et al.*, 2002). Other techniques, including exposure to solutions of potassium chloride (KCl) in *H. scabra* (Hartati and Pringgenies, 1998) and transfer of coelomic fluid from spawning individuals of *Bohadschia argus* (Mercier and Hamel, 2002) have also been reported to induce spawning in mature individuals. Finally, Mercier *et al.* (2007) demonstrated that *Isostichopus fuscus* spawns at the new moon around sunset without the need for any additional trigger.

In an attempt to bypass the vagaries of spawning and develop *in-vitro* techniques, some experimental studies have explored the artificial induction of oocyte maturation in some deposit-feeding sea cucumbers with planktotrophic development (Maruyama, 1986; Chen *et al.*, 1991; Léonet *et al.*, 2009). Various substances have been proposed to induce the breakdown of the follicle cells and/or the germinal vesicle, such as 1-Methyladenine (1-MA), Dithiothreitol (DTT), 2,3-Dimercapto-1-propanol (BAL), and L-cysteine (L-cyst). The substance 1-MA is known as a natural inducer of sea star oocyte maturation that acts

in the follicle cells, resulting in ovulation and the breakdown of the germinal vesicle (Kanatani, 1969). Similarly, DTT and BAL have been found to mimic the action of 1-MA (Kishimoto and Kanatani, 1973); whereas L-cyst presumably has a direct effect on the activation of maturation-promoting factors in the follicle cells (Mita, 1985). Contrary to studies in sea stars, the chemical induction of oocyte maturation in sea cucumbers has yielded inconsistent results. Maruyama (1980) reported maturation in 100% of the oocytes in the sea cucumbers *H. leucospilota* and *H. pardalis* with DTT, BAL and L-cyst; oocytes were fertilized and developed normally up to the auricularia larvae (6 d post fertilization). On the other hand, Kishimoto and Kanatani (1980) showed that DTT induced maturation in only 20% of the oocytes in *A. japonicus*; although maturation rates increased to 90% following pre-treatment with pronase (i.e. an enzyme that catabolizes proteins). Chen *et al.* (1991) achieved 84% of maturation in the oocytes of *Actinopyga echinites* after exposure to DTT; however, the development of the larvae until the pentactula stage was abnormal (asymmetric). High maturation rates were also achieved when oocytes of *H. scabra* were exposed to DTT, BAL, and L-cyst (with 100%, 80%, and 40% success, respectively) (Léonet *et al.*, 2009). Although, 1-MA induced oocyte maturation and gamete shedding in gonad fragments of the hermaphroditic sea cucumber *Leptosynapta inhaerens* (Ikegami *et al.*, 1976) and maturation in 40% of oocytes isolated from *H. scabra* (Léonet *et al.*, 2009), it failed to induce maturation in the oocytes of *P. californicus* (Stevens, 1970), *H. leucospilota* (Maruyama, 1980), and *A. japonicus* (Kishimoto and Kanatani, 1980). A natural complex named maturation induction fraction (MIF) extracted from oocytes of the sea urchin *Tripneustes gratilla* was reported to induce maturation in up to 90% of oocytes

in *H. scabra* and 22-99% in thirteen other species of deposit-feeding sea cucumbers from the Indian Ocean and Mediterranean Sea, with fertilization rates >90% (Léonet *et al.*, 2009). Moreover, extracts of the radial nerve of sea cucumbers (Maruyama, 1985) and a peptide from the buccal ring of *A. japonicus* (Kato *et al.*, 2009) and its synthetic derivative NGLWY-amide, also called cubifrin-L (Fujiwara *et al.*, 2010), have also been shown to induce oocyte maturation in *H. leucospilota*, *H. pervicax*, *H. moebi*, *H. pardalis*, and *A. japonicus*. However, the activation mechanisms of these chemical agents are still unclear and require further investigation. Importantly, suspension-feeding sea cucumbers (order Dendrochirotida) have been particularly understudied in this respect, especially species with lecithotrophic development (Mercier and Hamel, 2009) like the focal species.

The sea cucumber *Cucumaria frondosa* (Holothuroidea: Dendrochirotida) is widely distributed in cold waters of the Arctic and Atlantic oceans, occurring from the eastern coast of Canada to northern Russia (Hamel and Mercier, 2008a). This species has been commercially harvested for seafood since the 1980s (Hamel and Mercier, 2008b) and has been proposed to have potential for aquaculture in North America (Nelson *et al.*, 2012). However, *C. frondosa* differs from all previously cultured sea cucumbers; it is a cold-water species that feeds on live suspended plankton (Singh *et al.*, 1999), and produces large, yolky oocytes, which develop into maternally-provisioned lecithotrophic (non-feeding) larvae (Hamel and Mercier, 1996a). Spawning of *C. frondosa* in the Gulf of St. Lawrence was reported to broadly coincide with the annual increase in phytoplankton concentration during spring; release of gametes was directly observed at sunrise in the field (Hamel and Mercier, 1995) and in the laboratory (Mercier and Hamel, 2010). Considering the

expansion of sea cucumber aquaculture around the world, techniques that provide useful insights into the mediation of spawning and oocyte maturation in dendrochirotids and/or lecithotrophic species must be developed to ensure a reliable supply of fertilizable oocytes and, consequently, help improve production.

The present study investigated several potential stimuli that might induce spawning in *C. frondosa*, including thermal shock, live phytoplankton, commercial phytoplankton paste, desiccation, sperm from conspecifics, as well as exposure to or injections of solutions of potassium chloride and serotonin. The number of males and females releasing gametes, number and quality of oocytes released, and survival rates of embryos were assessed. Experimental trials were also conducted to evaluate the potential of 1-MA, DTT, BAL, and L-cyst to artificially induce maturation of oocytes in *C. frondosa*.

3.3 Material and Methods

3.3.1 Collection of broodstock and holding tanks

Adult sea cucumbers weighing 7.8 ± 1.9 g immersed weight (~385 g wet weight) and measuring 14.0 ± 1.9 cm ($\pm$ sd; n = 30) contracted body length were hand collected by divers in Bay Bulls (Avalon Peninsula, 47°17'44.6'' N: 52°46'8.9'' W), eastern Canada, at depths between 5 and 10 m, during the peak of gonad maturation for *C. frondosa* in Newfoundland (Mercier and Hamel, 2010). A sub-sample of 12 males and females were analyzed to confirm the maturity stage based on the colour of the gonad and the oocytes size distribution (Hamel and Mercier, 1996c; Gianasi *et al.*, 2017). Individuals were kept in three 500-L holding tanks (~150 sea cucumbers in each) with running seawater (50 L h⁻

[1]) at ambient temperature (~0°C). Light was provided through large windows and photoperiod fluctuated naturally. Plankton food present in the ambient seawater was available to sea cucumbers. Only healthy and undamaged sea cucumbers displaying normal pigmentation and feeding response, firm attachment to the substrate and no skin lesion were selected for the trials.

3.3.2 Spawning induction

3.3.2.1 Experimental setup

Each treatment was replicated in three independent tanks, each containing 20 sea cucumbers (total of 60 sea cucumbers per treatment). Spawning tanks (150 L) were supplied with running filtered seawater (20 L h^{-1}) at ambient temperature (~0ºC, natural temperature at which spawning occurs naturally in the field) and photoperiod (12L/12D). Light was provided by a set of fluorescent bulbs (spectrum of 450 to 780 nm) to a maximum intensity of 150 lx (measured with a light meter) at the water surface which covered the average light intensity in the field from 10 to 100 m depth (Kampa, 1970) where *C. frondosa* is most abundant. The filtration system that supplied seawater to experimental tanks were equipped with sand bed (15 µm), ultraviolet and foam fractionation filters which removed essentially all the suspended particles; therefore, no additional source of plankton (potential spawning stimulus) was available in the incoming water. Tanks were randomly distributed in the experimental setup. An attempt to equally distribute males and females (1:1) in each tank was made based on the gonopore morphology which is visible when

tentacles are deployed (Hamel and Mercier, 1996a). Sea cucumbers were acclimated in the spawning tanks 3 days before the beginning of the trials.

3.3.2.2 Induction and monitoring

Because investigation of spawning induction has never been attempted in a cold-water lecithotrophic sea cucumber, stimuli were chosen based on previous studies of spawning induction in tropical and temperate species (Battaglene *et al.*, 2002; Liu *et al.*, 2015) and to represent a wide range of environmental parameters that might be involved in triggering spawning in cold-water echinoderms (Starr *et al.*, 1992; Mercier and Hamel, 2009). Treatments tested included temperature shocks, desiccation, live phytoplankton, phytoplankton paste, sperm from conspecifics, exposure to and injection of potassium chloride and injection of serotonin (Table 3.1).

Temperature shocks were conducted by transferring sea cucumbers from the spawning tank (~0ºC) to another tank that was either at 5ºC or 10ºC for 1 h, before being returned to their tank. The control group was transferred to a tank with the same water temperature and returned to the original tank. Desiccation was conducted by placing sea cucumbers in empty trays where they remained exposed to air at room temperature (~15ºC) for either 1 or 2 h before returning to original tanks. The control group was not exposed to air; individuals were only handled while submerged.

Live phytoplankton (diatom *Chaetoceros muelleri*) and a commercial phytoplankton paste were tested as spawning inducers. The diatom *C. muelleri* was cultivated in 400-L polyethylene bags illuminated 24 h daily by a set of fluorescent light bulbs (daylight and cool light). Nutrients A and B and sodium metasilica (Kent Marine Pro-

Culture) were added to the culture weekly. The commercial phytoplankton paste (Shellfish Diet 1800, Reef Mariculture) consisted of a mix of *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, and *T. pseudonana* and was kept refrigerated at 4°C at all times. For both phytoplankton forms, the concentrations tested to induce spawning in each experimental group was 1 x 10^5 cells ml^{-1} (Gianasi *et al.*, 2017) and 1 x 10^6 cells ml^{-1}; whereas the control group was exposed only to added seawater (Table 3.1). The water flow was interrupted for 2 h and aeration was added to insure that the phytoplankton concentration was maintained long enough in suspension to allow sea cucumbers to extend their feeding tentacles, as per Gianasi *et al.* (2017).

Sperm of conspecifics was tested at two concentrations (2 x 10^6 and 10 x 10^6 spermatozoa ml^{-1}) within the range found in the water column during spawning of *C. frondosa* in the St. Lawrence Estuary (Hamel and Mercier, 1995). Three mature males were selected haphazardly from the holding tanks (described above); their sperm was extracted by pressing a glass rod on the surgically collected tubules, and mixed with seawater in a beaker (1 L). Aliquots (10 µl) were collected to determine the concentration of spermatozoa; dilutions were performed as needed to reach the desired experimental concentrations, which were added to experimental tanks. The overall procedure took ~20 min. Only seawater was added in the control group (Table 3.1). The water flow was also interrupted for 2 h and aeration provided as mentioned above.

In another treatment, individuals were either bathed in a KCl solution or injected with it directly into the coelomic cavity (Table 3.1). For the former procedure, sea cucumbers were transferred from their original tank to a KCl bath at a concentration of 1 x

10^{-5} M or 1 x 10^{-2} M for 1 h; whereas the control group was only exposed to a bath containing seawater. The concentrations of KCl were chosen based on the ones that induced spawning in *H. scabra* (Hartati and Pringgenies, 1998). Another experimental group received injections of 20 ml of 0.5 M or 1 M solution of KCL (in filtered seawater) into the coelomic cavity (mid body length, dorsal side) while submerged in seawater. The control group was only punctured with a needle.

Another group of individuals was injected with serotonin in the coelomic cavity (as described above) using 1 ml of 2 x 10^{-3} M or 2 x 10^{-2} M; whereas the control group was only punctured with the needle (Table 3.1). These solutions were chosen based on serotonin concentrations used to induce spawning in giant clams (Braley, 1985). Injections were performed with individuals submerged in the holding tanks and solutions were prepared with filtered seawater.

Each induction was performed once daily (at 8 am) for a total of 5 consecutive days. After the stimulation, tanks were monitored hourly for a total of ten hours daily for evidence of spawning. When spawning occurred, the following data were recorded: number of days since the stimulation was first performed, time of the day, posture of individuals spawning, number and sex of individuals spawning, broadcast synchronization between sexes and replicate tanks, duration of spawning, and the number of oocytes released in each treatment.

3.3.2.3 Quality and survival rates of embryos

When spawning had ceased (i.e. no gamete release detected over a period of 2 h), eggs were gently skimmed from the surface of the water into a 10-L bucket filled with the same seawater as in the holding tanks (~0ºC). The number of oocytes released was

calculated by collecting five 50-ml aliquots and performing counts under a stereo-microscope (Leica M205FA). Fertilization was confirmed by the elevation of fertilization envelop and/or cleavage, and eggs/embryos were then transferred to five 4-L incubators with running ambient seawater (20 L h^{-1}) at a density of 5 eggs/embryos ml^{-1}.

The onset of development (0 d post fertilization) and the survival of blastulae (5 d post fertilization) and gastrulae (20 d post fertilization) were monitored. A subsample of 200 eggs/embryos was collected from each incubator (n = 5 per treatment, total of 1,000 propagules per treatment) and photographed under the stereo-microscope (mentioned above). A new developmental stage was scored when >50% of the embryos had reached it.

3.3.3 Artificial induction of oocyte maturation and fertilization *in vitro*

Surgically extracted oocytes were exposed to different concentrations of 1-Methyladenine (1-MA), Dithiothreitol (DTT), 2,3-Dimercapto-1-propanol (BAL), and L-cysteine (L-cyst) to determine whether these substances can induce final oocyte maturation in *C. frondosa* (Table 3.1). Concentrations tested (Table 3.1) were chosen based on those that induced the highest number of oocyte maturation in *H. scabra* (Léonet *et al.*, 2009). Solutions were prepared with filtered seawater (5 µm) the day before the initial trial; they were kept refrigerated at 4ºC and renewed every 5 days (Maruyama, 1985).

Oocytes were obtained from females haphazardly selected from holding tanks (see above). The gonads were dissected, and oocytes removed by gently pressing a glass rod over them towards the gonopore. Oocytes were collected in a Petri dish and gently rinsed in running ambient seawater at the same temperature as holding tanks (~0ºC) for 5 min. A subsample of oocytes (n = 100) was then checked under the stereo-microscope (mentioned

above) for colouration, shape, floatation (buoyancy), and size. Only large (>550 µm Feret diameter), floating, reddish oocytes with intact plasma membrane and follicle cells were used in the trial. Oocytes were transferred to a 24-well culture plate where they were exposed to each tested solution (Table 3.1). Each well (4 ml) represented one treatment with 100 oocytes. Treatments were repeated 10 times at an interval of 1 day using a new female each day (100 oocytes x 10 times = total of 1,000 oocytes per treatment). Culture plates were half submerged in a water bath to keep water temperature constant (~0ºC).

Following the onset of each treatment, oocytes were photographed under a stereo-microscope and the number that had shed follicle cells was determined every 15 min for 2 h. Because the cytoplasm of oocytes in *C. frondosa* is yolky and reddish in colour (i.e. opaque rather than transparent), it is not always possible to directly observe the germinal vesicle breakdown (GVBD). Since only mature oocytes that undergo GVBD can be fertilized, fertilization *in vitro* was used to help (indirectly) determine the number of oocytes that underwent GVBD. Thus, oocytes were removed from the tested solutions after 2 h, rinsed in seawater (~0ºC) and placed in clean seawater inside a new culture plate. A mix of spermatozoa collected from 3 males was added to each well, using a ratio of 20 spermatozoa per oocyte. The fertilization rate in each treatment was determined by the elevation of the fertilization envelop. Control group consisted of oocytes exposed only to seawater.

3.3.4 Statistical analysis

Data were tested for normality and equal variance using Kolmogorov-Smirnov and Levene's tests ($\alpha = 0.05$), respectively. The number of male and female sea cucumbers that

spawned in each treatment was Log_{10}-transformed to achieve normality and compared among treatments using two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. The total number of oocytes released was compared among treatments using one-way ANOVA followed by Holm-Sidak pairwise tests. The number of eggs at the onset of development (0 d post fertilization), of blastula embryos (5 d post fertilization), and of gastrula larvae (20 d post fertilization) undergoing normal development was compared among treatments using one-way repeated measure ANOVA followed by Holm-Sidak pairwise tests. Survival rates of gastrula larvae (20 d post fertilization) were assessed among treatments with logrank survival analysis ($\alpha = 0.05$), using Kalpan-Meier estimator, followed by Holm-Sidak multiple comparison tests. Finally, the number of oocytes that lost follicle cells after 2 h of exposure was compared among treatment solutions using one-way ANOVA followed by Holm-Sidak multiple comparison tests. Data in the text are expressed as mean ± standard error. Statistical analyses were conducted with SigmaPlot V11.0.

3.4. Results

3.4.1. Induction of spawning

Spawning occurred on the fifth day of consecutive daily stimulations in three of the treatments: live phytoplankton, phytoplankton paste, and sperm. Spawning was first detected in all tanks of the live phytoplankton treatments (at both 1×10^5 and 1×10^6 cells ml^{-1}) where the first signs of spawning were detected at ~07:30 (30 min after the lights came on) and the last at ~15:30; the overall duration of the spawning event was about 7 h.

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In the phytoplankton paste and sperm treatments, sea cucumbers started to spawn later in the morning at ~10:30, but the last spawning was also at ~15:30, so the spawning event for these treatments was shorter (5 h). Although spawning started at different times among experimental groups, the order of spawning between sexes was consistent among treatments; males began to spawn first and females started to release oocytes ~30 min later. During spawning, males and females did not broadcast their gametes continuously in the water column; but rather in discreet bouts, at intervals of 10-15 min. Males and females had tentacles deployed in the water column; however, the fine tentacle ramifications were not fully extended. No evidence of spawning was detected in the treatments involving temperature shock, desiccation, exposure to and injection of potassium chloride, and injection of serotonin, or in any of the control groups.

Overall, there were significant differences in the proportion of spawning males and females ($F_{5,59} = 11.3$, $p = 0.013$; Fig. 3.1a) and the number of oocytes released ($F_{5,26} = 16.3$, $p > 0.001$; Fig. 3.1b) among the live phytoplankton, phytoplankton paste and sperm treatments. The highest proportion of females spawning and oocytes released were recorded in the live phytoplankton treatment at a concentration of 1×10^5 cells ml^{-1} ($56 \pm 8.4\%$ of females spawning; $19,200 \pm 3,400$ oocytes female^{-1}), followed by the more concentrated live phytoplankton treatment of 1×10^6 cells ml^{-1} ($43 \pm 6.8\%$ spawning and $16,100 \pm 4,500$ oocytes female^{-1}). Both concentrations of phytoplankton paste gave intermediate results, with $26 \pm 4.3\%$ of females spawning and $11,000 \pm 2,500$ oocytes female^{-1} released at 1×10^5 cells ml^{-1} and $26 \pm 4.3\%$ and $7,300 \pm 2,400$ oocytes female^{-1} released at 1×10^6 cells ml^{-1} (Figs. 3.1a and 3.1b). Among these three treatments, the lowest

proportions of spawning females and number of oocytes released occurred under sperm exposure, with $16 \pm 4.9\%$ and $3,000 \pm 895$ oocytes female^{-1} at 2×10^6 spermatozoa ml^{-1} and $16 \pm 4.9\%$ and $1,700 \pm 425$ oocytes female^{-1} at 10×10^6 spermatozoa ml^{-1}, respectively (Figs. 3.1a and 3.1b). Conversely, in males, spawning responses were strongest ($63 \pm 6.7\%$) following the sperm treatment at 2×10^6 spermatozoa ml^{-1} (Fig. 3.1a). Live phytoplankton at 1×10^5 and 1×10^6 cells ml^{-1} ($43 \pm 4.7\%$) and phytoplankton paste at 1×10^5 cells ml^{-1} ($46 \pm 6.8\%$) induced similar proportions of males to spawn. Phytoplankton paste at 1×10^6 cells ml^{-1} and sperm at 10×10^6 spermatozoa ml^{-1} induced the lowest proportion of males to release gametes ($26 \pm 4.7\%$; Fig. 3.1a).

The quality of eggs/embryos at time 0 d post fertilization (early cleavage) was significantly better in both live phytoplankton treatments than in the phytoplankton paste and sperm treatments ($F_{5,61} = 6.9$, $p = 0.007$; Fig. 3.2a). At the onset of development, good quality eggs exhibited reddish colouration, intact plasma membrane, and clear fertilization envelop (Fig. 3.3a), while early embryos showed symmetric cleavage of blastomeres (Figs. 3.3b and 3.3c); these characters defined 100% of the propagules obtained with the two concentrations of live phytoplankton tested and $78 \pm 6.8\%$ and $82 \pm 7.1\%$ of the propagules in the phytoplankton paste treatments at 1×10^6 and 1×10^5 cells ml^{-1}, respectively (Figs. 3.2a). The sperm treatments at 10×10^6 and 2×10^6 spermatozoa ml^{-1} resulted in the lowest production of good-quality eggs and early embryos ($43 \pm 8.7\%$ and $61 \pm 11.2\%$, respectively; Fig. 3.2a) among all successful groups, with the majority of them displaying abnormal lipid granules in the interstitial space between plasma membrane and fertilization

envelop (Fig. 3.3f), turbid and degrading cytoplasm with uneven distribution of yolk (Fig. 3.3g), or early embryos showing irregular-abnormal cleavage (Fig. 3.3h).

At 5 d post fertilization (hatched blastula stage), live phytoplankton treatments at 1 x 10^6 and 1 x 10^5 cells ml^{-1} still showed the highest proportion of propagules with normal development (77 ± 3.8% and 85 ± 4.4%, respectively, Fig. 3.2a); whereas the proportions of normal blastulae dropped to 55 ± 4.2% at 1 x 10^6 cells ml^{-1} and 61 ± 5.1% at 1 x 10^5 cells ml^{-1} in the phytoplankton paste treatments. The proportion of normal blastulae in the sperm treatments were even lower, i.e. 11 ± 8.6% and 18 ± 8.8% at 2 x 10^6 and 10 x 10^6 spermatozoa ml^{-1}, respectively ($F_{5,59}$ = 8.6, p = 0.011; Fig. 3.2a), resulting in high mortalities following a rapid degradation of the oocyte (Fig. 3.3i). Normal blastulae presented reddish colouration and a round-shaped form covered with cilia (Fig. 3.3d); whereas abnormal blastulae displayed an irregular shape and cleavages and sometimes no cilia (Figs. 3.3j and 3.3k). Survival rate of blastula embryos was 93 ± 3.8% and 82 ± 4.1% for live phytoplankton treatments at 1 x 10^5 and 1 x 10^6 cells ml^{-1}, respectively; 75 ± 4.3% and 74 ± 3.2% for phytoplankton paste at 1 x 10^5 and 1 x 10^6 cells ml^{-1}, respectively; and only 19 ± 6.8% and 21 ± 7.8% for sperm treatments at 2 x 10^6 and 10 x 10^6 spermatozoa ml^{-1}, respectively (Fig. 3.2b). All embryos obtained after the sperm treatments died within a maximum of 10 days of development (before the gastrula stage; Fig. 3.2b).

After 20 d of development, the survival rate of gastrulae was higher in the live phytoplankton treatment at 1 x 10^5 cells ml^{-1} (64 ± 5.8% of survival) than in all other treatments (χ^2 = 9.8, df = 5, p = 0.01; Fig. 3.2b). Gastrulae survival rates for the live phytoplankton treatment at 1 x 10^6 cells ml^{-1} and both concentrations of phytoplankton

paste (1×10^5 and 1×10^6 cells ml^{-1}) were $50 \pm 7.1\%$, $43 \pm 13\%$, and $38 \pm 8.6\%$, respectively (Fig. 3.2b). Gastrulae undergoing normal development showed reddish colouration and a bean-shaped form covered in cilia (Fig. 3.3e), whereas abnormal gastrulae displayed oval-shaped forms and damaged surface (Fig. 3.3l).

3.4.2. Artificial oocyte maturation

Oocytes extracted from ovaries of *C. frondosa* were surrounded by semi-transparent follicle cells (Fig. 3.4a). When these oocytes were exposed to various treatments, Dithiothreitol (DTT) at 10^{-1} M resulted in the highest proportion of follicle cells breakdown (i.e. ovulation; $48 \pm 25\%$) among all treatments ($F_{12,112} = 14.7$, $p < 0.001$; Fig. 3.5). Solutions of DTT at 10^{-3} M and 10^{-2} M induced intermediate proportions of ovulation ($26 \pm 6\%$ and $19 \pm 8\%$, respectively; Fig. 3.5). All other treatments (10^{-6}, 10^{-5} and 10^{-4} M of 1-MA; 10^{-4}, 10^{-3} and 10^{-2} M of BAL; 10^{-2}, 10^{-1} and 10^{0} M of L-cyst) induced the breakdown of follicle cells in only a few oocytes (2-6%) similar to the control group (Fig. 3.5).

When it occurred, the process of ovulation started ~25 min after exposure to the experimental and control conditions, with the oocyte forming a protrusion at the future site of extrusion (Figs. 3.4b and 3.4c). The rupture of the follicle cells occurred ~10 min later, on the side where the elongated protrusion on the oocyte surface occurred (Fig. 3.4d). Oocytes acquired a peanut-shaped form ~40 min after exposure when 50% of the oocyte surface were now free of follicle cells (Fig. 3.4e). The follicle cells gradually shrank and wrinkled on the side opposite the initial protrusion and oocytes acquired an oval shaped form ~45 min after exposure to the tested solutions (Figs. 3.4f and 3.4g). The follicle cells

clumped adjacent to the oocyte ~50 min after exposure and were easily detached thereafter; oocytes then regained their normal round shape (Fig. 3.4h).

The exposure of oocytes to 1-MA did not cause noticeable changes of the oocyte appearance. When ovulation occurred, it followed similar patterns as the control with no visible alteration of the process (Figs. 3.4a to 3.4h); whereas of the 48% of oocytes that shed their follicle cells under DTT treatments, ~20% showed abnormal shapes (Fig. 3.4i) and never resumed their normal round shape. All oocytes exposed to L-cyst and BAL showed irregular surface with granular cytoplasm (Fig. 3.4j). Oocytes exposed to BAL also displayed a rapid change from red to pale yellow and disintegrated cytoplasm (Figs. 3.4k and 3.4l) after 2 h of exposure. None of the oocytes that shed follicle cells after exposure to 1-MA, DTT, BAL, L-cyst, and seawater (control) could be fertilized.

3.5 Discussion

The addition of live phytoplankton emerged as the most efficient method to induce spawning in *C. frondosa* among all the treatments tested in the present study, both in terms of quantity and quality of eggs/embryos generated, which is broadly consistent with the fact that spawning of *C. frondosa in situ* and in mesocosms generally coincides with the annual spring algal bloom (Hamel and Mercier, 1995; Mercier and Hamel, 2010). Here, live phytoplankton induced females to release the highest number of oocytes and produce the healthiest eggs/embryos. These results support that live phytoplankton is a potent trigger of spawning in *C. frondosa*, which may act during the natural spawning window and even before the culmination of oogenesis, as suggested by Gianasi *et al.* (2017) who

noted individuals spawning two months in advance of their normal breeding period during a laboratory trial involving the use of phytoplankton as food for adults. These results also imply that the trigger is not only received by gamogenetically mature individuals, but also by individuals with gonads in the growth stages of gametogenesis (Gianasi *et al.*, 2017). There is apparently no direct link between gamete maturation and an increase in the perception of the spawning cue, such as phytoplankton concentration, with the caveat that if not used at the appropriate time this cue can trigger the release of non-mature gametes. Phenolic compounds secreted by diatoms were determined to act as the spawning inducer in sea urchins from a nearby study region in eastern Canada (Starr *et al.*, 1992). The adaptive value of this phytoplankton cue is presumably tied to feeding larvae; i.e. increase in primary productivity during spring algal blooms in temperate seas is proposed to provide favourable conditions for the development and survival of embryos and larvae due to the abundance of plankton food in the water column (Starr *et al.*, 1990). Although *C. frondosa* produces lecithotrophic (non-feeding) larvae, which do not require external nutrients during their pelagic phase (Hamel and Mercier, 1996a), phytoplankton has been identified as an important source of food for newly-settled juveniles (Gianasi *et al.*, 2018). In contrast, similar concentrations of live phytoplankton (1.5×10^5 cells ml^{-1}) failed to induce spawning in the deposit-feeding sea cucumber *Holothuria fuscogilva* (Battaglene *et al.*, 2002). Deposit-feeding tropical species presumably rely on other cues to synchronise their spawning, i.e. lunar cycle and temperature (see review by Mercier and Hamel (2009). Moreover, spawning in *C. frondosa* always occurred after 5 consecutive days of induction, suggesting that a constant prolonged stimulus is required to trigger the release of gametes.

This might be an adaptive strategy to prevent the release of gametes during brief blooms of phytoplankton outside the optimal season, which could compromise the survival of juveniles.

The concentration of live phytoplankton used to induce spawning in *C. frondosa* emerged as a secondary factor. Although both concentrations of the live phytoplankton (1 x 10^5 and 1 x 10^6 cells ml^{-1}) induced comparable proportions of males and females to spawn and resulted in good quality eggs and embryos overall, the number of gamete released as well as survival rates up to the gastrula stage (20 d post fertilization) were greater at the lowest concentration tested. This optimal concentration corresponds to the phytoplankton level that *C. frondosa* likely encounters in its natural habitat during spring blooms in eastern Canada (Pepin *et al.*, 2007). On the other hand, 1 x 10^6 cells ml^{-1} represented an algal biomass ~10 times greater, which might have caused some stress either to the spawning individuals or directly to the gametes, resulting in lower embryonic survival. Therefore, rather than a direct relationship between phytoplankton concentration and spawning, there seems to be a threshold beyond which the spawning response in *C. frondosa* is not increased, and perhaps even depressed. High phytoplankton density used to feed larvae of the sea cucumber *H. scabra* was suggested to alter water pH and concentration of un-ionised ammonia (Morgan, 2001). Thus, a change in seawater chemistry may have led to the comparatively lower survival of embryos at the highest live phytoplankton concentration during the present study.

Although the commercial phytoplankton paste tested also induced spawning in males and females of *C. frondosa*, the number of oocytes released, the quality of developing

eggs/embryos, and embryo survival rates were lower than in the live phytoplankton treatments. This result may suggest that the biochemical composition of microalgae (e.g. phenolic compounds as described by Starr et al., (1992) in the commercial phytoplankton paste is not as potent as that of live phytoplankton, resulting in a weaker spawning response. In addition, processing and bottling might have introduced other chemicals to the commercial paste (e.g. citric, ascorbic and larixinic acids and sodium alginate), which might have accumulated in the experimental tanks and affected the individuals and the quality of their gametes to some degree, since groups induced with phytoplankton paste showed poorer egg quality and lower survival rates (especially at the gastrula stage). While bottled phytoplankton has never been tested previously, prepared solutions of dried algae (*Schizochytrium* sp. and *Spirulina* sp.) have been reported to trigger spawning in 10-35% of tropical deposit-feeding sea cucumbers such as *H. fuscogilva* (Battaglene *et al.*, 2002) and *H. scabra* (James *et al.*, 1994; Pitt and Duy, 2005). However, the quality of eggs and the larval survival rates were not assessed or compared to results obtained with live phytoplankton. The widespread use of commercial phytoplankton products can be attributed to their year-round availability, relatively low cost and low bacterial contamination, and consistent biochemical composition. However, while commercial phytoplankton products can clearly induce spawning in sea cucumbers, including *C. frondosa*, the present study shows that the response and the quality of eggs and embryos might be suboptimal.

Exposure to conspecific sperm induced spawning in few males and females of *C. frondosa*. However, among all successful treatments, females released the smallest

number of oocytes after exposure to sperm, and the embryos died within 10 d post fertilization. Although both concentrations of spermatozoa initially added to the spawning tanks were within the range reported ($2–18 \times 10^6$ spermatozoa ml^{-1}) during a mass spawning of *C. frondosa* in the Gulf of St. Lawrence (Hamel and Mercier, 1996b), the presence of sperm drastically built up as soon as males started to spawn (even before the first females spawned). This overabundance of sperm could have been a source of stress for the females, resulting in only limited release of oocytes. It is also suspected that the high concentration of spermatozoa in the tanks favoured polyspermy, causing the abnormal development and mortality recorded at the blastula stage. Despite some limitations, this trigger deserves further study since the use of sperm from conspecifics combined with phytoplankton has been shown to successfully induce spawning in green sea urchins (Starr *et al.*, 1992).

Although temperature shocks and desiccation have been the most successful techniques to induce spawning in tropical (Battaglene *et al.*, 2002; Purcell *et al.*, 2012) and temperate sea cucumbers (Liu *et al.*, 2015), they did not induce any spawning response in *C. frondosa*. This result might be related to the fact that *C. frondosa* undergoes gametogenesis and spawning during some of the coldest seawater temperatures of the annual cycle, around 0°C (Hamel and Mercier, 1995), and/or because the temperature shock tested remained within the thermal range that populations of *C. frondosa* can experience during seasonal turnovers. Injections and exposure of KCl solutions commonly used to trigger spawning in sea urchins (Okada *et al.*, 1984) also failed to induce spawning in *C. frondosa*. Only one study has reported spawning of a sea cucumber (*H. scabra*) after exposure to KCl solutions for ~30 min; however, the larval survival rates were lower than

when using thermal shocks as a trigger (Hartati and Pringgenies, 1998). As expected, serotonin did not trigger spawning in *C. frondosa*, even though it is a powerful spawning inducer in several bivalve species (Gibbons and Castagna, 1984), suggesting that this neurotransmitter does not act on the nerve system of holothuroids.

The only chemical inducer of oocyte maturation that showed any promise with *C. frondosa* is DTT at 10^{-1} M, which triggered $48 \pm 25\%$ of the oocytes to shed their follicle cells. However, it apparently did not induce germinal vesicle breakdown (GVBD), which prevented the oocytes from becoming fertilizable. Previous studies of artificial induction of oocyte maturation in sea cucumber reported success in some deposit-feeding species (Maruyama, 1980; Chen *et al.*, 1991; Léonet *et al.*, 2009). However, those species possess much smaller oocytes (~150 µm) that are poorly provisioned, leading to the development of feeding (planktotrophic) larvae. Chemical inducers can presumably penetrate into the cytoplasm of such oocytes more easily to induce GVBD and, consequently allow final oogenetic maturation and competency. However, mature oocytes of *C. frondosa* are large (>550 µm) and rich in yolk, in order to sustain the development of the lecithotrophic (non-feeding) larvae for ~45 d until settlement of juveniles. These characteristics likely impeded the uptake or translocation of the tested chemicals, preventing the final maturation process. Although the likelihood of success is limited, other chemicals that successfully induced oocyte maturation in planktotrophic sea cucumber species, such as MIF (Léonet et al. 2009) and cubifrin-L (Fujiwana et al. 2011), might be tested in the future on *C. frondosa*. However, the search for a chemical inducer of oocyte maturation may require a better

understanding of the processes regulating the breakdown of the follicle cells and germinal vesicle in the oocytes of *C. frondosa*, and of lecithotrophic species in general.

3.6 Acknowledgement

3.7 References

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3.8 Tables

Table 3.1: Overview of the stimuli tested to trigger spawning and of the chemicals used to artificially induce oocyte maturation in the sea cucumber *Cucumaria frondosa.*

Experiments	Stimuli/ Chemicals	Level of exposure	Description
Induction of spawning	Temperature shock	5°C 10°C	Exposed to water temperature 5°C or 10°C warmer for 1 h, then returned to tanks (0°C).
	Desiccation	1 h 2 h	Exposed to air for 1 or 2 h, then returned to tanks.
	Live phytoplankton	1×10^5 cells ml^{-1} 1×10^6 cells ml^{-1}	Addition of live phytoplankton (diatom *Chaetoceros muelleri*) to tanks.
	Phytoplankton paste	1×10^5 cells ml^{-1} 1×10^6 cells ml^{-1}	Addition of commercial phytoplankton paste (Shellfish Diet 1800) to tanks.
	Sperm	2×10^6 spermatozoa ml^{-1} 10×10^6 spermatozoa ml^{-1}	Addition spermatozoa from three *C. frondosa* males to tanks.
	Exposure to KCl	1×10^{-5} M 1×10^{-2} M	Exposed to KCl for 1 h, then returned to spawning tanks.
	Injection of KCl	20 ml at 0.5 M 20 ml at 1 M	Injected with KCl into the coelomic cavity while submerged in tanks.
	Injection of serotonin	1 ml at 2×10^{-3} M 1 ml at 2×10^{-2} M	Injected with serotonin into coelomic cavity while submerged in tanks.
Induction of oocyte maturation	1-Methyladenine (1-MA)	10^{-6} M 10^{-5} M 10^{-4} M	Exposure of oocytes isolated from ovaries to three concentrations of 1-MA for 2 h.
	Dithiothreitol (DTT)	10^{-3} M 10^{-2} M 10^{-1} M	Exposure of oocytes isolated from ovaries to DTT for 2 h.
	2,3-Dimercapto-1-propanol (BAL)	10^{-4} M 10^{-3} M 10^{-2} M	Exposure of oocytes isolated from ovaries to BAL for 2 h.
	L-cysteine (L-cyst)	10^{-2} M 10^{-1} M 10^0 M	Exposure of oocytes isolated from ovaries to L-cyst for 2 h.

3.9 Figures
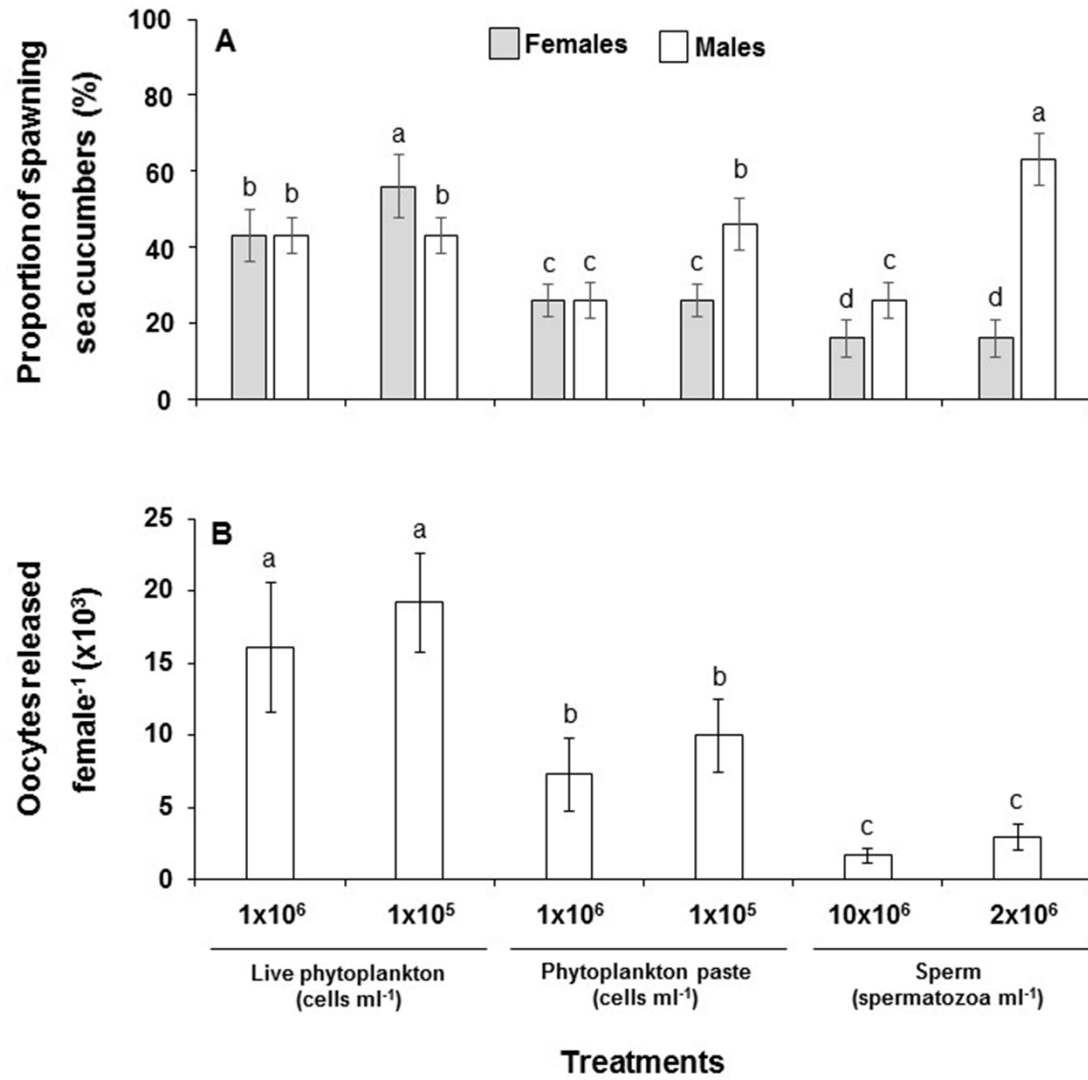
Figure 3.1 (previous page): Spawning of the sea cucumber *Cucumaria frondosa* induced with live phytoplankton, phytoplankton paste, and sperm from conspecifics. (A) The proportion of males and females that spawned varied significantly among treatments. (B) Average number of oocytes released by females undergoing spawning in each treatment. No evidence of spawning was observed in the treatments involving thermal shock, desiccation, KCl, and serotonin, or in control groups. Data is shown as mean $\pm$ se (n = 3). Means with different letters are significantly different. See text for full statistics and Table 3.1 for description of each treatment.
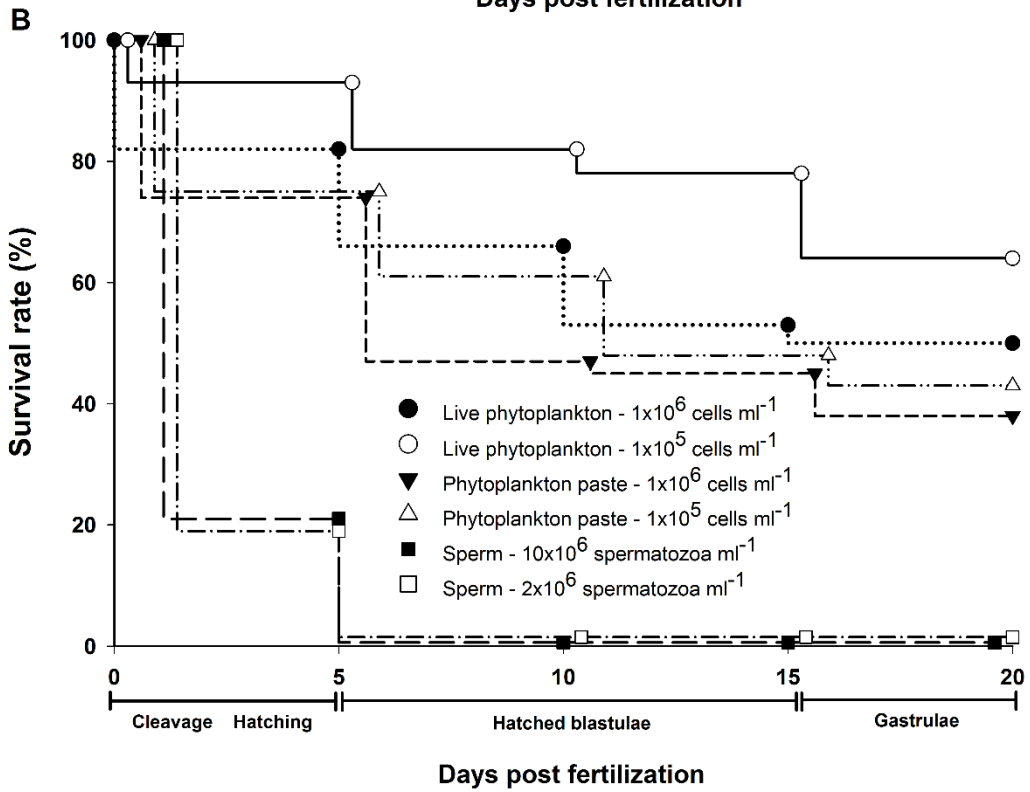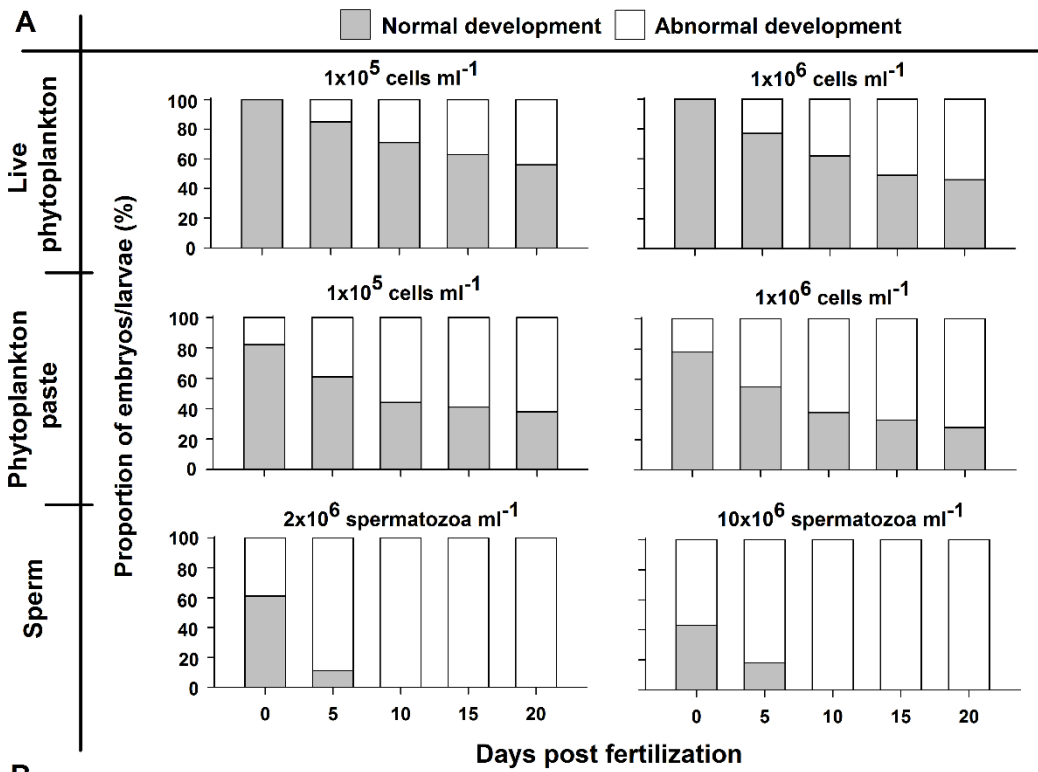
Figure 3.2 (previous page): Quality and survival of eggs and embryos of the sea cucumber *Cucumaria frondosa* induced to spawn with live phytoplankton, phytoplankton paste, and sperm from conspecifics. (A) Proportion of fertilized oocytes (0 d post fertilization), blastulae (5 d post fertilization), and gastrulae (20 d post fertilization) undergoing normal and abnormal development. (B) Survival rates of fertilized oocytes (0 d post fertilization), blastulae (5 d post fertilization), and gastrulae (20 d post fertilization). No evidence of spawning was observed in the treatments involving thermal shock, desiccation, KCl, and serotonin, or in control groups. See Table 3.1 for description of each treatment.
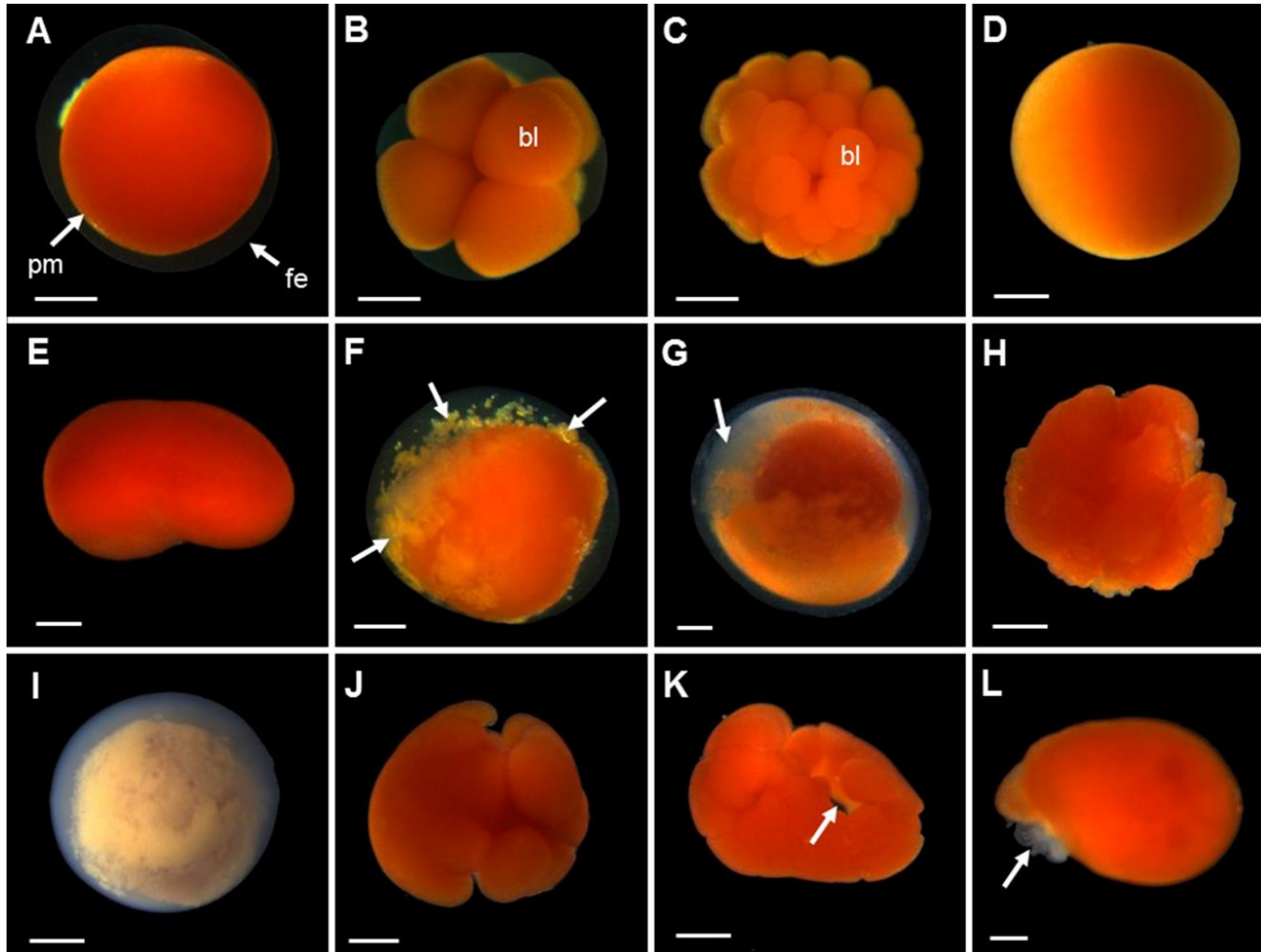
Figure 3.3 (previous page): Illustrations of fertilized oocytes (0 d post fertilization), blastula (5 d post fertilization), and gastrula embryos (20 d post fertilization) of the sea cucumber *Cucumaria frondosa* undergoing normal and abnormal development. (A) Normal fertilized oocyte displaying red colouration, clear fertilization envelop (fe) and plasma membrane (pm). (B) Normal cleavage with 8 symmetric blastomeres (bl). (C) Normal cleavage and adhesion of blastomeres (bl). (D) Normal blastula embryo with red colouration and round shape covered in cilia. (E) Normal gastrula embryo with red colouration, bean-shaped form covered in cilia. (F) Abnormal blastula embryo displaying lipid granules (arrows) in the interstitial space between plasma membrane and fertilization envelop observed in the phytoplankton paste treatments. (G) Abnormal blastula embryo displaying turbid (arrow) and degrading cytoplasm observed chiefly in the phytoplankton paste and sperm treatments. (H) Abnormal blastula embryo showing irregular cleavage observed in the sperm treatment. (I) Dead blastula embryo with degraded cytoplasm. (J) Abnormal hatched blastulae with anarchic cleavages, irregular shape and no cilia observed in the sperm treatment. (K) Abnormal hatched blastulae displaying incomplete compaction (arrow) and anarchic cleavages. (L) Abnormal gastrula embryo with irregular shape and damaged blastoderm (arrow). Scale bars represent 100 µm.
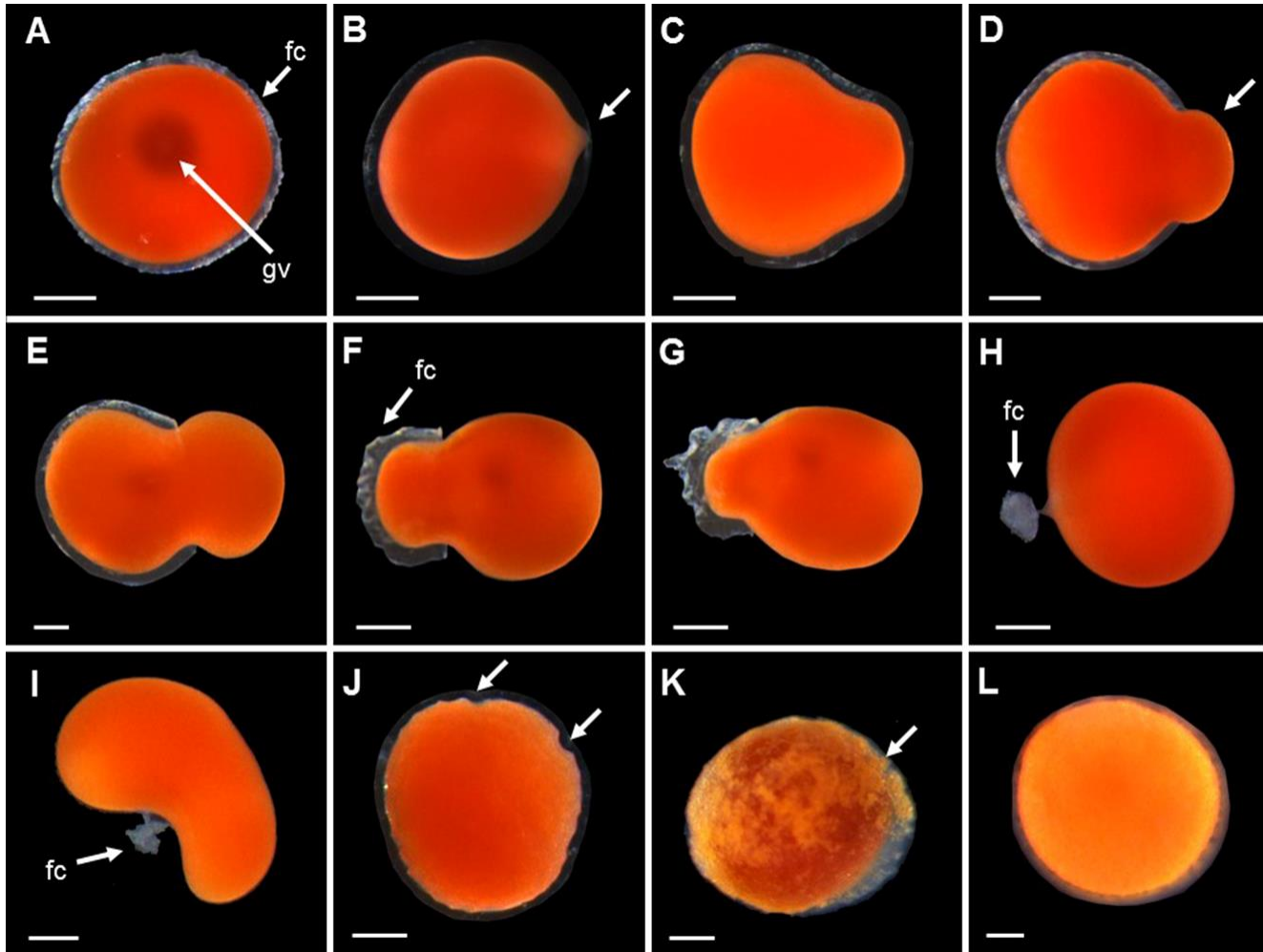
Figure 3.4 (previous page): Ovulation process in oocytes of the sea cucumber *Cucumaria frondosa*. (A) Oocytes surrounded by follicle cells (fc) and displaying the germinal vesicle (gv). (B) Breakdown of follicle cells starting, with a protrusion of the oocyte (arrow). (C) Enlargement of oocyte protrusion. (D) Breakdown of follicle cells ~35 min after exposure when ~20% of the oocyte surface area is no longer covered by follicle cells. (E) Oocyte acquire a peanut-shape form ~40 min after exposure, when 50% of surface is outside of the follicle cells. (F) Oocyte gradually leaving the follicle cells (fc), which starts to shrivel on the opposite side. (G) Oocyte acquires an oval-shaped form ~45 min after exposure, as follicle cells continue to shrink. (H) Follicle cells (fc) clump near the oocyte ~50 min after exposure; oocyte regains its normal round shape. (I) Abnormally-shaped oocyte after the breakdown of the follicle cells (fc) observed in Dithiothreitol treatments. (J) Oocytes surrounded by the follicle cells showing granular cytoplasm (arrows) after exposure to L-cysteine. (K) Oocyte displaying irregular surface with yellowish granules (arrow) in the interstitial space between plasma membrane and follicle cells after exposure to 2,3-Dimercapto-1-propanol. (L) Yellowish oocyte indicating degradation of cytoplasm and death after exposure to L-cysteine and 2,3-Dimercapto-1-propanol for 2 h. Scale bars represent 100 µm.
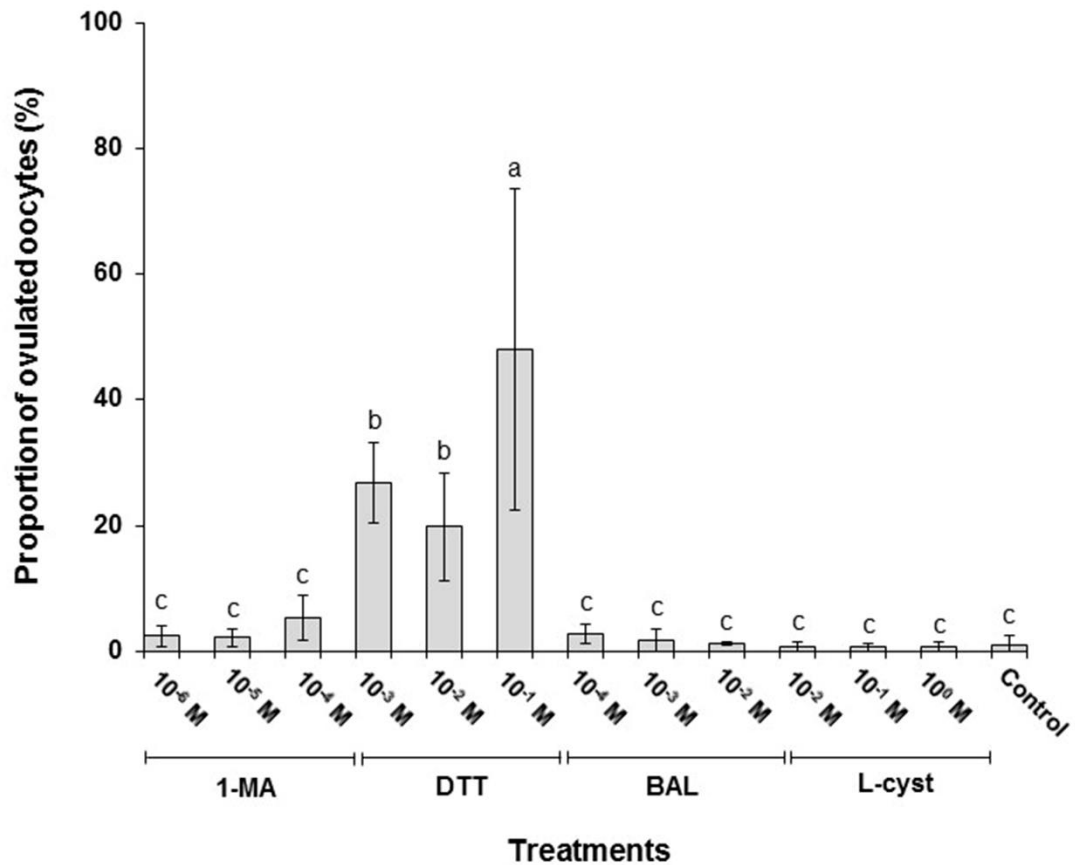
Figure 3.5: Proportion of oocytes that lost their follicle cells after 2 h of exposure to different concentrations of 1-Methyladenine (1-MA), Dithiothreitol (DTT), 2,3-Dimercapto-1-propanol (BAL), and L-cysteine (L-cyst). Data is shown as mean ± se (n = 10). Means with different letters are significantly different. See text for full statistics and Table 3.1 for description of each treatment.

Chapter 4. Full Allogeneic Fusion of Embryos in a Holothuroid Echinoderm[3]

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4.1 Abstract

Whole-body chimaeras (organisms composed of genetically distinct cells) have been directly observed in modular/colonial organisms (e.g. corals, sponges, ascidians); whereas in unitary deuterostosmes (including mammals) they have only been detected indirectly through molecular analysis. Here we document for the first time the step-by-step development of whole-body chimaeras in the holothuroid *Cucumaria frondosa*, a unitary deuterostome belonging to the phylum Echinodermata. To our knowledge, this is the most derived unitary metazoan in which direct investigation of zygote fusibility has been undertaken. Fusion occurred among hatched blastulae, never during earlier (unhatched) or later (larval) stages. The fully fused chimaeric propagules were 2-5 times larger than non-chimaeric embryos. Fusion was positively correlated with propagule density and facilitated by the natural tendency of early embryos to agglomerate. The discovery of natural chimaerism in a unitary deuterostome that possesses large externally-fertilized eggs provides a framework to explore key aspects of evolutionary biology, histocompatibility, and cell transplantation in biomedical research.

Keywords: Fusion, sea cucumbers, chimaera, genetic heterogeneity, allorecognition

4.2 Introduction

Intraorganismal genetic heterogeneity (IGH), and the natural occurrence of chimaeras, is perplexing (Pineda-Krch and Lehtilä, 2004), because it challenges the traditional biological notion of unit of selection, which relies on genetic homogeneity (Santelices, 1999). While IGH remains a biological puzzle, stem-cell-derived chimaeras have proven useful to study complex *in vivo* processes of developmental biology and regenerative medicine (Wu *et al.*, 2016; Behringer, 2007). However, artificially-produced interspecies chimaeras are the subject of ethical discussions, whereas the study of spontaneous intraspecific chimaeras may hold untapped potential (Behringer, 2007). So far, whole-body fusion among conspecifics has been documented chiefly in modular/colonial organisms with the capacity to reproduce asexually, such as sponges (Maldonado, 1998), corals (Puill-Stephan *et al.*, 2009; Barki *et al.*, 2002), bryozoans (Hughes *et al.*, 2004) and ascidians (Westerman *et al.*, 2009; Bishop and Sommerfeldt, 1999; Rinkevich, 2005), whereas its occurrence in unitary organisms has only been evidenced indirectly in mammals (Strain *et al.*, 1995; Rinkevich, 2001; Ross *et al.*, 2007; Haig, 1999) through microsatellite DNA markers, and directly in brooded propagules of sea anemones (Mercier *et al.*, 2011) (a basal group of marine invertebrates). Here, we provide evidence of full allogeneic fusion among embryos of another unitary metazoan that sits closer to humans in the animal tree of life (in the deuterostome clade), the holothuroid *Cucumaria frondosa* (Echinodermata: Holothuroidea).

Cucumaria frondosa is distributed throughout the Arctic and North Atlantic oceans (Hamel and Mercier, 2008). It is a suspension-feeding gonochoric species with an

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obligatory sexual reproduction. During the annual breeding season, oocytes and spermatozoa are released and fertilization occurs externally (Hamel and Mercier, 1996a, 1996b). The large maternally-provisioned (yolky) oocytes/eggs are buoyant and develop into non-feeding (lecithotrophic) larvae over ~45 d, until metamorphosis and settlement (Hamel and Mercier, 1996a). While feeding (planktotrophic) larvae of certain holothuroids and other echinoderms have been shown to undergo budding (Balser, 1998; Eaves and Palmer, 2003), embryonic fusion in an echinoderm species has never been recorded.

As evidence supporting the occurrence of natural chimerism accumulates across many taxa, chimaeric entities are receiving considerable attention in bids to explore the unit of selection, genetic heterogeneity and allorecognition mechanisms. The present study explores the development of chimaeras in *C. frondosa* embryos and constitutes the first direct investigation of fusibility in an echinoderm and in a unitary deuterostome.

4.3 Methods

4.3.1 Initial detection of fusion and formation of chimaeras

Given that chimaerism had never been documented in echinoderms, the occurrence of paired embryos (attached to each other) was initially thought to be a case of larval cloning by asexual budding, previously reported in planktotrophic larvae of Echinoidea and Holothuroidea (Eaves and Palmer, 2003). To investigate the phenomenon, 15 pairs of embryos showing evidence of external tissue connection were incubated in PVC culture vessels (5 cm diameter) in which walls were removed and replaced with 800-µm mesh to allow water circulation. The vessels were placed inside a 24-L tank with running ambient

seawater. Pictures and measurements of the embryos were taken daily under a motorized stereo-microscope (Leica M205FA) for a total of seven days, which confirmed the progressive fusion of embryos and the development of whole-body chimaeras. Based on these initial observations, experiments were devised to explore morphological changes in embryonic shape and developmental time as well as to assess the prevalence of chimaeras among the offspring population (see below). Experiments were conducted during two spawning seasons (2016 and 2017) following the same experimental protocol, but with propagules from distinct progenitors in order to confirm the findings.

4.3.2 Egg collection and incubation

Similarly sized adults of *C. frondosa* weighing 4.8 ± 0.8 g immersed weight (~290 g wet weight) and measuring 14 ± 1.4 cm contracted body length (± sd; n=30) were hand collected by divers from two sites along the Avalon Peninsula, Newfoundland, eastern Canada (47°17'44.6" N: 52°46'8.9" W and 47°05'22.7" N: 52°55'10.0" W), at depths between 5 and 10 m. In the laboratory, visual inspection confirmed that holothuroids were healthy; displayed normal pigmentation, firm attachment to the substrate, and no skin lesions. They were acclimated in two 500-L tanks (~100 individuals each) with running ambient seawater (40 L h^{-1}) at a temperature of 3 ± 1°C. An attempt to equally distribute males and females (1:1) in each tank was made based on gonopore morphology (Hamel and Mercier, 1996a). Continuous input of new seawater in the tanks provided natural plankton and particulate organic matter as food sources. Light was provided through fluorescent bulbs with a maximum intensity of 150 lux and photoperiod adjusted weekly to match natural fluctuations.

Upon spontaneous spawning during the normal spring breeding season, buoyant eggs were gently collected from the tanks. Egg density was estimated by collecting five 50-ml aliquots and performing counts under a stereo-microscope (Leica M205FA). Fertilization was confirmed by the elevation of the fertilization envelop and/or cleavage and eggs/embryos were then transferred to three incubators, each containing 0.8 egg ml^{-1}. Since the propagules of *C. frondosa* float on the surface of the water, their density was also calculated based on the surface area they covered, resulting in a concentration of 14 egg cm^{-2}. Incubators consisted of 5-L round black plastic vessels placed inside a 500-L tank with running ambient seawater (40 L h^{-1}), i.e. the same seawater source used in parental tanks. In order to ensure a constant water flow inside the incubators, four equal openings (40 cm^2 each) were made on the walls of the vessels and covered with a 400-µm mesh. Light was provided as mentioned previously.

4.3.3 Developmental kinetics and frequency of chimaerism in the propagule population

Embryonic and larval development were monitored by collecting a subsample of 200 propagules from each of the 3 incubators daily until the settlement of the pentactula larvae (for a total of 45 measurements). Propagules were measured at their maximum length and photographed under the previously described microscope. A new developmental stage was scored when >50% of the embryos/larvae had reached it. In order to identify the life stage(s) during which chimaeric fusion may appear, and to quantify fusion rates in the offspring population, each incubator was examined daily for evidence of fusion (for total

of 45 observations). Owing to the large size of unitary embryos in *C. frondosa* (~700 µm), the initial stages of fusion were large enough to be visually identified with a magnifying glass and separated from unitary propagules (figure 4.1). Fusing embryos were photographed under the stereo-microscope (described above) and the frequency of chimaerism determined.

When identified, chimaeric embryos were collected and distributed into three separate culture vessels (1 vessel per incubator) as described above, and their development further monitored. Among all chimaeras collected, 15 were sampled from each vessel (15 chimaeras x 3 culture vessels, for a total of 45 chimaeras) daily and pictures were taken under the stereo-microscope (see above). The rotational swimming of chimaeric and non-chimaeric embryos was documented as an indicator of health since it is expected that healthy propagules exhibit circular path movement through their development at the blastula, gastrula, and vitellaria stages (Hamel and Mercier, 1996a). The 9,600 propagules studied measured 500 to 1,300 µm and their size frequency distribution (50-µm bins) was established. Size classes and developmental tempo of chimaeric embryos were compared with non-chimaeric blastulae and gastrulae using one-way analysis of variance (ANOVA; $\alpha=0.05$) followed by pairwise Holm-Sidak tests.

4.3.4 Histology of chimaeric embryos

In order to better understand the cellular organization of fusing embryos, chimaeras at various stages of fusion were preserved in 3% formaldehyde and processed using standard histological procedures for methacrylate embedding. Sections (3 µm) were stained

with haematoxylin and eosin and digitalized using an automated slide scanner (Axio Scan Z1) with a 20x objective.

4.3.5 Effect of density and agglomeration on the development of chimaeras

The hypothesis that high propagule density might favour fusion was tested across two density treatments (high, low). Each treatment consisted of three 50-ml glass vials placed inside a 24-L tank with running ambient seawater. The low and high density treatments were chosen to respectively represent half and twice the initial egg density in the incubators where chimaerism had already been detected (see above). The low density treatment was prepared using 0.4 egg ml^{-1} or 7 egg cm^{-2}, whereas the high density treatment consisted of 1.6 egg ml^{-1} or 28 egg cm^{-2}. These values are in line with the concentration of eggs that can occur after natural mass spawning of *C. frondosa* in the field, which was reported to reach ~0.2 egg ml^{-1} 17 h post release while the majority of eggs were dispersed in the water column and in the process of accumulating at the surface (Hamel and Mercier, 1996b). The occurrence of fusion was monitored in all the treatments daily for 21 d post fertilization. Chimaeric embryos were identified as mentioned previously, and their total number was compared between treatments using Student's *t*-test.

Moreover, it was hypothesized that the gregarious behaviour of blastulae at the surface of the water favoured physical contact and fusion. Hatched blastulae were released in a 2-L vessel at a density of 0.8 propagule ml^{-1} or 14 propagule cm^2 (same as in the initial egg incubators where fusion was recorded) and gently agitated to ensure uniform dispersion

in the water column. A digital camera (Olympus Tough TG-3) placed above the container took pictures every 10 s for a total of 15 min. Movement and agglomeration of the embryos were monitored.

4.4 Results

Fertilized oocytes (eggs) of *C. frondosa* floated at the surface of the water a few minutes after their natural release. They measured 700 ± 150 µm and cleavage started within a few hours post fertilization (figure 4.1). The successive cell divisions generated a blastula (equivalent to blastocyst in mammals) ~3 d post fertilization. Swimming blastulae hatched from the fertilization envelop ~5 d post fertilization, while still gregariously floating close to the water surface, sometimes in great numbers (electronic supplementary material, figure S.4.1).

Five different stages of fusion were determined based on morphological characters, including maximum length (figures 4.2a and 4.2b) and shape, as well as percent fusion over time. Fusion started to occur after hatching from the fertilization envelop, through the formation of a bond between the blastoderms of two touching embryos (figure 4.1; Fusion Stage 1). This early attachment was easily broken during gentle handling, but it provided enough strength for the paired embryos to spin and move together. The total length of embryos at Stage 1 of fusion was ~1300 µm (figures 4.1 and 4.2b). In Stage 2 (~9 d post fertilization), a stronger bond ~150 µm thick was established between blastulae (figure 4.1; Stage 2) and the chimaeric entity measured 1150 ± 40 µm (figure 4.2b). As fusion progressed to Stage 3 (~11 d post fertilization) the cellular connection between embryos

thickened to 400 µm and total length of the merging pair decreased (1050 ± 55 µm; figures 4.1 and 4.2b). At this stage, fusion was about one-third complete and embryos acquired a peanut shape (figure 4.1). Histology revealed that there were still two visible blastocoels, but that the blastoderms were completely fused (figure 4.3a).

Chimaeric embryos acquired a smoother oval shape when fusion was two-third completed in Stage 4, ~13 d post fertilization (figure 4.1). The bond thickened to 550 µm and there was a further decrease in overall length to 1000 ± 35 µm (figures 4.1 and 4.2b). After ~15 d post fertilization, non-chimaeric blastulae started to elongate and developed into gastrulae (normal development illustrated in figure 4.1) and reached a length of 930 ± 50 µm. Concurrently, full fusion (Stage 5) was completed in chimaeric propagules (figure 4.1), which displayed a round shape, homogenous ciliation, and a single blastocoel/gastrocoel (figure 4.3b). These whole-body chimaeras had a mean length of 980 ± 40 µm (figure 4.2b), which was significantly larger than non-chimaeric blastulae (700 ± 60 µm) and gastrulae (930 ± 30 µm; $F_{2,31}$=24.6, P=0.011). Moreover, their volume was 0.5 mm^3 which was roughly twice that of non-chimaeric blastulae.

Further fusion events were detected at Stage 3, either among chimaeras or between chimaeras and blastulae, resulting in multi-chimaeric propagules (figure 4.1). The frequency of occurrence of these multi-chimaeras was low (0.02% of the population). However, some multi-chimaeras composed of three entities reached the full fusion stage (figure 4.1; Stage 5), resulting in notably large propagules with a length of 1100 ± 38 µm and a volume of ~0.7 mm^3 (~5 times the volumetric size of non-chimaeric blastulae). Multi-chimaeras involving more than 3 entities failed to develop.

Apart from directly monitoring fusion events, we measured the size structure of embryos at the post-hatching blastula stage and detected two dominant cohorts (figure 4.2a). The smaller cohort (maximum length of 700 ± 60 µm, volume of ~0.18 mm^3) included those that did not undergo allogeneic fusion. The second cohort was represented by larger embryos (1120 ± 51 µm; figure 4.2a), subsamples of which were microscopically shown to be the result of fusion (figure 4.1). Confirmed fusion occurred in $8.6 \pm 0.6\%$ of the entire propagule population (9,600 embryos). It was seen in all independent cultures of *C. frondosa* (n=12) across two separate breeding seasons (2016 and 2017) involving progenitors collected from different locations, supporting the fact that allogeneic fusion in this species is a natural widespread phenomenon. The post-metamorphic just-settled juveniles still displayed marked size variations, between 910 and 1160 µm in length.

4.5 Discussion

Natural fusion of early life stages has only been reported previously in cnidarians and sponges. In brood-protected larvae and embryos of the sponge *Chalinula* sp. and the sea anemone *U. felina*, natural fusion occurred in 40% and 3% of the propagules, respectively (Ilan and Loya, 1990; Mercier *et al.*, 2011). Fusion rates at later stages (newly-settled polyps) were 78% in the soft coral *Clavularia hamra*, 90% in *Nepthea* sp., 40% in *Heteroxenia fuscescens*, and 80% in *Parerythropodium fulvum fulvum* (Barki *et al.*, 2002). Natural fusion was also documented among modular adults including, 3-5% of colonies in the coral *Acropora millepora* (Puill-Stephan *et al.*, 2009), 40-90% of colonies in four

species of soft corals (Barki *et al.*, 2002), and 8-73% of colonies in botryllid ascidians (Ben-Shlomo *et al.*, 2008).

The mechanisms that lead to the establishment of chimaeras revolve around allorecognition (Scofield *et al.*, 1982; Brown and Rodriguez-Lanetty, 2015). Studies have identified allogeneic responses controlling fusion or rejection in sponges; however, the molecular mechanism is still poorly understood (Fernandez-Busquets and Burger, 1999). According to grafting trials in hydroids, fusion may occur among colonies when they share at least one of the histocompatibility loci *alr1* and *alr2* (Nicotra *et al.*, 2009). In ascidian, fusion among oozoids is controlled by the self-recognition fusibility/histocompatibility (Fu/HC) locus (Weissman *et al.*, 1990; De Tomaso *et al.*, 2005; Litman, 2005). Allograft studies suggest that echinoderms have a non-adaptive, activation type immune response based on coelomocyte activity; however, the specificity of the response is still unclear (Gross *et al.*, 1999). Hence, whether fusion in *C. frondosa* occurs among embryos that share at least one histocompatibility locus (i.e. between full or half siblings) or non-related embryos remains to be determined through controlled fertilization and molecular analyses. Such information might provide insights into the potential use of chimaeric embryos produced by *C. frondosa* in biomedical research, especially with the recent publication of the holothuroid genome (Zhang *et al.*, 2017).

Allogeneic fusion has been associated with maturation of the alloimmune system. Natural chimaeras have been detected among free-swimming larvae and embryos of sponges (Ilan and Loya, 1990). In the brooding sea anemone *U. felina*, fusion of either embryos or larvae seemed to occur inside the brooding female (Mercier *et al.*, 2011). In

soft corals, fusion occurred among young polyps a few weeks after settlement (Barki *et al.*, 2002). Although the stages and mechanisms involved in the formation of *natural* chimaeras in mammals are still elusive (Ross *et al.*, 2007; Haig, 1999), interspecies chimaeras produced *artificially* are generated only during the blastocyst stage (Wu *et al.*, 2016). Similarly, natural fusion in *C. frondosa* only occurred among hatched blastulae; no evidence of fusion was found in either earlier (unhatched) or more developed embryonic and larval stages (i.e. gastrula, vitellaria, and pentactula). This result indicates that the alloimmune system might not be completely developed in blastula embryos of *C. frondosa*, preventing them from recognizing genetically distinct (non-self) tissues/embryos. This also suggests that allorecognition mechanisms in echinoderms may develop within a brief window during early ontogeny, i.e. between just-hatched blastulae and full development of gastrulae, similar to evidence from mammals. Fusion at earlier stages (unhatched embryos) was presumably prevented because the elevated fertilization envelope acted as a physical barrier. The present study documented the very early process of natural fusion in *C. frondosa*, with the appearance of a yellowish bond linking two blastulae, which may be the first evidence of alloimmune interaction, leading to the development of a tissue connection between blastoderm cells. Although this bond might represent the first communication between fusing embryos, its nature and composition remain unclear. Further studies using molecular markers may help characterize its origin. Rejection was never detected, unlike reports in corals where a visible inflammatory response occasionally developed in the contact area, sometimes leading to the death of one of the partner (Puill-Stephan *et al.*, 2009).

After hatching, blastulae of *C. frondosa* showed a strong tendency to aggregate with each other at the surface of the water column (electronic supplementary material, figure S.4.1); a phenomenon that was not seen in the older stages where no fusion was detected. Moreover, experimental trials revealed that the formation of chimaeras was density dependent. There were statistically higher proportions of confirmed chimaeras in the high-density blastula treatment ($1.6 \pm 0.2\%$) than in the low-density treatment ($0.6 \pm 0.3\%$; t_2=3.5, P=0.02). While this result indicates that, all else being equal, higher propagule density favoured fusion in embryos of *C. frondosa*, these small-scale experimental trials were not optimal for fusion. The maximum fusion rates (8.6%) were obtained at medium density in the much larger culture vessels (5 L) where 4000 propagules were incubated, likely because it increased the chance of embryos sharing fusibility/histocompatibility loci. Aggregation of hatched blastulae at the surface of the water may involve the hyaline layer, which is an extracellular matrix surrounding early echinoderm embryos that is composed of glycoproteins and is necessary for blastomere adherence (Citkowitz, 1971). Although the nature and interactions of these adhesive molecules remain uncertain, they may favour contact agglomeration of blastulae leading to the first step of fusion in *C. frondosa*.

On a fundamental level, the ecological and evolutionary benefits of different life-history strategies in echinoderms have been investigated on theoretical grounds. It has been suggested that larval cloning in echinoderms might be an adaptation to increase survival under favourable conditions of water temperature and food concentration (Eaves and Palmer, 2003; Vickery and McClintock, 2000) and to minimize visual detection and predation by reducing propagule size (Vaughn and Strathmann, 2008). Clonal propagation

may also provide evolutionary advantages by preventing internal conflicts of genetically distinct cell lineages (Grosberg and Strathmann, 2007). Conversely, the evolutionary role of chimaerism (which is essentially the opposite of cloning) is rather controversial, fueling discussions about the potential benefits of fusion (Pineda-Krch and Lehtilä, 2004; Pérez-Portela *et al.*, 2013). Fusion between allogeneic entities is expected to confer genetic variability, developmental synergism, and immediate increase in size and survivorship (Buss, 1982; Hennige *et al.*, 2014). An overall gain in fitness has been documented in multi-chimaeric ascidians (Rinkevich and Shapira, 1999; Pérez-Portela *et al.*, 2013), corals (Puill-Stephan *et al.*, 2009), and sea anemones (Mercier *et al.*, 2011). Our results confirmed that fully fused chimaeras are significantly larger than singletons, with a volume 2-5 times greater than non-chimaeric blastulae. This difference in size is still clear in post-metamorphic juveniles. Hence, the occurrence of chimaerism may explain reports of high variability in the size of just-settled juveniles in *C. frondosa* (Hamel and Mercier, 1996a; Gianasi *et al.*, 2018) and other holothuroids (Qiu *et al.*, 2015), suggesting that fusion among embryos may be common in holothuroid species from other climes and with different modes of development. Because *C. frondosa* produces maternally-provisioned lecithotrophic larvae that do not feed until settlement (when the mouth and anus are formed), a size increase through feeding can be ruled out. The greater volume of chimaeric embryos may increase buoyancy, allowing them to disperse efficiently during the long pelagic phase (~45 d). Moreover, larger juveniles that are a product of fusion may gain competitive advantage during the settlement phase due to increased resistance to water turbulence and predation (Buss, 1982), enabling chimaeric juveniles to better cope in

dynamic competitive habitats where food and space are limited. Although chimaerism may provide potential benefits, early zygote fusion before the germline is sequestered may also allow these deuterostomes to develop germ cell competition/parasitism. The occurrence of cell-lineage parasitism was previously reported in chimaeric ascidians, and sexual parasitism was documented in anglerfish (Rinkevich, 2011).

Overall, *Cucumaria frondosa* emerges as a promising model organism for investigating intraspecific chimaeras since natural fusion occurs among large free-swimming blastulae (i.e. not inside the body or womb, as in sea anemones and mammals). The potential costs/benefits of chimaerism in this unitary species definitely deserve further investigation due to the strategic position of Echinodermata within the deuterostome lineage (Lowe *et al.*, 2015). Unlike more primitive clades, such as colonial and unitary cnidarians, where visual evidence of incomplete fusion can occur (electronic supplementary material, figure S.4.2), demonstrating the existence of chimaeric holothuroids in the wild will require the development of appropriate molecular markers and techniques. Meanwhile, in light of recent genomic advances (Zhang *et al.*, 2017), the present discovery offers a unique framework to explore cellular self-recognition mechanisms and develop our understanding of the evolution of immune systems in higher metazoans.

4.6 Acknowledgments

4.7 References

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4.8 Figures
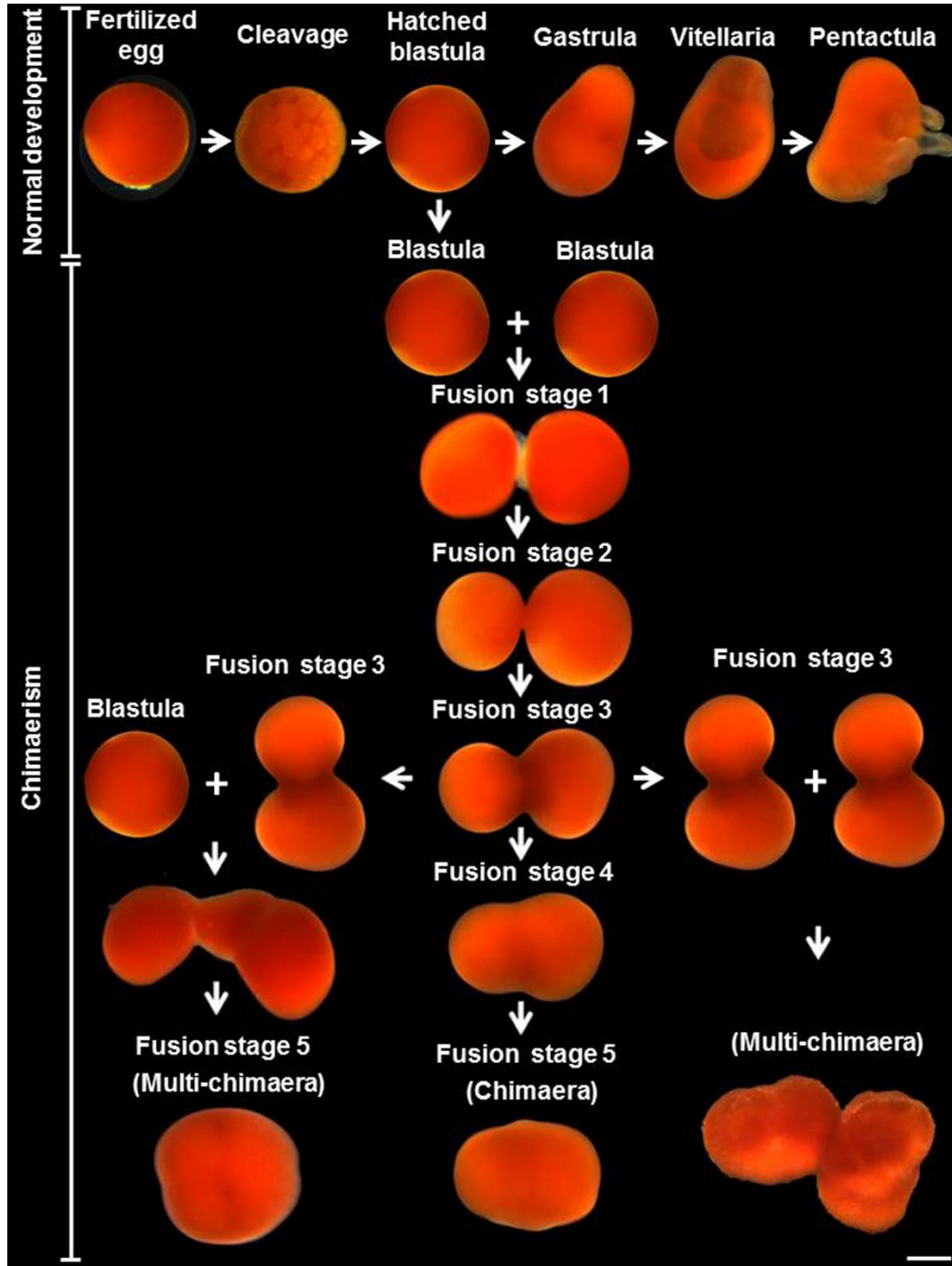
Figure 4.1 (previous page): Normal development and formation of chimaeras in embryos of the holothuroid *Cucumaria frondosa*. Fertilized eggs started cleavage within a few hours post fertilization. The successive cell divisions generated a blastula embryo which hatched from the fertilization envelop 5 d post fertilization. The free-swimming ciliated blastula started to elongate and developed into gastrula larva 15 d post fertilization. After 30 d, the primary tentacles and two ambulacral podia were visible in the vestibule and the embryo became vitellaria. After 45 d post fertilization, the pentactula larva was ready to settle on the substrate. Allogeneic fusion strictly occurred between hatched blastula embryos. The first stage of fusion started 5 d post fertilization with the development of yellowish bond between two embryos. In Stage 2, the blastoderms of both partners were connected by a narrow tissue connection. This connection between embryos thickened progressively through Stages 3 and 4. Full fusion (Stage 5) was generally completed inside 15 d post fertilization. Some individuals at Stage 3 fused again to form multi-chimaeras, i.e. organisms composed of three (left path) or more (right path) embryos. Scale bar represents 300 µm. See figure 4.2b for length of each stage of fusion.
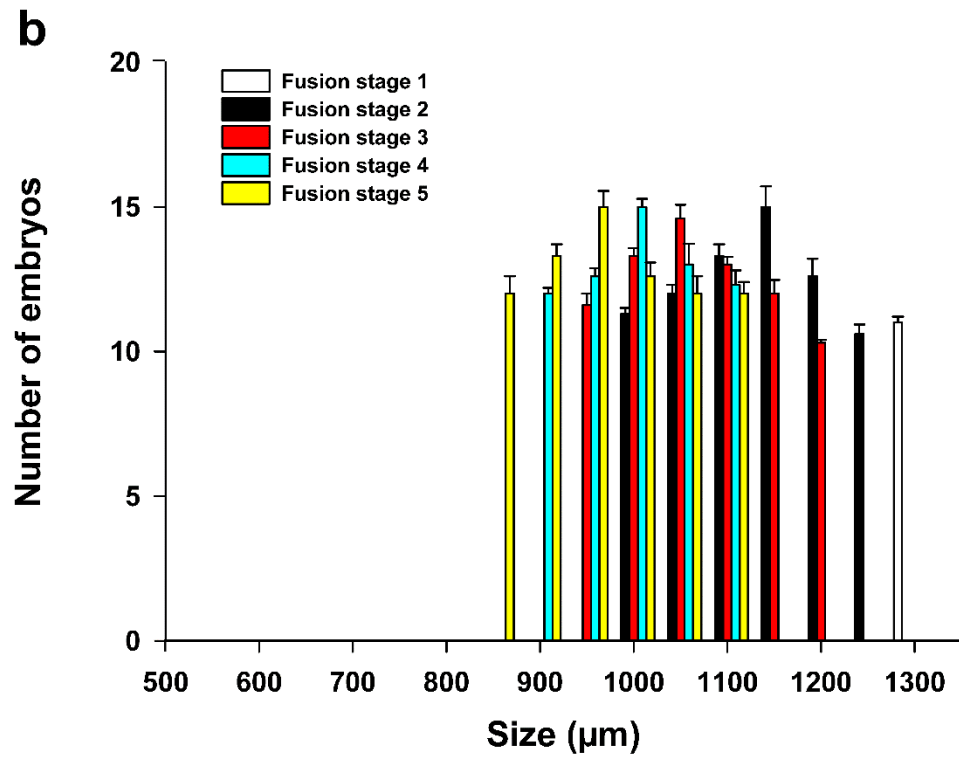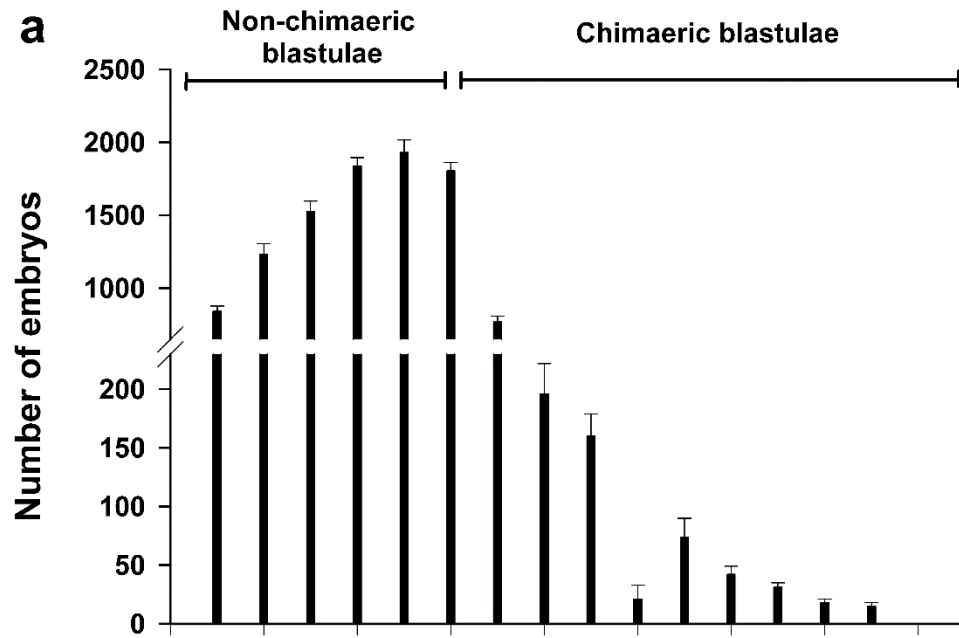
Figure 4.2 (previous page): Size of chimaeric and non-chimaeric embryos of *Cucumaria frondosa*. (**a**) Size frequency distribution of non-chimaeric and chimaeric embryos. (**b**) Size structure of chimaeric blastulae in the 5 stages of fusion described in figure 4.1. Data shown as mean ± se (n=3).
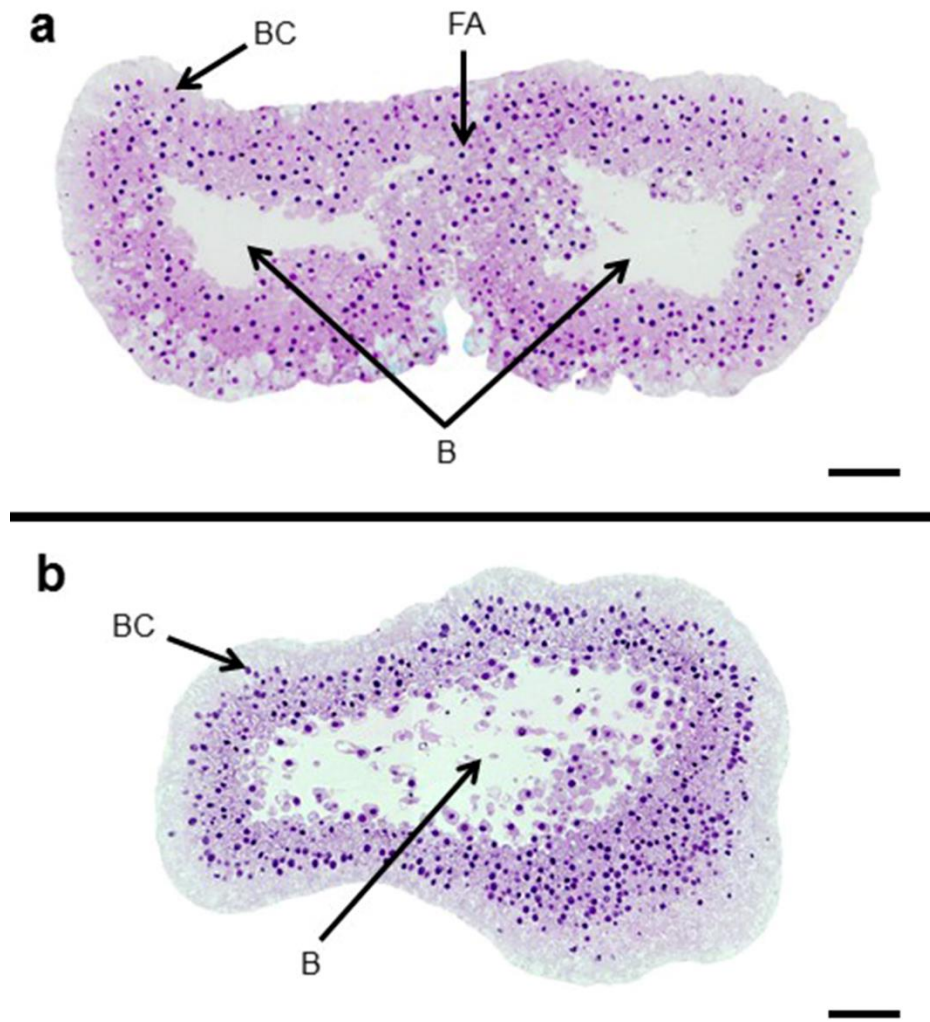
Figure 4.3: Histological sections of chimaeric embryos of the holothuroid *Cucumaria frondosa*. (**a**) Fusing embryos (Stage 3) can be individually distinguished by the presence of two distinct blastocoels (B); however, blastoderm cells (BC) are mingling at the fusing area (FA). (**b**) Fusing embryos at Stage 5 of fusion are no longer distinguishable from each other and exhibit a single blastocoel (B), indicating complete fusion. Scale bars represent 100 µm. See figure 4.1 for illustrations of each stage of fusion.

4.9 Supplementary Material
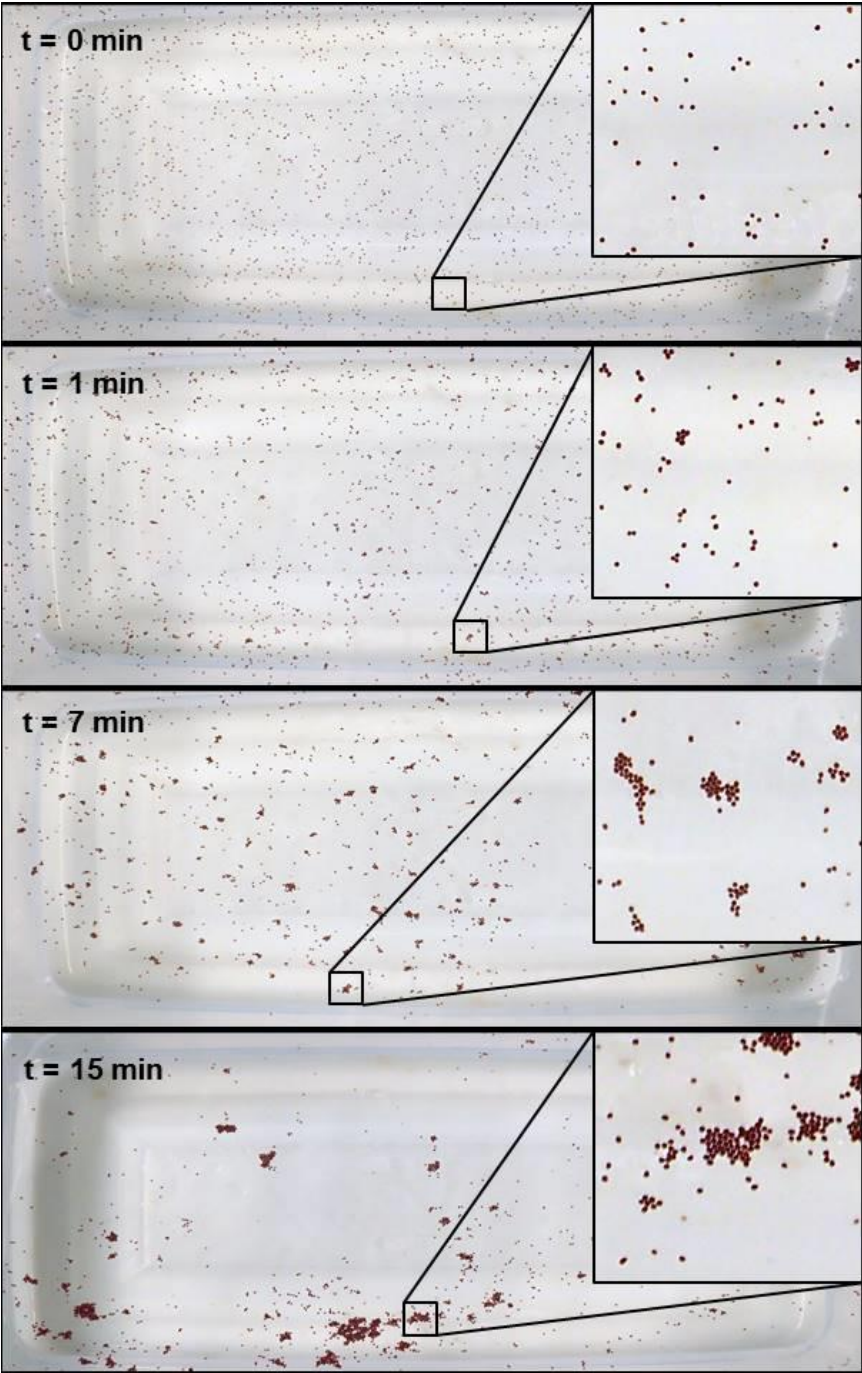
Figure S.4.1 (previous page): Agglomeration of recently hatched blastulae of *Cucumaria frondosa* at the surface of the water 0, 1, 7, and 15 min after initial mixing. When embryos were mixed at time 0, individual blastulae were homogeneously distributed and no clusters were apparent. However, as the turbulence decreased over time and embryos started to float on the surface; blastulae began to pair up, ~1 min post mixing. After 7 min, all blastulae were on the surface of the water, which favoured the formation of clusters, each composed of 10-150 embryos. Large clusters composed of 100-500 blastulae formed after 15 min. At the end of the experiment, >80% of the blastulae were agglomerated to some degree.
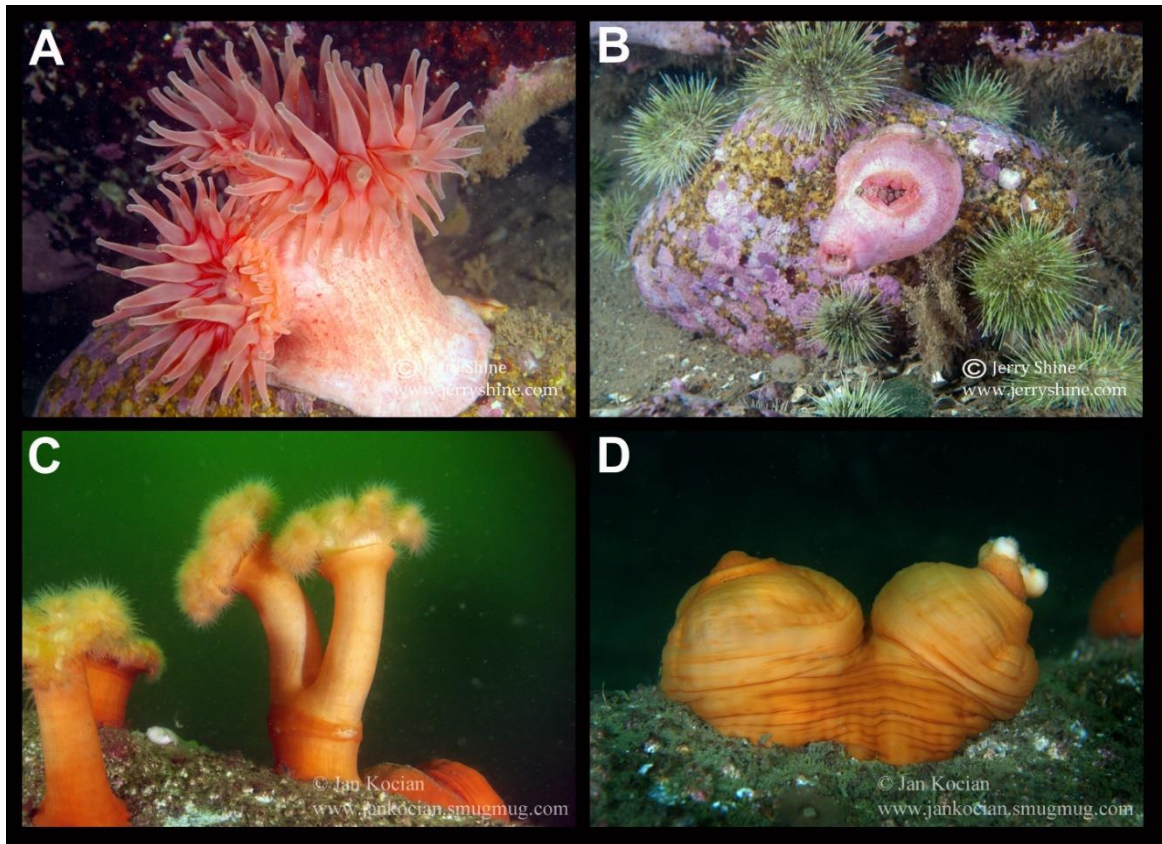
Figure S.4.2: Evidence of chimaerism in unitary animals in the marine environment. Photos of chimaeric sea anemones captured by underwater photographers. A-B) Multi-headed individuals of the genus Urticina photographed off Eastport, Maine, USA. C-D) Bi-headed individuals of the genus Metridium photographed near Whidbey Island, Washington, USA.

Chapter 5. Morphometric and Behavioural Changes in the Early Life Stages of the Sea Cucumber *Cucumaria frondosa*[4]

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5.1 Abstract

The present study examined morphological and behavioural development in post-settled juveniles of the commercial sea cucumber *Cucumaria frondosa*, in an effort to assist captive breeding and conservation initiatives. Juveniles developed all 10 tentacles within 16 months; they had 9 podia and measured 4.6 mm in length after 21 months. Scaling between body length and number of podia was isometric, whereas dorsal tentacle metrics showed negative allometric scaling, indicating that growth was accompanied by a decreasing tentacle to body size ratio. Dorsally-oriented tentacles developed and ramified faster, showing 6 ramifications after 21 months, when ventrally-oriented tentacles only displayed one. This asynchrony underlines the distinctive roles of dorsal vs. ventral tentacles during the early months. The former were strictly used to capture plankton from the water column; whereas the latter were used for anchorage and feeding on deposited material. Ossicles of the body wall increased in length and thickness, and became slightly curved and rounded in older juveniles. Light intensity and water flow tolerance increased with age; from 25 lux and 5 cm s^{-1} in 1-month-old individuals, to >50 lux and 10 cm s^{-1} in 6, 12, and 21-month-old individuals, consistent with migration from sheltered to more exposed locations. Moreover, 1 and 6-month-old juveniles preferred rock substrates and black or red background colours; whereas 12 and 21-months-old individuals favoured substrates of coralline algae and a red background, also indicative of increasing affinity with sunlit environments and a shift from benthic to planktonic feeding. Juveniles of all age classes were feeding (i.e. had deployed tentacles) 24 h a day and commonly sought vertical surfaces. Together, the findings shed light on the early juvenile ecology of this

cold-water suspension-feeding sea cucumber. Considering the expansion of sea cucumber fisheries and growing interest in the aquaculture of *C. frondosa*, this work also provides a framework for improved stock management and culture protocols.

Keywords: Echinoderm; sea cucumber; juvenile; growth; behaviour; age.

5.2 Introduction

Holothuroids, or sea cucumbers (Echinodermata: Holothuroidea), are ubiquitous members of the epibenthic fauna and constitute an important component of both nearshore (Purcell *et al.*, 2016) and deep-water (Brandt *et al.*, 2007) communities. They have long been commercially harvested for seafood and as a source of nutraceutical products (Purcell *et al.*, 2014; Xia and Wang, 2015). However, sea cucumber fisheries have a poor history of sustainability, typically undergoing a boom and bust pattern, resulting in the collapse of wild stocks (Purcell *et al.*, 2013). Declines in the natural populations of high-value sea cucumbers have encouraged the emergence of new fisheries around previously unexploited species and the development of aquaculture programs worldwide (Hamel and Mercier, 2008; Purcell *et al.*, 2012; Yang *et al.*, 2015). The expansion of sea cucumber farming and the ongoing concerns over the status of the natural populations have intensified research on aspects of sea cucumber biology and ecology; with studies primarily focusing on the reproductive biology of adults (Battaglene *et al.*, 2002; Ramofafia *et al.*, 2003; Leite-Castro *et al.*, 2016), larval settlement, substrate preferences (Mercier *et al.*, 2000b) and growth rates post settlement (Ramofafia *et al.*, 1997; Yang *et al.*, 2005). There is still a relative scarcity of literature dedicated to the earliest benthic life stages, especially in suspension-feeding species (order Dendrochirotida) and temperate-cold habitats, which has hindered advances in sea cucumber fisheries management and aquaculture.

Direct observation of newly-settled sea cucumber juveniles (i.e. recruits) in the field is limited, partly due to their small size, cryptic nature, and the general paucity of information on nursery habitats (Shiell, 2004). Studies combining field and laboratory

approaches have recorded the morphological development and distribution of juveniles in a limited number of species, most of them deposit feeders in the order Holothuriida (formerly Aspidochirotida). Young and Chia (1982) identified 20-mm juveniles of *Polus chitinoides* on shaded rock walls in the San Juan Islands, Washington. Cameron and Fankboner (1989) investigated recruitment habitats of *Parastichopus californicus* in British Columbia (Canada) and observed juveniles (3-10 mm) on dense mats of red algae and in crevices of rock walls at depths of 2 to 20 m. McEuen and Chia (1991) compared the development of embryos and larvae of the temperate species *Psolus chitonoides* and *Psolidium bullatum* from Washington (USA) and briefly described the ossicle structure, and development of tentacles and podia in juveniles up to one year old (~1.2 mm) under laboratory conditions. In tropical holothuriids, a size-related distribution with depth was observed in the Solomon Islands where 1.7-mm recruits of *Holothuria scabra* were found attached to seagrass leaves before migrating to the surrounding soft substrate when reaching about 11 mm; juveniles 10-40 mm long occurred on muddy-sandy substrates inside seagrass beds, whereas individuals 40-150 mm long were mostly recorded on sandy substrate in deeper areas (Mercier *et al.*, 2000a). Post-settled juveniles of one dendrochirotid have also been studied; Guisado *et al.* (2012) described the embryonic development, larval morphology, and juvenile growth of *Athyonidium chilensis* up to 5 months (~1.5 mm) in Valparaiso (Chile).

Juveniles of three sea cucumber species (all holothuriids) have also been shown to respond to a wide range of stimuli. Mercier *et al.* (1999) found that *H. scabra* juveniles responded to light intensity by burrowing at sunrise and emerging at sunset to feed. Similar

patterns of daily activity were found in 30-g juveniles of *Apostichopus japonicus* which sought shelter during the day and fed during the night (Dong *et al.*, 2010a). Moreover, changes in water temperature and salinity influenced the daily feeding and burrowing activity in juveniles of *H. scabra* (Mercier *et al.*, 1999) and *A. japonicus* (Chen *et al.*, 2007). Substrate selection by early life stages has also been documented in some of these species. When presented with sediment of various grain sizes and organic content, individuals of *H. scabra* ranging from 10 to 140 mm in length favoured medium-sand grain size and organically rich sediment (Mercier *et al.*, 1999). Similarly, juveniles of *Actinopyga echinites* measuring 15 to 71 mm in length displayed a preference for dark substrate composed of limestone or dead corals (Wiedemeyer, 1994). Apart from sediment composition, substrate colour was shown to affect the behaviour and body colour of juvenile sea cucumbers. Dong *et al.* (2010b) found that green, red, and blue artificial shelters attracted slightly more 24-g juveniles of *A. japonicus* than white, black, and yellow shelters. Furthermore, Jiang *et al.* (2015) reported that although juveniles of *A. japonicus* (~1.5 g) grew faster on a yellow background, the highest proportion of individuals that retained the desirable red body colour (for high market value) was observed on blue background.

The sea cucumber *Cucumaria frondosa* (Holothuroidea: Dendrochirotida) is widely distributed in cold-water habitats from the Arctic Ocean to Cape Cod (USA) as well as along the coast of northern Europe and Russia (Hamel and Mercier, 2008). This species is the focus of a commercial fishery in the North Atlantic (Hamel and Mercier, 2008) and was recently proposed as a candidate for multi-trophic aquaculture (Nelson *et al.*, 2012). Unlike

the most commonly studied species of sea cucumber, *C. frondosa* is a passive suspension-feeder that captures food particles from the water column with its ten ramified tentacles (Singh *et al.*, 1998). Adults are mostly found on rocky substrates from the lower intertidal zone to ~300 m depth; however, the highest abundance usually occurs between 20 and 100 m (Hamel and Mercier, 2008; So *et al.*, 2010). Recruitment of *C. frondosa* in the field is poorly understood, although a few small individuals were found in shallow areas, underneath rocks, between branches of coralline algae and attached to mussel shells (Hamel and Mercier, 1996; Medeiros-Bergen and Miles, 1997). So far, only one study has documented the movements and behaviour of early recruits in the laboratory, following a settlement preference experiment (Hamel and Mercier, 1996). Growth rates of *C. frondosa* juveniles are considered to be slow and largely influenced by water temperature and food availability (Hamel and Mercier, 1996; So *et al.*, 2010).

While previous work has determined the influence of environmental parameters on pelagic embryos and larvae of *C. frondosa* (Hamel and Mercier, 1996), no study has quantified and critically appraised the appearance and development of morphological structures and behaviours within the first few months of benthic life. Considering the expansion of sea cucumber fisheries and aquaculture around the world, including eastern Canada, basic knowledge must be collected to provide a solid basis for the development of effective stock management and culture protocols. The present study investigated morphometric changes occurring on a monthly basis in *C. frondosa*, from newly-settled recruits to juveniles 21 months old. Focal metrics included body length, number and size of tentacles, tentacle ramifications, number of ventral and dorsal ambulacral podia, tentacle

insertion rates, ossicles, and skin pigmentation. Experimental trials were also conducted to assess ontogenetic changes in the response of various age groups to different light intensities, substrate types, background colours, current speeds, as well as to tease out their daily activity patterns.

5.3 Material and Methods

5.3.1 Collection and spawning of adults

Adult sea cucumbers weighing 7.0 ± 1.5 g immersed weight (~390 g wet weight) and measuring 11.0 ± 1.7 cm ($\pm$ sd; n = 30) contracted body length were hand collected by divers in Bay Bulls (Avalon Peninsula, 47°17'44.6'' N: 52°46'8.9'' W), eastern Canada, at depths between 5 and 10 m. Individuals were kept in two 500-L holding tanks (~100 sea cucumbers in each) with running seawater (50 L h^{-1}) at ambient temperature (~1°C). Light was provided through large windows and photoperiod fluctuated naturally. Planktonic food present in the ambient seawater was available to sea cucumbers. Males and females spawned naturally in March, and fertilization was confirmed under an automated stereo-microscope (Leica M205FA) from the elevation of the fertilization envelop and/or cleavage.

5.3.2 Rearing of juveniles

Fertilized oocytes (eggs) of *C. frondosa* were incubated in three rearing vessels (~0.4 egg ml^{-1}) which consisted of 4-L round plastic containers with black bottom and walls, placed inside a 40-L tank supplied with running ambient seawater (20 L h^{-1}). In order

to ensure a constant water flow from the 40-L tanks into the rearing tanks, four equally spaced holes (40 cm^2 each) were made on the walls close to the bottom of the round containers and covered with 1-mm mesh. The meshed openings were cleaned weekly to maintain optimum water circulation. Light was provided through large windows and followed natural photoperiod (from 15L/9D in the summer to 8L/16D during winter). Temperature of the water fluctuated naturally between 14°C at the end of summer and -1°C in the winter.

The development of embryos and larvae was monitored every 3 days until the settlement of pentactulae. All individuals, from newly-settled to 21 months of age, will henceforth be referred to as juveniles. At the onset of the study, each rearing vessel contained ~150 juveniles (representing a density of 37 juveniles L^{-1}). The system that supplied seawater to the rearing tanks did not have any filtration; therefore, natural plankton (e.g. small ciliates, flagellates, diatoms and copepod nauplii) and suspended organic material present in ambient seawater was available as food for the juveniles. In an attempt to mimic the natural habitat, rocks (n = 3; measuring ~5 X 3 cm) were spread evenly inside the rearing tanks to provide shelter and/or substrate for attachment to the juveniles. These rocks were conditioned in seawater for over 1 month in order to form a natural biofilm on their surface prior to adding them to the rearing tanks.

5.3.3 Assessment of morphometrics

In order to develop hatchery techniques and design adequate rearing tanks, knowledge of the early life stage is needed, specially post settlement. Therefore, measurements were recorded monthly in juveniles (n = 30) selected haphazardly from one

of the rearing tanks. The juveniles were detached manually, without any chemicals, to avoid either damaging them or changing their behaviour. For measurements, individuals were placed in a 20-ml Petri dish kept inside a shallow water bath (Boekel GD120L) to maintain temperature similar to that in the rearing tanks. Anchorage to the dish post transfer occurred within 2 min. Pictures of the juveniles were taken using an articulated macroscope (Leica DMS1000) and analysed using the software Leica LAS EZ® following protocols described below. The transfer and imaging procedures took ~20 min per individual, before it was returned to the rearing tank.

Body length (dorsally, from the base of the tentacles to the anus) was measured on attached individuals with fully deployed tentacles and the number of tentacles in the mouth counted (Table 5.1). Because tentacles were at different level of development during the initial growth phase, measurements of size and number of ramifications focused on the two longest and most developed tentacles positioned dorsally. Tentacle length was assessed from the base to the apex when fully deployed in the water column. As the tentacles developed, ramifications radiated from the principal stalk to form primary ramifications, which further ramified into secondary and tertiary ramifications (Table 5.1). In an attempt to standardize the ramification metrics, measurements only considered the number of primary ramifications, excluding the apex, emerging from the two longest tentacles on the dorsal side of the juvenile. The number of ambulacral podia on the ventral and dorsal side of the juveniles was determined. Skin pigmentation was defined as the presence of colour

patches in any location of the body. Finally, relationships among morphometric parameters was estimated using the power function equation

$$y=ax^b;$$

where y is the body length, a is the y-intercept, x is the metric of interest (tentacle ramifications, tentacle length, number of ambulacral podia), and b is the allometric coefficient (Poot-Salazar *et al.*, 2014).

Scanning electron microscopy (SEM) was used to characterize the changes in the ultrastructure of the ossicles on the body wall of juveniles at 1, 6, and 12 months post settlement (Table 5.1). A total of 6 juveniles per age class were collected haphazardly and preserved in 10% neutral buffered formalin. Individuals were washed three times for 5 min in each of the following solutions: phosphate buffered saline (PBS), distilled water, 0.1% Tween 80, distilled water. They were subsequently rinsed for 10 min in a graded series of ethanol baths. Final rinse in 100% ethanol was performed 3 times and ethanol removed from the sample and replaced by 2 ml of hexamethyldisilazane (HMDS). They were completely submerged for 2 min and gently swirled. HMDS was then replaced and left for 30 min in a covered Petri dish. Finally, excess HMDS was removed and dishes with juveniles were placed inside a desiccator for 48 h before being transferred to the carbon-coated stubs using an eyelash brush under a dissecting microscope (Nikon SMZ745T). SEM was performed using a Phenom ProX at an acceleration voltage of 10 kV. Photos

were taken with the software Phenom ProSuite at magnifications varying from 200 to 16,000x.

5.3.4 Assessment of behaviour

5.3.4.1 Tentacle retraction/insertion rate

The movement of the tentacles, including their periodic insertion into the mouth (tentacle insertion rate), has been used as a proxy for feeding activity and as an indicator of food intake in *C. frondosa* (Holtz and MacDonald, 2009). The movement of the tentacles in newly-settled juveniles up to 5 months old was characterized by a retraction towards the mouth (without insertion); however, when juveniles reached 6 months old, they were able to fully insert the tentacles into the mouth. Both types of tentacle behaviours were associated with feeding and quantified over time. Once morphometric measurements were completed, tentacle retraction/insertion rates were recorded monthly from 1 to 21-month-old juveniles (n = 30) as the number of tentacles retracted/inserted in the mouth per minute (Table 5.1).

5.3.4.2 Daily activity

In order to characterize daily activity in juveniles of different ages over a 24-h period, individuals were exposed to optimal conditions of light intensity, substrate type, background colour, and water flow (determined experimentally; see sections below). Juveniles were positioned in the center of 4-L round vessels (19 cm diameter, 15 cm height), on the bottom of which circular grids were drawn at 2-cm intervals to help monitor position and displacement. These vessels were placed inside a water bath with running

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ambient seawater to keep similar temperature as in the rearing tanks. Juveniles were grouped by age classes that were expected to show different responses to environmental parameters. For each age class (1, 6, 12, and 21 months post settlement), three vessels holding five juveniles each were used. The number of juveniles with tentacles deployed was recorded every 4 h over 24 h; the interval between observations was chosen to minimize stress that might alter the normal behaviour of juveniles. The locomotor speed of juveniles was also monitored since individuals may move in their natural habitat to find food, microhabitat or to escape from predators (Legault and Himmelman, 1993). The speed at which juveniles moved to different locations of the vessel and their final position (as an indication of their preference for the tank walls or vertical substrates vs tank bottom or horizontal substrates) were recorded hourly for a total of 24 h with a digital camera (Olympus® TG-3). The locomotor speed was calculated based on the distance traveled by the juvenile in body length per unit of time. Data for speed was assessed for normality and homogeneity using Kolmogorov-Smirnov and Levene's tests and statistically compared among age classes after 4, 12, and 24 h in the experimental tanks. Two-way ANOVA (speed x age) was used followed by pairwise Holm-Sidak multiple comparison tests ($\alpha = 0.05$).

5.3.5 Experimental determination of response to stimuli

Trials were conducted after 1, 6, 12, and 21 months post settlement in order to identify changes in behaviour as the juveniles aged (Table 5.1). Except when otherwise mentioned, experiments were performed in 20-ml vessels (3 cm in diameter) half submerged in a water bath supplied with running ambient seawater. LED lights with adjustable brightness (Aled Light®; spectrum of 400-780 nm) were positioned above the

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experimental setup, providing uniform light to each dish. Photoperiod was adjusted to follow natural fluctuations in the field ranging from 15L/9D in the summer to 8L/16D in the winter. Preferred light intensity was used for each age class of juveniles as determined during the light response trials (see below).

For 1, 6, and 12-month-old juveniles, treatments consisted of three vessels, each containing two juveniles, except when otherwise mentioned. Treatments were run in triplicates and haphazardly rotated in the experimental setup in order to minimize tank and environmental effects. Individuals were selected haphazardly from their holding tanks. Due to the low number of 21-month-old juveniles available, limited data were gathered and their behaviour was only assessed qualitatively. Experiments ran for a maximum of 24 h, eliminating the need for feeding, which could have altered behaviours.

5.3.5.1 Response to light

The response of juveniles to different light intensities was evaluated in all age classes (1, 6, 12, and 21 months post settlement) in order to identify optimal light conditions for the rearing tanks. Five light gradients were generated by placing the light source on one side of the experimental vessels (using the light system and vessels described previously). Light gradients tested included 0-0 (front-back of dish), 25-18, 50-36, 75-55, and 100-77 lux (measured with a Traceable light meter). These ranges of intensities were chosen based on a previous study, which indicated that post-settled juveniles of *C. frondosa* have a preference for low-light and shaded areas (Hamel and Mercier, 1996). Treatments also covered the probable range of light intensities recorded in the field between 5 and 100 m depth (Kampa, 1970).

Vessels were marked with four cardinal points (North, South, East, and West) and four intercardinal areas (Northeast, Southeast, Southwest and Northwest) in order to monitor the position of juveniles across 8 quadrants. Treatments were separated with a black vinyl tarpaulin to avoid bleeding of light. At the onset of a trial, juveniles were placed in the center of the vessel and their position monitored with digital cameras (previously described) every 30 min for 24 h (for a total of 48 pictures). The number of juveniles in each quadrant was compared among treatments using Watson-Williams F-tests ($\alpha = 0.05$). Rose diagrams were created to visualize the position of juveniles and circular statistical analysis was performed in the software Oriana[®].

5.3.5.2 Response to substrate type and background colour

Previous studies indicated that juveniles of tropical and temperate sea cucumbers may have a preference for substrate type (Wiedemeyer, 1994; Mercier *et at.*, 1999) and substrate colour (Jiang *et al*. 2015). Therefore, to optimize rearing conditions for *C. frondosa* juveniles, experiments were designed to assess whether individuals had the ability to express substrate selection and background colour preference. Treatments were chosen based on previous reports of just-settled and early juveniles of *C. frondosa* being attached to hard structures such as rocks, with and without coralline algae, gravel (Hamel and Mercier, 1996), and mussel shells (Medeiros-Bergen *et al.*, 1995).

Substrates tested included: (1) coralline algae (rhodoliths *Lithothamnion glaciale*), (2) mussel shells (*Mytilus edulis*), and (3) rocks; all representing similar surface (~3 mm long and 2 mm wide, or 6 mm^2). Two different substrates were offered in a pairwise design by spreading them in equal semi-circles covering the entire bottom of the vessel

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(representing a surface area of 6 cm^2 each). Juveniles were then placed between the two paired substrates, using the samples sizes and replication scheme described in the general experimental section. Control treatments consisted of the same vessels without any substrate (other than the bare bottom), where juveniles were placed on an imaginary intersecting line.

The preference of juveniles for background colour was tested using white, black, and light red backgrounds. The red and black backgrounds were used to mimic natural components of *C. frondosa* habitat, i.e. coralline algae, rocks, and mussel shells. The white background was used for comparative purposes since it is an unusual colour in habitats where juveniles of *C. frondosa* occur (So *et al.*, 2010). All backgrounds were tested in a pairwise design, as per previously outlined methods. Background colours were applied underneath the transparent bottom, each covering the entire half of the vessel (6 cm^2). Juveniles were then placed between the two colours. Controls were designed as mentioned previously.

Experimental vessels were illuminated using the preferred light intensity for each age class of juveniles (determined during the light response trials). The position of juveniles was recorded using the setup described above, with pictures taken hourly for 24 h (for a total of 214 photographs per treatment). Treatments were compared by assessing the number of juveniles on each substrate or background colour at the end of the experimental period using paired *t*-tests ($\alpha = 0.05$). Normality and homogeneity were verified by using Kolmogorov-Smirnov and Levene's tests, respectively and transformations using $\log(x)$, arc $\sin(x)$ or $\sqrt{x}$ were carried out where necessary.

5.3.5.3 Response to current strength

In the rearing tanks, the water flow ensures a constant supply of oxygen and nutrients to the individuals and the removal of wastes (e.g. ammonia); however, the strength of the current might affect juvenile behaviour. Therefore, the preference for water flow was tested in *C. frondosa* from all age classes (see above) by measuring the deployment and retraction of tentacles under current speeds of 0, 5, 10, and 20 cm s^{-1}. Juveniles were placed in the center of thermos-regulated 4-L round vessels (described previously) with black bottom and walls (identified as preferred colour in a previous experiment) under the optimal light conditions previously determined. Ambient seawater was filtered (100 μm mesh) to remove large particles that could stimulate the deployment of tentacles (Hamel and Mercier, 1998). An overhead variable stirrer (i.e. lab egg stirrer, IKA® RW11) was positioned in the center of each container to generate a unidirectional clockwise flow (measured with the displacement of dyes). Individuals were initially introduced under static conditions to permit their anchorage to the bottom. The flow was initiated 15 min after the confirmation of firm attachment to the substrate. The deployment of tentacles after being transferred to the experimental vessels started within ~30 s in 1 and 6-month-old juveniles and ~15 min in 12-month-old juveniles (as determined during preliminary experiments). The control group consisted of juveniles exposed to the stirrer paddle without any motion.

Each treatment, composed of one vessel holding five juveniles under a set flow, was repeated three times at intervals of 4 days. The time needed for juveniles to extend their tentacles was noted; thereafter, the number of juveniles with tentacles fully deployed was recorded every 30 min for a total of 6 h (12 measurements per trial, in triplicate, for a total

of 36 values per treatment). Magnifying lenses were used to monitor the tentacles of the juveniles during the experimental period. The number of juveniles with tentacles extended was compared among different flows at the end of the experiment (after 6 h) allowing them enough time to acclimate to the current regime. Data violated the assumptions for use of parametric statistics even after transformation and, for this reason, Kruskal-Wallis one-way ANOVA on ranks followed by Tukey tests was used ($\alpha = 0.05$).

5.4 Results

5.4.1 Morphometrics and tentacle movement

One-month-old juveniles measured 0.8 ± 0.3 mm (Fig. 5.1) and exhibited a uniform dark reddish colour (RGB values of R: 237, G: 89, B: 18) on the entire body wall, tentacles, and ambulacral podia, similar to the pigmentation of embryos and larvae (Fig. 5.2a). Individuals had 2 ambulacral podia, used exclusively for anchorage, and 5 primary tentacles (Fig. 5.1 and 5.2a, Table 5.2). Primary tentacles measured 0.3 ± 0.1 mm long, were not ramified and had flattened adhesive tips, which were used for both anchorage and feeding (Fig. 5.3a). In the feeding posture, the body of one-month-old juveniles was in a horizontal position relative to the substrate (i.e. lying down) and tentacles were in contact with the substrate (not erect in the water column). Tentacles were typically touching or pushing the substrate, or exhibiting writhing and sweeping movements. When tentacles were contracted, they were found close to the mouth, but remained outside of it and were surrounded by protruding ossicles of the body wall forming a ridge around the mouth (Fig. 5.4a). The movement of the tentacles toward the mouth was thus characterised by a straight

retraction motion bringing the food close to the mouth (but not in it). Tentacles were fully extended at all times, except for feeding motions (Fig. 5.2a). The tentacle retraction rate was 22 ± 1.7 tentacle min^{-1} and did not display any consistent retraction order (Fig. 5.1). The majority of one-month-old juveniles (~85%) were found in shaded areas of the tank such as underneath or on the side of rocks. Ossicles in the shape of fenestrated plates covered the entire body wall surface (Fig. 5.4a), each measuring 120 ± 18 µm long and 3.5 ± 0.4 µm thick. Ossicle presented 6 ± 0.8 large central holes, without any knobs or denticles on either the inner or outer surface (Fig. 5.4b). A closer look revealed that they bore multiple smaller cavities on their surface (Fig. 5.4c) and were covered by a thin dermal tissue layer (Fig. 5.4a).

Upon reaching 2 months of age, the juveniles were 1.2 ± 0.3 mm long (Fig. 5.1). The first ramification of the tentacles occurred in three (out of five) dorsally oriented tentacles; whereas the two tentacles closest to the substrate retained non-ramified flattened tips (Fig. 5.2b and 5.3b). Concomitantly, there was an increase in tentacle length to 0.45 ± 0.2 mm. A third ambulacral podium also developed on the left side and anteriorly to the two primary podia (Fig. 5.1).

At 4 months, juveniles measured 1.5 ± 0.5 mm long, and started to grow a sixth tentacle (Fig. 5.1) between a non-ramified and a ramified tentacle on the ventral side (Fig. 5.2c). New tentacles always emerged in this manner and developed their first ramification ~2 weeks after their emergence (Fig. 5.2c). A second ramification developed from the main stalk among dorsally oriented tentacles (Fig. 5.3c), which had grown to 0.6 ± 0.3 mm long. A fourth ambulacral podium appeared on the right side (Fig. 5.1). Up to that age, the colour

of the body (RGB values of R: 205, G: 76, B: 35) as well as the feeding and cryptic behaviours followed those described for the 1-month-old juveniles.

When juveniles were 6 months old, they measured 1.8 ± 0.3 mm long and their body wall colour changed to semi-transparent light orange (Fig. 5.2d). These juveniles possessed 5 ramified dorsal tentacles (0.9 ± 0.5 mm long with 3 ramifications, Fig. 5.1 and 5.3d) and 2 non-ramified ventral tentacles. The number of ambulacral podia increased to a total of 5, with the fifth appearing on the left side. At that age, individuals presented an erect posture in relation to the substrate (i.e. $90°$ with the substrate) and tentacles were generally fully deployed upward in the water column (Fig. 5.2d). The ambulacral podia located posteriorly were responsible for most of the anchorage and the still non-ramified tentacles (located closer to the substrate) remained used solely to assist locomotion (Fig. 5.2d). The movement of the tentacles for feeding also changed. While tentacles of younger juveniles displayed a retraction motion bringing them close to the mouth, the tentacles of six-month-old juveniles bended in a semicircular movement before being inserted into the mouth in the typical fashion of adult dendrochirotid sea cucumbers (Fig. 5.2e). Juveniles showed tentacle insertion rates of 19 ± 3.4 tentacle min^{-1} (Fig. 5.1). Tentacles were inserted into the mouth without any consistent order and were rarely seen touching the substrate as documented in younger juveniles. The semi-transparent light-orange body colour (RGB values of R: 245, G: 234, B: 195) allowed the observation of food in the intestine for the first time as light brownish patches moving from the anterior to the posterior end of the juvenile (Fig. 5.2f) and defecation was noted (Fig. 5.2f). Although juveniles were still entirely covered with ossicles (Fig. 5.4d), the latter became oval, slightly curved and grew

to 190 ± 11 µm long and 7 ± 1.4 µm thick; the number of central holes also increased to 12 ± 1.2 per ossicle (Fig. 5.4e). Sharp knobs and denticles rimed the outer edge of the ossicles (Fig. 5.4e), which lost their porosity (Fig. 5.4f), although a thin layer of dermal tissue still covered them (Fig. 5.4d and 5.4e). Although the majority of the juveniles (~70%) still occurred underneath or attached to the sides of rocks, some of the largest individuals were observed on top of the rocks and in more illuminated areas.

At 8 months, juveniles were 2.4 ± 0.5 mm long and developed a yellowish colouration around the mouth (Fig. 5.2g), which covered ~20% of the body surface in ~30% of the juveniles. Individuals still displayed 5 ambulacral podia, 5 ramified dorsal tentacles and 2 non-ramified ventral tentacles. Dorsally oriented tentacles reached 1.1 ± 0.3 mm long and displayed a fourth ramification (Fig. 5.1 and 5.3e). There was still no sign of ramification of the two ventral tentacles closest to the substrate.

Twelve-month-old juveniles reached 3.6 ± 0.6 mm in length (Fig. 5.1, Table 5.2). The first dorsal podium appeared (Fig. 5.2h), and juveniles had a total of 8 tentacles and 7 ambulacral podia (Fig. 5.1 and 5.2h). Dorsal tentacles were 2.2 ± 0.4 mm long and had 5 ramifications (Fig. 5.3f). Although individuals were still entirely covered with ossicles, the latter increased in size to 210 ± 24 µm long and 12 ± 2.1 µm thick (Fig. 5.4g). Ossicles were still slightly curved and had 12 ± 2.4 holes; however the knobs and denticles at their outer edges were no longer sharp, but more rounded (Fig. 5.4h). Ossicles presented a denser structure (Fig. 5.4i), with a thicker layer of dermal tissue covering them (Fig. 5.4g). The majority of juveniles (~65%) were observed in more open areas, exposed to light and stronger water current, usually on the rocks or attached to the walls of the tank.

When juveniles were 16 months old, they measured 3.8 ± 0.8 mm long, possessed 8 ambulacral podia, 1 dorsal podium, and 10 fully-grown tentacles (Fig. 5.1). Eight of these tentacles were ramified, but the two tentacles closest to the substrate were not branched yet and were still used only to assist locomotion. Dorsal tentacles measured 2.8 ± 0.7 mm and showed 6 levels of ramifications (Fig. 5.3g). Skin pigmentation appeared between tentacles and presented a much darker shade, resembling the dark brownish colour of adults (Fig. 5.2i). The 16-month-old juveniles had a tentacle insertion rate of 16 ± 4.1 tentacle min^{-1} (Fig. 5.1).

At 21 months of age, juveniles measured 4.5 ± 0.8 mm long, had a light brownish colour (RGB values of R: 193, G: 143, B: 86) over the entire body (Fig. 5.2j), and 10 tentacles (Fig. 5.1, Table 5.2). Dorsal tentacles measured 3.1 ± 0.6 mm long and still had 6 ramifications. The tip of the 2 ventral tentacles (Fig. 5.2b) started to elongate and formed their first primary ramification (Fig. 5.3h), not simultaneously but ~5 days apart. Once ramified, these ventral tentacles lost their flattened adhesive tips and were no longer used for anchorage or locomotion (Fig. 5.3h). The tentacle insertion rate decreased to 14 ± 5.1 tentacle min^{-1}. Individuals showed a total of 9 ambulacral podia; 5 on the left side and 4 on the right side of the body (Fig. 5.1). The majority of juveniles were found on rocks in lighted areas, similarly to 12-month-old individuals.

The number of ambulacral podia increased proportionally with body length, following isometric scaling (Supplementary Material Fig. S.5.1). A negative allometric relationship was detected between body length and dorsal tentacle metrics, including tentacle length and number of tentacle ramifications, indicating that growth was

accompanied by a generally decreasing tentacle to body size ratio (Supplementary Material Fig. S.5.1).

5.4.2 Daily activity

Immediately after their introduction into the monitoring arena, juveniles started moving around over a period that varied across age classes (Fig. 5.5). In detail, the locomotor speeds 4 h post-release did not differ statistically among age classes and individuals moved at an average speed of ~3.1 body lengths h^{-1}. However, after 12 h post-release, 12-months old juveniles moved significantly faster (6.1 ± 0.7 body lengths h^{-1}) than 1 and 6-months old individuals (~3.5 body lengths h^{-1}; $F_{2,22} = 43.3$, $p < 0.001$; Fig. 5.5). At the end of the trial, 24 h post-release, no statistically difference was found among the locomotor speeds of juveniles of different ages (~2.7 body lengths h^{-1}) and their final position was always on a vertical surface, such as the walls of the tank or the sides of rocks. Juveniles had their tentacles deployed (i.e. in the feeding posture) day and night.

5.4.3 Response to stimuli

5.4.3.1 Response to light

Juveniles displayed increasing tolerance to light intensity with age (Table 5.2). The threshold condition above which a photonegative response was elicited in ≥50% of the juveniles was 25 lux in 1-month-old juveniles, 50 lux in 6-month-old juveniles, and 75 lux in individuals 12-21 months old (Fig. 5.6).

Specifically, 1-month-old juveniles exposed to light gradients with a maximum intensity of 25 lux did not display any clear phototactic response, moving both towards and

away from the light source with no consistent net distribution, similar to movements in dark (0 lux) conditions (Fig. 5.6). When exposed to a gradient with a peak intensity of 50, 75 or 100 lux, 65-91% of the juveniles moved away from the light source ($F_{4,23} = 38.7$, $p < 0.001$; Fig. 5.6).

Six-month-old juvenile exposed to either 0 or 25 lux moved in all directions with no clear net distribution pattern (Fig. 5.6). When exposed to 50 lux, 35% of the individuals moved to the sides of the vessels, while 48% moved away from the light source ($F_{4,36} = 44.9$, $p < 0.001$). When exposed to intensities of 75 and 100 lux, 72% of the individuals were seen moving away from the light source ($F_{4,12} = 22.4$, $p < 0.003$; Fig. 5.6).

Twelve-month-old juveniles moved indiscriminately towards or away from the light source when exposed to 0, 25, and 50 lux. However, a significant pattern was observed at intensities of 75 and 100 lux, where 65% of the juveniles moved away from the light ($F_{4,21} = 12.7$, $p < 0.011$; Fig. 5.6). Similarly, 21-month-old juveniles (75%) moved away from the light source when exposed to intensities of 75 and 100 lux.

5.4.3.2 Response to substrate type and background colour

Overall, juveniles showed strong substrate and background colour preferences that shifted slightly with age (Table 5.2). Pair-wise comparisons revealed that both 1 and 6-month-old juveniles favoured rocky substrates and black or red background colours. However, 12 and 21-month-old juveniles showed a preference for substrates of coralline algae and red background. Mussel shells and white backgrounds were the least preferred by all juveniles (Fig. 5.7 and 5.8). Statistical results are detailed below.

Specifically, the proportion of juveniles on rocks (~65%) was statistically higher than on coralline algae (~35%) for both 1-month ($t = 4.3$, df = 16, p < 0.001) and 6-month old juveniles ($t = 5.7$, df = 16, p < 0.001; Fig. 5.7). Their proportions on rocks (~82 and ~70%, respectively) were still significantly higher than on mussel shells (~18 and ~30%; $t = 4.3$, df = 16, p < 0.001 and $t = 3.3$, df = 16, p < 0.001, respectively). Moreover, the proportion of juveniles on coralline algae (~65%) was statistically higher than on mussel shells for both 1-month ($t = 5.2$, df = 16, p < 0.001) and 6-month old individuals ($t = 4.8$, df = 16, p < 0.001; Fig. 5.7).

The proportion of 12-month-old juveniles on coralline algae (58-75%) was significantly higher than on rocks (~42%; $t = 5.6$, df = 16, p < 0.001) and mussel shells (~25%; $t = 2.1$, df = 16, p < 0.001). Moreover, the proportion of 12-months-old juveniles on rocks was statistically higher than on mussel shells ($t = 2.0$, df = 16, p < 0.001; Fig. 5.7). Similarly, 21-month-old juveniles favoured coralline algae, on which 75% of the juveniles were found. The remaining juveniles opted for rock substrates and none of them were found on mussel shells.

When background colours were opposed, no significant difference was found in the proportion of 1 and 6-month-old juveniles on black (60%) vs red (40%) backgrounds (Fig. 5.8). However, there were statistically higher proportions of 1 and 6-month-old individuals on black (100% and ~82%; $t = 0.54$, df = 16, p < 0.001 and $t = 2.3$, df = 16, p < 0.001, respectively) and red (100% and 75%; $t = 0.79$, df = 16, p < 0.001 and $t = 3.3$, df = 16, p < 0.001, respectively) than on white (0-25%), background for both age classes.

The proportion of 12-month-old juveniles on red backgrounds (~65%) was statistically higher than on black (35%) and white (30%) colours ($t = 5.2$, df = 16, $p < 0.005$ and $t = 4.4$, df = 16, $p < 0.002$, respectively). When comparing black vs white backgrounds, the proportion of juveniles on the black (65%) was significantly higher than on the white (35%; $t = 3.5$, df = 16, $p < 0.001$; Fig. 5.8). When 21-month-old juveniles were given the choice between red or black, the majority (75%) was also found on the red background. Control treatments conducted during all trials showed no significant difference in the proportion of individuals between the two bare/uncoloured halves of the experimental vessels.

5.4.3.3 Response to current

Overall, there was an increase in tolerance to flow with age (Table 5.2). A significant difference was found in the proportion of 1-month-old juveniles with tentacles extended among current speeds tested (H = 22.4, df = 3, $p < 0.001$). Under flows of 0 and 5 cm s^{-1}, all individuals (100%) had their tentacles extended at all times (Fig. 5.9). However, at flow of 10 cm s^{-1}, this proportion decreased significantly to $65 \pm 9\%$ after 6 h. At 20 cm s^{-1}, another significant decreased was observed in tentacles extended ($45 \pm 7\%$) when compared to 10 cm s^{-1} (Fig. 5.9).

The proportions of 6 and 12-month-old juveniles with tentacles extended after 6 h of exposure to currents of 0 ($86 \pm 8\%$ and $80 \pm 9\%$, respectively), 5 ($100 \pm 0\%$ and $86 \pm 11\%$), and 10 cm s^{-1} ($100 \pm 0\%$ and $86 \pm 7\%$) were not significantly different (Fig. 5.9). However, at 20 cm s^{-1}, the proportion of juveniles with tentacles extended was significantly lower ($46 \pm 12\%$ and $53 \pm 7\%$, respectively) than at 10 cm s^{-1} (H = 38.3, df = 3, $p < 0.001$,

Fig. 5.9). Similarly, 100% of 21-month-old juveniles had their tentacles extended at flow of 10 cm s^{-1}; however, only 50% extended their tentacles when exposed to a flow of 20 cm s^{-1}.

5.5 Discussion

One of the most striking morphological changes over the first 21 months of benthic life in *C. frondosa* was the asynchronous growth and ramification of dorsal and ventral tentacles. Primary tentacles of newly-settled recruits were unramified and visually similar to ambulacral podia; both displaying flattened and adhesive tips, which were used for anchorage and locomotion. A high number of anchoring appendages (combining podia and tentacles) may help stabilize recruits and early juveniles in high-energy environments. While field records of recruits and juveniles are rare; one study found small individuals (35 to 130 mm) of *C. frondosa* on hard substrates in areas of high turbulence at shallow depths ranging from the lower intertidal area to 20 m (Hamel and Mercier, 1996). As seen here, primary tentacles with an appearance and function similar to ambulacral podia have been noted in newly-settled juveniles of other suspension-feeding sea cucumbers, *P. chitonoides* and *P. bullatum* (McEuen and Chia, 1991), as well as juveniles of the deposit-feeding species *A. japonicus* (Hu *et al.*, 2010; Qiu *et al.*, 2015). When not used to attach to the substrate, primary tentacles of *C. frondosa* were constantly sweeping the substrate to collect deposited materials, which contrasts with older juveniles and adults that display a strict suspension-feeding behaviour (Hamel and Mercier, 1996). This finding indicates that

deposits on the substrate may, unexpectedly, be an important source of food for early recruits of dendrochirotid sea cucumbers.

The ramification of tentacles was a slow and gradual process that started in tentacles located on the dorsal side of the oral cavity, which probably play the greatest role in capturing suspended particles. Concurrently with tentacle development, the addition of ambulacral podia starting from 6 months of age gave more anchoring strength and stability to the juveniles so that they could adopt an erect posture, which facilitated the deployment of the ramified dorsal tentacles in the water column. Tentacles located on the ventral side remained podia-like until all 10 oral tentacles had developed after 16 months. Ventral tentacles lost their role in attachment (and started ramifying and being used in feeding) only after 21 months, when individuals had already developed 9 ambulacral podia and were much more difficult to detach mechanically from the substrate.

The transition from feeding on benthic matters to feeding exclusively on suspended particles in *C. frondosa* was also accompanied by a shift in the skin pigmentation of juveniles. New recruits showed a uniform dark reddish pigmentation on the entire body surface, similar to the colour of unspawned oocytes and developing embryos/larvae. The yolky oocytes of *C. frondosa* are mainly composed of sterols (Verkaik *et al.*, 2016), and they develop into non-feeding (lecithotrophic) larvae (Montgomery *et al.*, 2017). The colour of early juveniles (1 to 6 months old) presumably reflects the inheritance of nutrients from the egg/larva, underlying the importance of energy stored during vitellogenesis, not only for metamorphosis and settlement, but also for early juveniles with a limited ability to capture food. Indeed, until 6 months of age, juveniles were not able to insert their tentacles

fully into the mouth due to their small size, suggesting lower feeding efficiency than older juveniles that exhibited the tentacle insertion behaviour of adults. Concomitant with the adoption of the typical feeding posture and strategy, the reddish colouration faded, turning into light orange after 6 months and light brown after 21 months, presumably reflecting pigment uptake from phytoplankton, which constitutes the main diet of *C. frondosa* (Hamel and Mercier, 1998). Similar colour polymorphism as a result of diet composition has been documented in sea stars (Harley *et al.*, 2006), lobsters (Chandrapavan *et al.*, 2009), and fishes (Mora *et al.*, 2006). Despite the apparent link between skin pigments and the start of exogenous feeding, intrinsic genetic control of colouration cannot be excluded, as discussed by Zhao (2015) in *A. japonicus*. The appearance of brown pigments in *C. frondosa* might be evidence of early melanin production in the tegument of juveniles (Xing *et al.*, 2017), which also coincided with the emergence of juveniles from under-surfaces or crevices, and consequent greater exposure to light (discussed below). Melanin provides protection against ultraviolet radiation (Nielsen *et al.*, 2006) and is known to be obtained in part through a diet of plankton (Tartarotti *et al.*, 2001).

Experimental trials showed that juveniles of *C. frondosa* in all age classes were very responsive to environmental parameters. Newly-settled juveniles were highly photonegative and moved away from even the faintest light source, which is consistent with the distribution of small individuals of *C. frondosa* in crevices and underneath rocks (Hamel and Mercier, 1996). Similar preferences for shaded and sheltered habitats were also documented in field surveys of small juveniles of the temperate sea cucumber *P. chitonoides* found in rock crevices (Young and Chia, 1982) but different from studies of

H. scabra where recruits and early juveniles occurred directly on sea grass leaves, generally in areas exposed to high light intensity (Mercier *et al.*, 2000a). Light levels can be perceived by the nerve system in the body wall (Motokawa, 1984) and this cue might be used in part as guidance for juveniles from different age classes to locate adequate habitats. The present study showed that 1-month-old juveniles tolerated only low light levels to 25 lux, indicative of sheltered areas, whereas 6-month-olds coped with 50 lux (i.e. exposed surfaces) and juveniles 12 months of age and beyond tolerated intensities up to 75 lux. The migration of juveniles from dark/sheltered to increasingly illuminated areas coincided with the acquisition of the planktonic feeding strategy as individuals developed more tentacles and ambulacral podia. Sensory cells located on the body wall and tentacles of juvenile sea cucumbers may play a role as chemo and mechanoreceptors, allowing them to analyse environmental stimuli (including light intensity) and select habitats with appropriate conditions (Cameron and Fankboner, 1984).

Another key ontogenetic pattern was a shift in the preference of juveniles for substrate type and background colour. Newly-settled recruits of *C. frondosa* favoured plain rocky substrates, consistent with previous laboratory experiments where larvae of *C. frondosa* settled preferentially on rocks (Hamel and Mercier, 1996). Rocks might provide a smooth and even surface for optimal adhesion of the limited number of ambulacral podia and primary tentacles found in recruits. At this early age, juveniles of *C. frondosa* also preferred dark backgrounds in shades of black or red, suggesting that not only the composition of the substrate, but also its colour might influence the distribution of early life stages, consistent with cryptic behaviour documented in a variety of sea cucumber

juveniles, which is often considered an anti-predator adaption (Shiell and Knott, 2008). As juveniles of *C. frondosa* aged, substrate and background colour preferences shifted to coralline algae and red backgrounds, respectively. Coralline algae are pink-red and grow on exposed hard surfaces where there is more light for photosynthesis and stronger water flow. Recently, sea stars were shown to have the capacity to use visual navigation (Garm and Nilsson, 2014), and this capacity may extend to sea cucumbers. Chemical cues could also be involved, since coralline algae are covered by specific biofilms composed of bacteria, fungi, and single-celled algae (Johnson *et al.*, 1991). It has been suggested that papillae at the tip of ambulacral podia and tentacles are involved in the sensory ability of sea cucumber juveniles to discriminate food items as well as substrate types (McEuen and Chia, 1991). Overall, coralline algae may be an indicator of optimal habitats where adults of *C. frondosa* abound, i.e. exposed, lighted areas, with medium current (Cotté and Simard, 2005). This view supports the idea that a tactile response is involved in selecting the type of substrate; whereas the background colour might be detected through light reflecting off the substrate towards sensory cells on the body wall of the juveniles. This result is also consistent with reports of *C. frondosa* juveniles (1-10 mm) on coralline algae in the Gulf of Maine (Medeiros-Bergen and Miles, 1997). A shift in substrate preference with age has previously been documented in the deposit-feeding sea cucumber *H. scabra*, whereby newly-settled juveniles were attached to seagrass leaves, whereas older juveniles preferred sandy substrates with medium-sized grains ~0.4 mm and organically-rich sediments (Mercier *et al.*, 1999, 2000b).

Monitoring of *C. frondosa* juveniles revealed that they keep their tentacles extended 24 h a day and may thus be feeding at all times. This finding contrasts with the feeding patterns of *C. frondosa* adults in the Gulf of St. Lawrence and the Bay of Fundy (eastern Canada), which showed a defined seasonality and tidal influence. They were seen with tentacles deployed and feeding during spring and summer months, but ceased feeding in winter (Singh *et al.*, 1999), and they fed mainly during changing tides (Hamel and Mercier, 1998). This cyclic feeding behaviour of *C. frondosa* adults was strongly related to the plankton concentration in the water column (Hamel and Mercier, 1998; Singh *et al.*, 1999); whereas juveniles might feed at all times to maximize food intake and, consequently, growth rates when water current allows the full extension of the tentacles. The continuous feeding of *C. frondosa* juveniles also contrasts with the rhythmic deposit-feeding and burrowing patterns found in holothuriid (aspidochirotid) sea cucumbers. For instance, *H. scabra* juveniles forage only at night and seek shelter during the day, in response to light levels and temperature (Mercier *et al.*, 1999). Importantly, dendrochirotids do not need to move in order to feed, like holothuriids do when ingesting sediment; instead their movement may help them find optimal areas for suspension feeding, or enable them to escape from a predator. Here, 21-month-old juveniles of *C. frondosa* moved at a faster pace than 1 and 6 months-old individuals, likely aided by a greater number of ambulacral podia. Juveniles of all age classes settled on vertical surfaces within 24 h and displayed minimal to no movement thereafter.

From very early on, juveniles of *C. frondosa* had ossicles covering the entire body wall, which might offer some protection from predation and other environmental factors.

Hamel and Mercier (1996) hypothesized that small juveniles of *C. frondosa* could be preyed upon by the sea star *Solaster endeca* and the green sea urchin *Strongylocentrotus droebachiensis*. This assumption was partially confirmed by laboratory experiments showing predation of young juveniles of *C. frondosa* by *S. endeca* (So *et al.*, 2010). Ossicles covering the entire body wall surface have also been detected in pentactula larvae of *Stichopus* sp. (Hu *et al.*, 2010) and settled juveniles of *P. chitonoides* and *H. scabra* (McEuen and Chia, 1991; Massin *et al.*, 2000). However, the putative importance of ossicles as a mechanism of defense in sea cucumber juveniles is still unclear. In older juveniles and adults, the density of ossicles on the body wall generally decreases, coinciding with greater mobility and more efficient escape responses (Legault and Himmelman, 1993; So *et al.*, 2010). The proportionally greater mineralization of juveniles may alternatively or additionally provide enhanced protection against UV rays, and fluctuations in light intensity, temperature, and salinity. In juveniles of sea stars, ossicles were shown to protect sensory cells against abrasion (Blake, 1983).

The growth of *C. frondosa* juveniles monitored in the present study was consistent with the trend previously obtained by So *et al.* (2010) under similar rearing conditions. However, these rates were slower than those reported from the St. Lawrence Estuary (eastern Canada), where juveniles reached a maximum length of 35 mm in 25 months, with an average monthly growth rate of ~1.5 mm (Hamel and Mercier, 1996). Despite similar laboratory setups, this growth rate is almost 10 times faster than both the present study and that of So *et al.* (2010). The discrepancy is most likely due to the higher food concentrations naturally available in the St. Lawrence (Plourde and Runge, 1993). In support of the

importance of food, a recent study showed that the growth of *C. frondosa* (~10 cm long contracted body) can be 5 times faster when a lipid-rich diet is provided regularly (Gianasi *et al.*, 2016).

The relationship between body length and feeding tentacle metrics is consistent with allometric scaling patterns in early juveniles of *C. frondosa*. In particular, there was negative allometry between body length and both the length and number of ramifications in the dorsal tentacles. However, the relationship between body length and number of ambulacral podia in juveniles of *C. frondosa* followed isometric scaling, i.e. a proportional increase in body length and number of podia.

The early benthic phase is among the most susceptible to mortality inside sea cucumber aquaculture programs, often on account of limited biological and ecological knowledge about this key life stage, as exemplified by struggles reported with the grow-out of *H. scabra* (Becker *et al.*, 2004; Lavitra *et al.*, 2009; Purcell *et al.*, 2012). Basic knowledge of the requirements of recruits and early juveniles (often referred to as seedlings) as well as their response to various stimuli, emerges as a crucial advantage in developing successful programs. Case in point: the cost-effective farming of *A. japonicus*, which had a total value of ~4 billion USD in 2015 (Zhang *et al.*, 2015). The present study offers the first characterization of morphological and behavioural development of early juvenile stages in *Cucumaria frondosa*, and in a temperate-cold dendrochirotid sea cucumber, providing preliminary but useful means of optimizing captive-breeding initiatives. Juveniles underwent important morphological and behavioural shifts as they aged. In the first months, they displayed few tentacles and ambulacral podia and showed a

preference for shaded areas with low current speed and dark substrate mainly composed of rocks. As they aged, they developed more tentacles and podia, no longer fled illuminated areas, tolerated stronger water flow, and preferred red substrates and coralline algae. This transition indicates that juveniles of *C. frondosa* evolve markedly different environmental requirements over the first two years of their life. Therefore, proper conditions of light intensity, substrate, background colour and current strength need to be provided in rearing tanks to optimize feeding, growth and development, and minimize stress and mortality. Moreover, juveniles of *C. frondosa* were shown to have the capacity to feed 24 h a day, which constitutes an advantage for aquaculture production. Further study is needed to investigate how growth and development of juveniles may be maximized through the manipulation of diet composition, water flow and water temperature.

5.6 Acknowledgements

5.7 References

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5.8 Tables

Table 5.1: Overview of parameters measured in juveniles of the sea cucumber *Cucumaria frondosa*. Morphometrics and tentacle retraction/insertion rates were measured monthly over 21 months, whereas behavioural responses were assessed in 1, 6, 12, and 21-month-old juveniles.

Study	Parameters measured	Description
Morphometrics	Body length	Measurement of the length of juveniles when anchored with tentacles deployed, taken on the dorsal side from the base of an oral tentacle to the anus.
	Number of tentacles	Number of tentacles around the buccal cavity.
	Tentacle stalk size	Length of the two longest oral tentacles positioned on the dorsal side of the juvenile, measured from the base of the tentacle in the buccal cavity to its apex when fully deployed in the water column.
	Number of primary tentacle ramifications	Number of primary lateral ramifications developing from the principal stalk, counted in the two longest oral tentacles positioned on the dorsal side of the juvenile.
	Number of ventral and dorsal ambulacral podia	Number of ambulacral podia used for attachment on the ventral and dorsal sides of the juveniles.
	Skin pigmentation	Natural colourings detected on the juveniles, including proportion of body wall occupied by each.
	Ultrastructure of the body wall	Changes in shape of dermal ossicles of the body wall.
Behaviour	Retraction/insertion rates	Number of tentacles inserted in the mouth in a given amount of time during feeding.
	Daily activity	Shifts in locomotor speed, position, and number of juveniles with tentacles deployed over 24 h, under optimal conditions of light, substrate, background colour, and water current.
Response to stimuli	Light intensity	Number of juveniles moving either towards or away from a light source. Light gradients were created with maximum intensities of 0, 25, 50, 75, and 100 lux on one side of the experimental vessels.
	Substrate	Number of juveniles on each of two paired substrates, among fragments of coralline algae, mussel shells, and rocks.
	Background colour	Number of juveniles on each of two paired background colours, among black, white, and red.
	Current strength	Proportion of juveniles with tentacles fully deployed under water currents of 0, 5, 10, and 20 cm s^{-1}.

Table 5.2: Summary of morphometrics and behaviours of juveniles of the sea cucumber *Cucumaria frondosa* at different ages.

Age (month)	Body length (mm)	Number of tentacles and ramifications	Number of ventral podia	Skin pigmentation	Ossicle structure	Light (lux)	Substrate preference	Background colour preference	Current (cm s⁻¹)
1	0.8 ± 0.3	5 non-ramified tentacles.	2	Uniform dark reddish colour on the entire body surface.	Flat plates without knobs. Ossicles with small cavities.	25	Rocks	Black and red	5
2	1.2 ± 0.3	Dorsally oriented tentacles with 1 ramification.	3						
4	1.5 ± 0.5	Total of 6 tentacles. Tentacles with 2 ramifications.	4						
6	1.8 ± 0.3	Total of 7 tentacles. Dorsally oriented tentacles with 3 ramifications	5	Semi-transparent light orange colour on the entire body surface.	Slightly curved with sharp knobs. No cavities on ossicles.	50	Rocks	Black and red	10
8	2.4 ± 0.5	Tentacles with 4 ramifications	5	Yellowish colouring appears around oral cavity and covers 20% of body surface.					
12	3.6 ± 0.6	Total of 8 tentacles. Dorsally oriented tentacles with 5 ramifications.	7		Rounded knobs at the edges. No cavities on ossicles.	75	Coralline algae	Red	10
16	3.8 ± 0.8	Total of 10 tentacles. Dorsal oriented tentacles with 6 ramifications.	8	Dark brownish colouring appears between tentacles.					
20	4.6 ± 0.9	First ramification of ventrally oriented tentacles.	8						
21	4.5 ± 0.8	10 ramified tentacles	9	Light brownish colour on the entire body surface.		75	Coralline algae	Red	10

5.9 Figures

Age (months)

Figure 5.1 (previous page): Monthly morphometric measurements of body length, number and length of dorsally oriented tentacles, number of tentacle ramifications, number of ambulacral podia, and tentacle retraction/insertion rate in juveniles of the sea cucumber *Cucumaria frondosa* from 1 to 21 months old. Data shown as mean ± sd (n = 30).

Figure 5.2 (previous page): Morphological development of juveniles of the sea cucumber *Cucumaria frondosa* from 1 to 21 months old. (A) One month-old juvenile showing primary tentacles (t) and ambulacral podia (ap). (B) Illustration of 3 ramified dorsally-oriented tentacles (dt) used for feeding and 2 non-ramified ventrally oriented tentacles (vt) used for both feeding and attachment. (C) Growth of a new tentacle (nt) occurred between ramified tentacles (t) and a non-ramified and ventrally oriented tentacle (vt). (D) Six-month-old juvenile displaying 7 tentacles (t) and 5 ambulacral podia (ap). Juveniles displayed an erect posture with posterior ambulacral podia (ap) ensuring anchorage. (E) Tentacles of six-month old juveniles are bent in a semicircular movement (arrow) and inserted into the month (mo) one at a time. (F) Six-month-old juveniles with organic particles in the tentacles (t). Ingested food (fo) is visible in the intestinal tract through the semitransparent body wall and juvenile is seen defecating (fc). (G) A yellowish skin pigmentation (pg) appeared around the oral cavity of eight-month-old juveniles. (H) The first dorsal podium is visible on the dorsal side of twelve-month-old juveniles. (I) Dark brownish skin pigmentation (pg) appeared between tentacles of sixteen-month-old juveniles. (J) twenty-one-month-old juvenile displaying 10 ramified tentacles (t) and 9 ambulacral podia (ap). Scale bar presents 0.5 mm.

Figure 5.3 (previous page): Tentacle ramifications in juveniles of the sea cucumber *Cucumaria frondosa*. (A) Unramified primary tentacles are present in one-month-old juveniles. (B) The first ramification of dorsal tentacle appeared when juveniles were two months-old. (C) The second ramification of dorsal tentacle became visible when juveniles were four months old. (D) Dorsal tentacles with 3 ramifications were present in six-month-old juveniles. (E) The development of the fourth ramification on the dorsal tentacles occurred when juveniles reached eight months of age. (F) Twelve-month-old juveniles showed 5 ramifications on dorsal tentacles. (G) Six ramifications were present on dorsal tentacles of sixteen-month-old individuals. (H) Ventrally oriented tentacles (vt) only started branching out when juveniles were twenty-one-month-old. Arrows indicate the number of ramifications in the tentacles. Scale bars present 0.5 mm.

Figure 5.4 (previous page): Scanning electron microscopy of the body wall of 1, 6, and 12-month-old juveniles of *Cucumaria frondosa*. (A) One-month-old juvenile showing fenestrated ossicles covered with very thin dermal tissue (arrow). (B) Ossicle of one-month-old juveniles are flat, without either knobs or denticles on the inner and outer surface. (C) Small holes and cavities (arrows) are present in ossicles of one-month-old-juveniles. (D) Six-month-old juveniles displaying large fenestrated ossicles covered by a thin layer of dermal tissue (arrow). (E) Sharp knobs and denticles present in the outer edge of the ossicles of six-month-old juveniles (arrows). (F) Ossicles of six-month-old juveniles displayed a solid structure without cavities (arrows). (G) Twelve-month-old juveniles armoured with large ossicle covered by a thick layer of dermal tissue (arrows). (H) Knobs and denticles on the outer surface of the ossicles showed rounded edges (arrows) of twelve-month-old juveniles. (I) Ossicles presented a solid structure without cavities (arrows) of twelve-month-old individuals. Scale bar represents 50 µm.

Figure 5.5: Locomotor speed of juveniles of the sea cucumber *Cucumaria frondosa* during

a period of 24 h. Data shown as mean ± se (n = 3).

Light intensity (lux)

| | 0 | 25 | 50 | 75 | 100 |

Age (months): 1, 6, 12

⬆ **Light source**

201
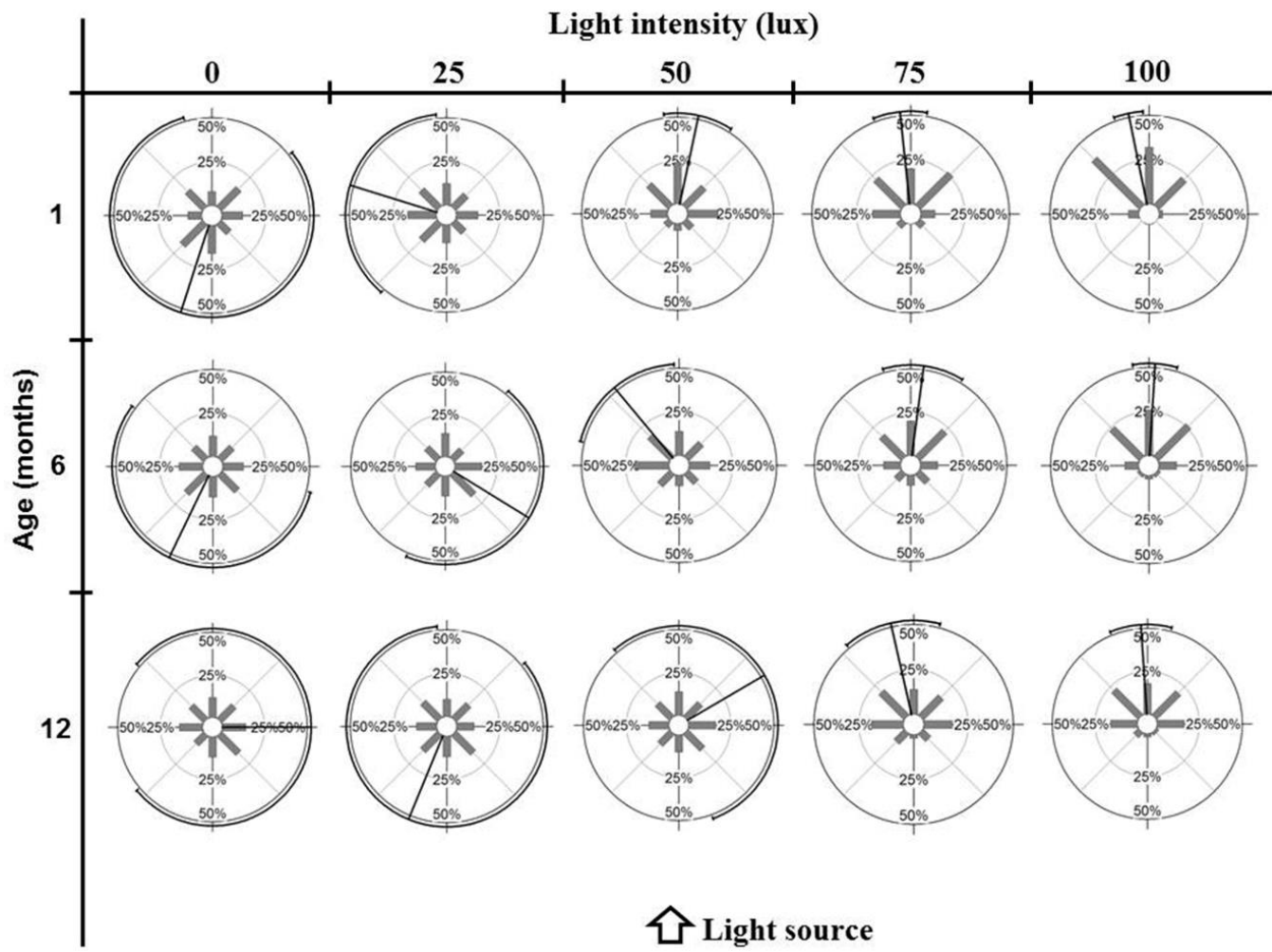
Figure 5.6 (previous page): Mean position of 1, 6, and 12-month-old juveniles of the sea cucumber *Cucumaria frondosa* exposed to light sources ranging from 0 to 100 lux. The light source was positioned South of the experimental Petri dishes. Bars inside the circles represent the proportion of juveniles in each position. The black line running from the center of the diagram to the outer edge represents the overall average position of juveniles with the lines outside the circles delimiting the standard deviation ellipse (n = 3).

Substrate

Rock x Coralline algae   Rock x Mussel shell   Mussel shell x Coralline algae

Age (months): 1, 6, 12

Proportion of juveniles (%)

Time (h)

☐ Middle   ■ Rock   ▨ Coralline algae   ▨ Mussel shell

203
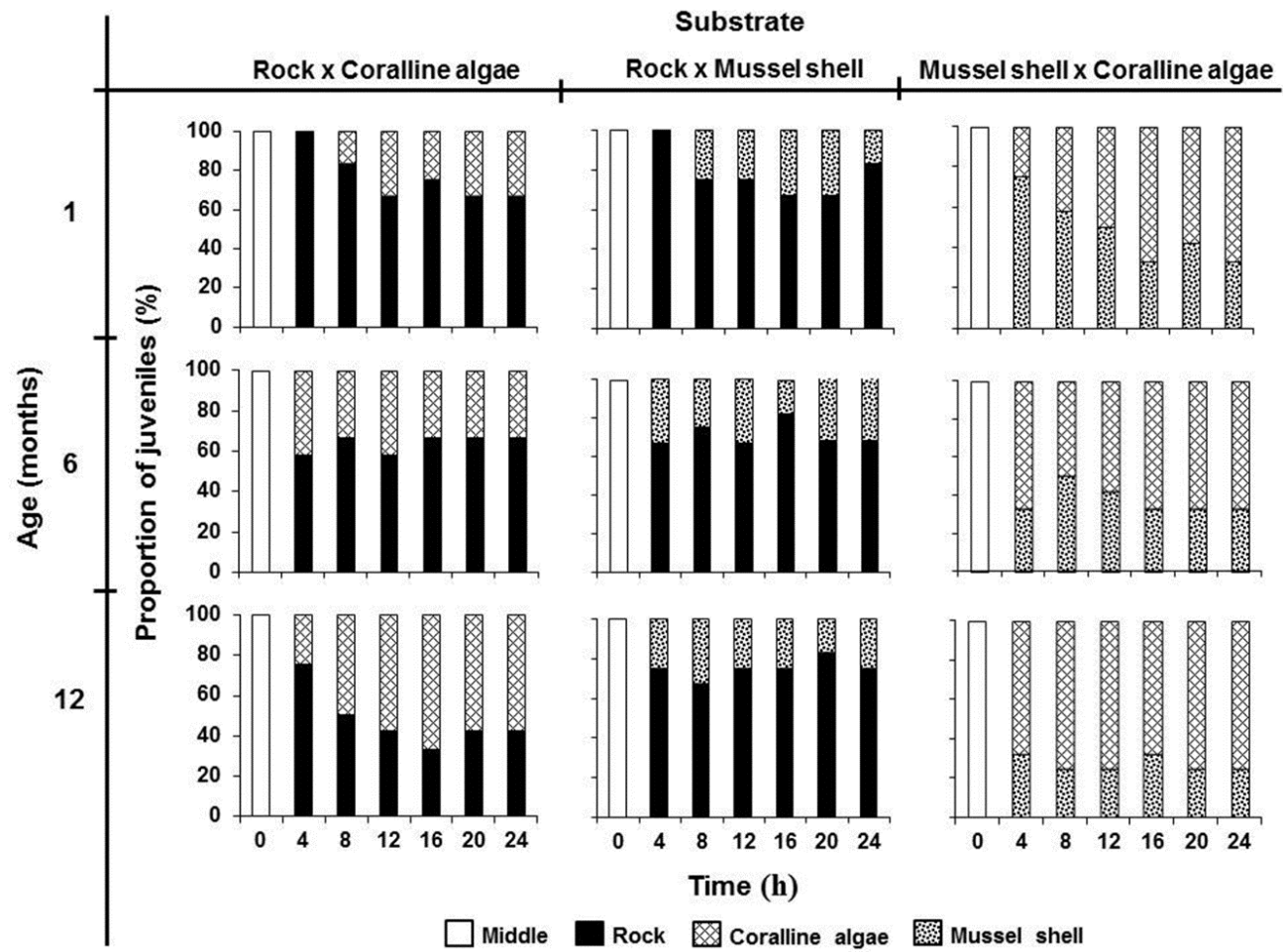
Figure 5.7 (previous page): Proportion of 1, 6, and 12-month-old juveniles of the sea cucumber *Cucumaria frondosa* found on coralline algae, mussel shells, and rocks during 24-h pair-wise tests of substrate selection. Juveniles were only seen between the two substrates (in the middle) at time 0.
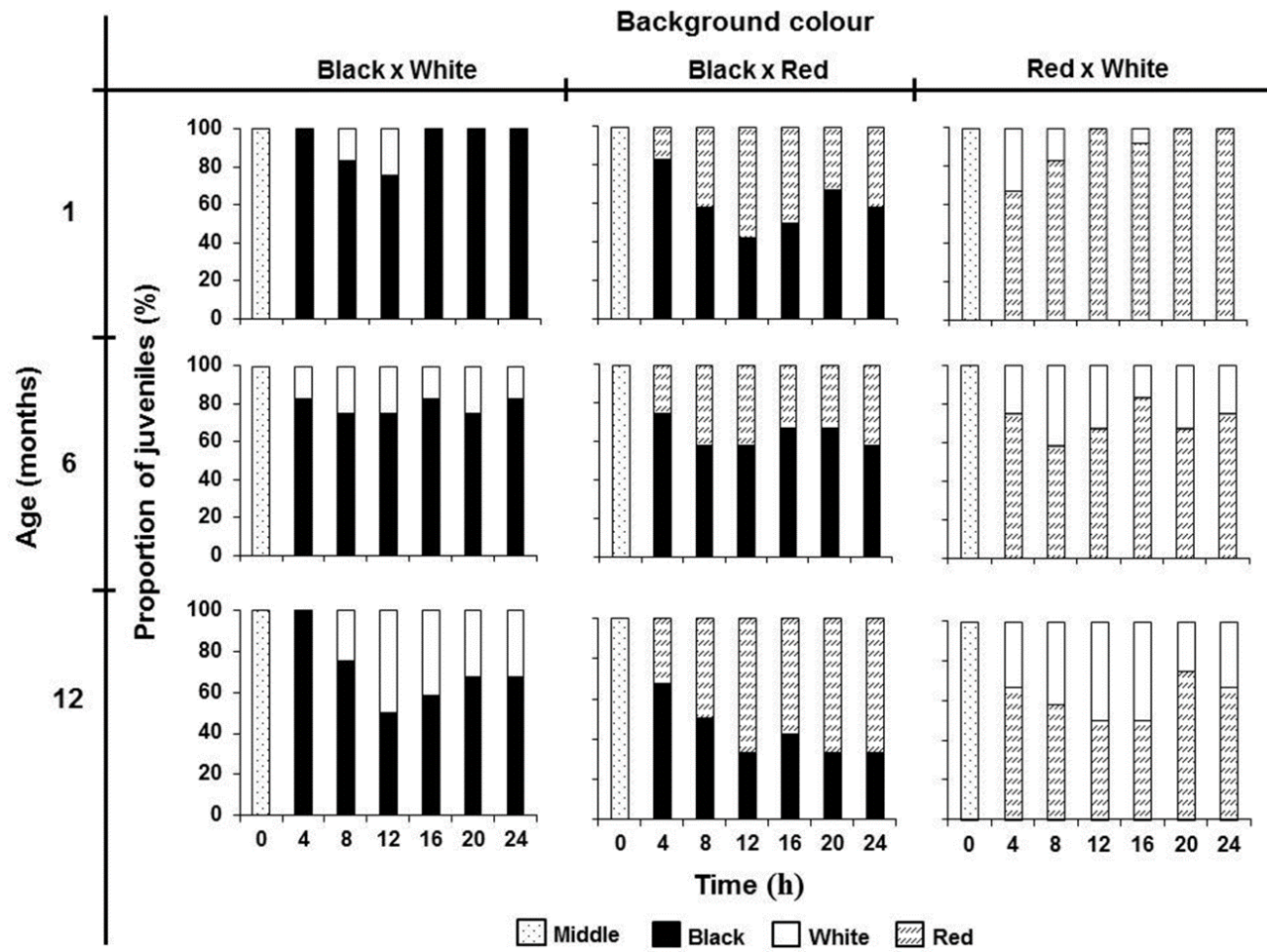
Figure 5.8 (previous page): Proportion of 1, 6, and 12-month-old juveniles of the sea cucumber *Cucumaria frondosa* found on black, red, and white background during 24-h pair-wise tests of background colour selection. Juveniles were only seen between the two colours (in the middle) at time 0.

Current speed (cm s^{-1})

Age (months)

Proportion of juveniles (%)

Time (h)

■ Tentacles extended □ Tentacle retracted

207
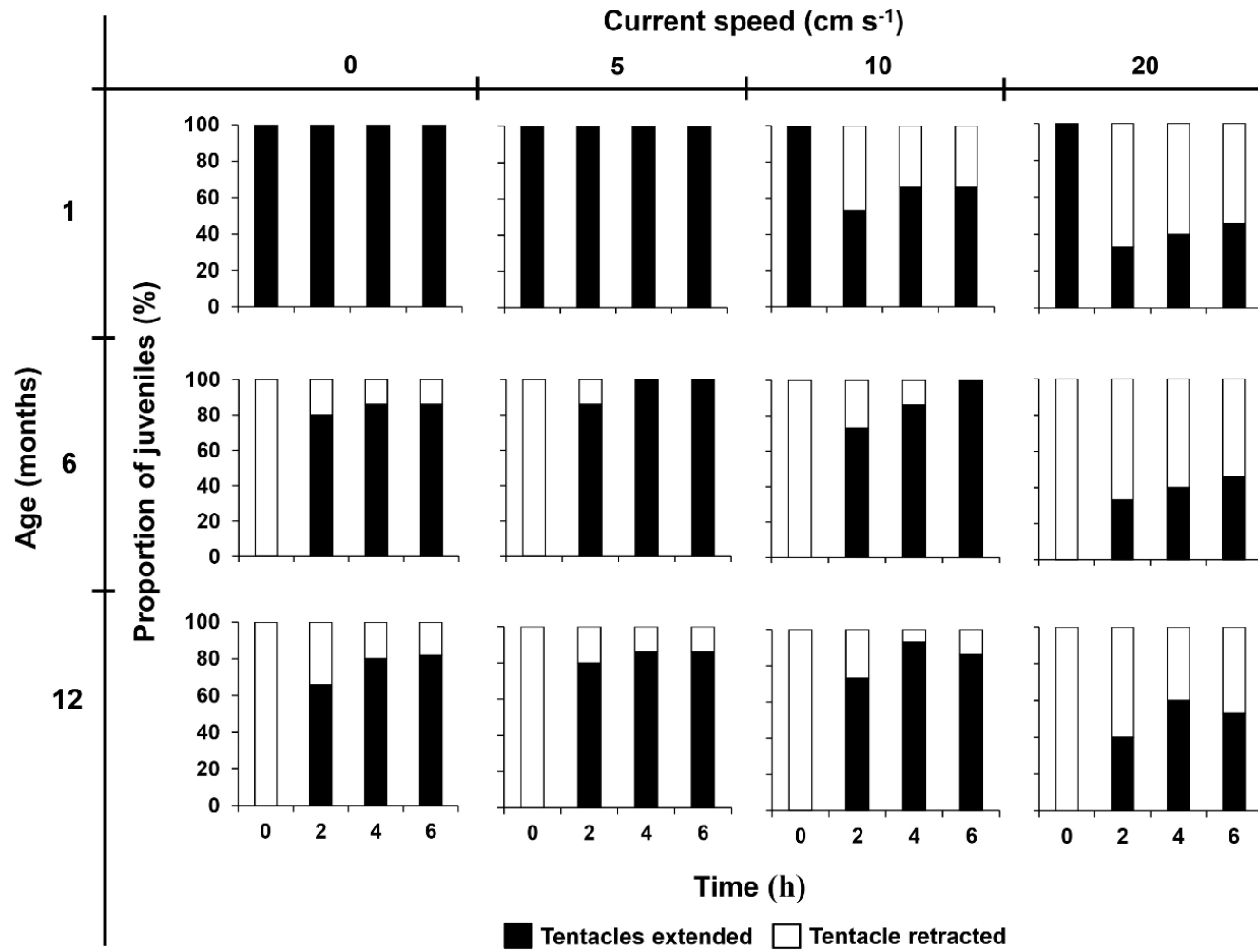
Figure 5.9 (previous page): Proportion of 1, 6, and 12-month-old juveniles of the sea cucumber *Cucumaria frondosa* with tentacles either extended or retracted when exposed to current speeds of 0, 5, 10, and 20 cm s^{-1} for a total of 6 h.

5.10 Supplementary Material
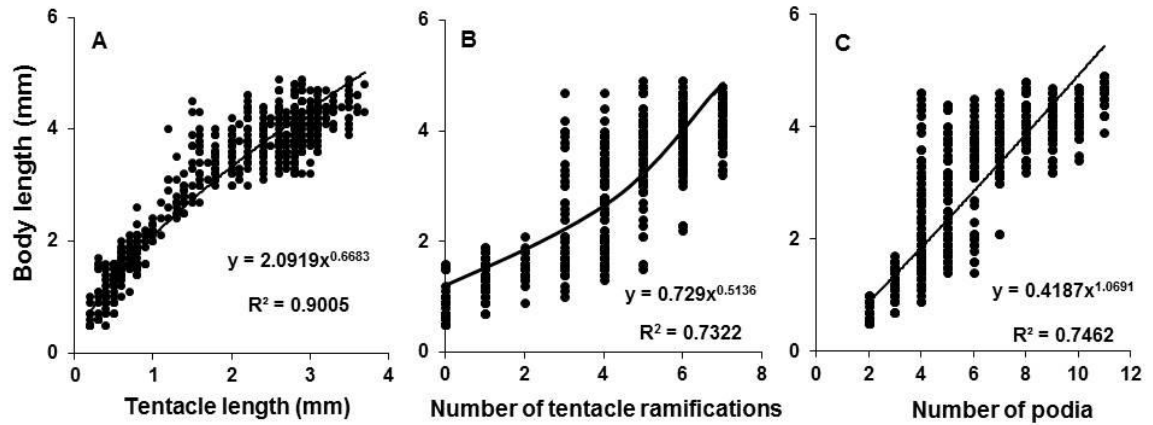
Figure S.5.1:Relationship between body length and (A) dorsal tentacle length, (B) number

of ramifications in dorsal tentacles, and (C) number of ambulacral podia.

Chapter 6. General Conclusions

6.1 Summary

Sea cucumber aquaculture is developing quickly around the word in order to supply the increasing demand for this luxury seafood in Asian countries. Many aquaculture initiatives have primarily focused on tropical/temperate deposit-feeding sea cucumbers such as *Holothuria scabra*, *Isostichopus fuscus* and *Apostichopus japonicus* (Battaglene *et al.*, 2002; Purcell *et al.*, 2012; Mercier and Hamel, 2013; Yang *et al.*, 2015). In recent years, interest has grown for boreal species such as *Cucumaria frondosa* (Hamel and Mercier, 2008; Nelson *et al.*, 2012). Unlike most commonly studied sea cucumbers, *C. frondosa* colonizes cold-water environments; it is a passive suspension feeder that captures food particles from the water column (Singh *et al.*, 1998) and produces large maternally-provisioned oocytes, which develop into lecithotrophic (non-feeding) larvae (Hamel and Mercier, 1996a). Considering the emerging focus on its potential as an aquaculture candidate, knowledge of the reproductive cycle, spawning, larval and juvenile development of *C. frondosa* in captivity must be collected to provide a solid foundation for the development of culture protocols. This study provides a comprehensive coverage on a broad range of topics relevant to the prospective aquaculture of *C. frondosa* in Newfoundland (eastern Canada); it begins with the conditioning of broodstock and optimization of gamete quality, followed by the development of spawning techniques, embryonic and larval development leading to the formation of chimaeras; and it concludes with a long-term study of the morphology and behaviour of juveniles over 21 months post settlement.

Chapter 2 explored aspects of broodstock conditioning by investigating how different water temperatures, photoperiods, and food concentrations influence gametogenesis, spawning, quality of gametes, and embryo survival rates in captivity. Males developed mature gonads across all treatments. However, among all environmental conditions tested, females exposed to ambient water temperature (~0°C), photoperiod (8-13 h light), and food supply (3 x 10^3 rotifers g^{-1} of sea cucumbers d^{-1}) showed the highest gonad indices prior to spawning, with ovaries becoming filled with large mature oocytes. These treatments also produced the highest proportions of spawning females, number of eggs released, and the best results for egg quality and embryo survival. Females exposed to 4-month advanced photoperiod (13-15 h light) also exhibited appropriate gonad maturation; however, the proportion of spawning females, the number of eggs released, and the survival of embryos were lower. Warmer water temperature (6 and 12°C) under ambient photoperiod resulted in delayed oogenesis and impeded spawning. Non-ambient photoperiods (24-h dark or light) also delayed oogenesis, resulting in low proportions of spawning females and poor survival of embryos. Females fed with a food concentration that was twice the ambient level developed mature ovaries and exhibited proportions of spawning females, number of egg released, egg quality and embryo survival similar to ambient controls. These findings indicate that the reproductive output of *C. frondosa* is optimized under environmental conditions that prevail during the normal gametogenic period (winter-spring), and that enhancement of gamete production using warmer water temperatures or advanced photoperiod may not be suitable in cold-water and/or lecithotrophic sea cucumber species. Moreover, the increase in food supply did not enhance

the reproductive output of *C. frondosa*, suggesting that natural winter-spring food concentrations might be abundant enough for maintenance of metabolism and production of gametes during oogenesis.

Chapter 3 explored methods that might be used to trigger spawning or artificially induce final oocyte maturation in *C. frondosa*. Live phytoplankton at 1×10^5 cells ml^{-1} induced the highest proportion of mature females to spawn, promoted the greatest oocyte release, best quality of eggs and highest survival of embryos. The higher concentration of live phytoplankton (1×10^6 cells ml^{-1}) resulted in slightly lower results, suggesting that high concentration of phytoplankton in spawning tanks may cause some stress either to *C. frondosa* adults or directly to the gametes. Therefore, rather than a direct relationship, there seems to be a threshold beyond which the spawning response in *C. frondosa* is not increased, and perhaps even depressed at high phytoplankton concentration. Phytoplankton paste yielded intermediate results, whereas conspecific sperm induced the lowest proportion of females to spawn and all resulting embryos died within 10 d post fertilization. For males, spawning was more readily induced by a low concentration of conspecific sperm, followed by live phytoplankton, and phytoplankton paste; the lowest spawning success was observed for a high concentration of conspecific sperm. Other methods commonly used with commercial species, including thermal shock, desiccation, potassium chloride (injection or bath), and serotonin (injection), did not induce spawning in *C. frondosa*. When oocytes isolated from ovaries were exposed to three different concentrations of 1-Methyladenine (1-MA), 2,3-Dimercapto-1-propanol (BAL), L-cysteine (L-cyst), and Dithiothreitol (DTT), only the latter (at 10^{-1} M) promoted ovulation;

the other treatments yielded poor results that were comparable to the control (seawater). However, ovulated oocytes obtained with DTT remained unfertilizable. Overall, spawning induction with live phytoplankton emerged as the most suitable and reliable technique to maximize the collection of healthy gametes from *C. frondosa*. Optimal protocols might be developed that could potentially be used to induce spawning in this species and in taxonomically close holothuroids. The apparently poor receptivity of lecithotrophic oocytes to chemical triggers of final maturation deserves further investigation.

Chapter 4 investigated allogeneic fusion among embryos and the formation of chimaeras in *C. frondosa*. This was the first direct documentation of chimaeric development in a unitary metazoan belonging to the deuterostome clade (which includes Echinodermata, Hemichordata and Chordata). The formation of chimaeras and its prevalence were studied in two distinct propagule populations originating from adult sea cucumbers collected from different locations around the Avalon Peninsula, Newfoundland. Fusion was detected in 8-9% of the propagules and occurred only among hatched blastula embryos, never during earlier (unhatched) or later (larval) stages, suggesting that the immune system in echinoderms develops within this narrow window. Multi-chimaeras (composed of three or more embryos) were also detected in low proportions (0.02% of the propagule population). The fully fused chimaeric propagules were 2-5 times larger than non-chimaeric embryos. Fusion was positively correlated with propagule density and facilitated by the natural tendency of early embryos to agglomerate at the water surface. Given the fact that several scientific questions have been raised on this topic, the discovery of chimaerism in an Echinodermata (sister clade to chordates) that possesses large

externally-fertilized eggs provides a new exciting experimental framework. It is valuable from an ecological standpoint and it also has the potential to expand our understanding of higher metazoans to help explore aspects of cell communication, transplantation, and evolution of the immune system.

Finally, Chapter 5 investigated morphometric changes during early developmental stages as well as responses of juveniles to different environmental conditions over time, from settlement to 21 months of age. Juveniles developed all 10 tentacles in 16 months; they grew 9 ambulacral podia and measured 4.1 mm long after 21 months. Dorsally-oriented tentacles captured suspended particles from the water column; whereas ventral tentacles were used both to collect deposited organic material and assist in attachment and locomotion. Scaling between body length and number of podia was isometric, whereas tentacle metrics showed allometric scaling patterns, indicating that growth was accompanied by a decreasing tentacle-to-body-size ratio. Recruits (1 to 6 month-old) tolerated low light intensities and water flows, and preferred substrates composed of rocks with black or red background colours. As juveniles aged (12 to 21 month-old) they tolerated higher light intensities and water flows, and preferred substrates of coralline algae and red backgrounds. The findings presented here provide novel information about early growth, morphology and behaviour of a cold-water suspension-feeding sea cucumber, which will help develop tools to enhance aquaculture programs, particularly those focused on rearing juveniles in captivity.

Taken together, the studies presented in this thesis provided new information on the biology and ecology of *C. frondosa* with an aim to improve and refine broodstock

conditioning, gamete supply and juvenile rearing in captivity. As sea cucumber aquaculture expands towards temperate and subpolar environments, these results should greatly help the development of aquaculture protocols for other cold-water sea cucumber species with a similar biology.

6.2 Future directions

A number of studies on the life cycle and ecology of *C. frondosa* have been conducted over the past 25 years (e.g. Hamel and Mercier, 1995, 1996a, 1996c; Mercier *et al.*, 2007; So *et al.*, 2010; Gianasi *et al.*, 2017); however, several aspects of its biology still need to be investigated within an aquaculture perspective. Additional research on this species could provide important information on broodstock conditioning, handling and incubation of eggs, embryos and larvae, as well as juvenile growth in captivity. Some areas that deserve further attention are outlined below.

Chapter 2 showed that gametogenesis and spawning of *C. frondosa* are affected by changes in the environmental conditions (water temperature and photoperiod) during a relatively short-term exposure (120 d). However, *C. frondosa* has an annual reproductive cycle whereby gametogenesis starts in early winter and spawning usually occurs in the spring, followed by a period of phagocytosis of the remaining gametes in the gonad tubules (Hamel and Mercier, 1995, 1996c). Therefore, the conditioning of *C. frondosa* broodstock in captivity under different water temperatures and photoperiods will need to be investigated over a longer term (full year) in order to definitely exclude the possibility of out-of-season gamete production and spawning. Sea cucumbers conditioned in seawater at

6°C and 12°C did not spawn, and low proportions of females released oocytes under advanced (13-15 h light) and constant photoperiod (24-h dark and light). The mechanisms involved in the perception of environmental parameters and how they are translated into spawning cues are still poorly understood in sea cucumbers (Mercier and Hamel, 2009); however, further studies can shed some light on this topic, which will help improve broodstock management. While previous study indicated that lipid-rich diets (i.e. cod eggs) enhanced the gonad development and gamete production in *C. frondosa* compared to individuals fed with low-lipid content diets (live diatoms) (Gianasi *et al.*, 2017), the type and quantity of food provided in the present work (rotifers enriched with a commercial rotifer diet) did not enhance the gamete production of *C. frondosa*. Other types of diets such as rotifers enriched with live phytoplankton or mixes of natural plankton typical of *C. frondosa*'s natural habitat (e.g. ciliates, flagellates, copepods, bacterial floc) could be tested. Moreover, as males and females of *C. frondosa* can be easily identified based on external sexual dimorphism (i.e. morphology of the gonopore) (Montgomery *et al.*, 2018, see Appendix), broodstock conditioning experiments could be conducted separately on the two sexes or in tanks combining different proportions of males and females. Inter-individual chemical communication has been observed in *C. frondosa* during gametogenesis, whereby mucus secreted by the sea cucumbers was shown to maintain gametogenic synchrony among conspecific (Hamel and Mercier, 1996b). In this context, the long-term effect of environmental parameters on gametogenesis and spawning can be determined in populations of males and females conditioned either separately or together.

The artificial induction of final maturation in oocytes surgically removed from sea cucumber ovaries had primarily been explored only in deposit-feeding species with small eggs and planktotrophic larval development (Maruyama, 1980, 1986; Léonet *et al.*, 2009). Despite various substances proposed to induce the breakdown of follicle cells and germinal vesicle in sea cucumber oocytes were tested in Chapter 3, only one chemical induced ovulation in *C. frondosa* and oocytes remained unfertilizable (i.e. final maturation was not achieved). The fact that *C. frondosa* oocytes are larger and possess a greater amount of yolk than planktotrophic oocytes might have impeded the translocation of these substances into their cytoplasm. Previous studies identified a compound named "Radial Nerve Factor" that acts through the follicle cells surrounding the oocytes to produce a maturation-inducing substance (referred to as MIS) which leads to germinal vesicle breakdown in several planktotrophic sea cucumber species (Maruyama, 1980, 1985). However, the pathway through which these compounds act on the germinal epithelium, follicle cells, and oocytes is still poorly understood. Therefore, understanding the mechanisms and substances involved in ovulation, germinal vesicle breakdown and final maturation of lecithotrophic oocytes would greatly enhance the capacity to produce sea cucumber juveniles in captivity. Moreover, other substances such as the so-called "Maturation Induction Fraction" (Léonet *et al.*, 2009) and Cubifrin-L (Fujiwara *et al.*, 2010) can be tested in the future on *C. frondosa* oocytes. Finally, investigations on the viability of *C. frondosa* oocytes can be undertaken to determine how long naturally-spawned oocytes remain fertilizable and the optimum time for fertilization of ovulated oocytes. Such data can help advance techniques of controlled fertilization in the laboratory.

Sea cucumber aquaculture (of tropical species) is characterised by high mortalities during embryonic and larval stages, resulting in variable juvenile production (Battaglene *et al.*, 1999; Purcell *et al.*, 2012; Mercier and Hamel, 2013). Generally speaking, the poor output of juvenile production can be associated with the quality of the eggs and larvae. However, very few studies have assessed the biological characteristics involved in offspring quality (Morgan, 2009). Therefore, quantitative methods that can assess egg, embryo and larval quality in sea cucumbers need to be developed. Chapters 2 and 3 of this thesis teased out some aspects of egg and embryo quality in *C. frondosa* based on size, colour, blastomere divisions, morphology, and swimming behaviour; however, detailed criteria must be developed for each stage of embryonic and larval development. Quality criteria should include the parameters mentioned above as well as fertilization rates since healthy gametes (oocytes and spermatozoa) should result in high fertilization rates, and the chemical composition of the eggs (e.g. lipids, fatty acids, vitamins, minerals), which might also reflect the amount of energy available for embryonic and larval development based on health of the broodstock. Finally, to fine tune in broodstock selection for optimal offspring production, molecular techniques that assess chromosomal anomalies during the final maturation of oocytes and early stages of embryonic development could be developed. Quality criteria for early development is an important aspect of hatchery management and will help determine whether an egg batch seems viable or not and thus should be incubated or discarded. Moreover, abnormalities observed in fertilized oocytes and blastula embryos of *C. frondosa* during experiments conducted in Chapters 2 and 3 could not be explained. These abnormalities included a yellowish substance in the interstitial zone between the

plasma membrane and the fertilization envelop in fertilized oocytes and early embryos, and the presence of blastomeres with recessed surfaces. Further studies still need to be carried out in order to understand the mechanisms leading to egg and embryo malformation since it compromises survival of larvae and newly-settled juveniles. Such studies can be conducted under controlled laboratory conditions whereby abnormalities in each developmental stage will need to be documented and the frequency of their occurrence in the propagule population noted. Moreover, patterns of swimming behaviour and histology of abnormal embryos and larvae might help clarify the drivers of malformations and mortalities.

Importantly, studies (and aquaculture) of sea cucumbers are currently focused on species with small oocytes and planktotrophic (feeding) larval development (Purcell *et al.*, 2012). However, *C. frondosa* produces large yolky oocytes which support the development of the lecithotrophic (non-feeding) larvae (Hamel and Mercier, 1996a). Therefore, the developmental studies mentioned above will be instrumental in providing adequate foundation to both fundamental and applied work. In addition, in the context of hatchery settings, the large size and lipid-rich nature of oocytes in *C. frondosa* mean that dying/dead eggs quickly result in poor water quality even in flow-through incubators due to their slow degradation, which likely favours bacterial proliferation. Moreover, dying/dead eggs are also positively buoyant and float on the surface of the water, together with normal/healthy eggs, which makes their removal difficult. This situation was frequently observed in all experiments conducted in Chapters 2 and 3 and required sustained monitoring to remove decaying eggs from each incubator. Therefore, improvements to the incubation methods of

C. frondosa eggs will be required before the production of juveniles can be scaled up. Some techniques used in the incubation of halibut eggs might be tested. In order to remove the dead eggs, a saltier solution (~38 psu) is added to the incubator for a short period of time (~15 min) making the dead eggs sink to the bottom of the tank where they are easily removed from the incubators (Moksness *et al.*, 2008). However, because eggs of *C. frondosa* have a very different structure (no chorion), the effect of a rapid change in salinity on embryonic and larval development will first need to be investigated in *C. frondosa*. Moreover, methods for egg disinfection can be explored as a way to minimize bacterial proliferation in the incubators. Studies on egg disinfection can be performed by gently removing them from the incubators and submerging them into a chemical solution (e.g. diluted formaldehyde, hydrogen peroxide, peracetic acid, or iodine-based solutions) for a range of durations while the incubators are cleaned and rinsed. Eggs can then be returned to the disinfected incubators where development is carried on. This procedure can be adopted to minimize bacterial proliferation on the incubator's walls as well as on the fertilization envelope in order to optimize embryo survival and improve hatching rates. Further studies will need to be conducted to determine which chemical compound would be more suitable to disinfect eggs of *C. frondosa*, assess the level of tolerance for each developmental stage, suitable concentration, time of immersion, and any side effect on the embryonic and larval development, and hatching and survival rates.

The discovery of chimaerism in embryos of *C. frondosa* was somewhat unexpected and was only revealed when dead/dying eggs were being manually removed from the incubators with the help of a magnifying lens. Whole-body fusion among conspecifics has

been documented mainly in modular/colonial organisms such as sponges (Maldonado, 1998), corals (Barki *et al.*, 2002), and ascidians (Rinkevich *et al.*, 2016), whereas its occurrence in unitary organisms had only been evidenced indirectly in mammals (e.g. Ross *et al.*, 2007) and directly in brooded propagules of sea anemones (a basal group of marine organisms) (Mercier *et al.*, 2011). While Chapter 4 documented the step-by-step process of full embryonic fusion in *C. frondosa*, it also raised several questions related to histocompatibility and the ecological benefits of chimaerism. For instance, whether fusion occurs among embryos that share at least one histocompatibility locus (i.e. between full or half siblings) or completely non-related embryos remains to be determined using controlled fertilization and molecular analysis. Moreover, the identification of chimaeric individuals in wild populations would greatly help the understanding of the ecological importance of chimaerism in *C. frondosa*. However, such information might only be gathered following the development of suitable molecular markers and techniques. Meanwhile, the recently published genome of the sea cucumber *Apostichopus japonicus* (Zhang *et al.*, 2017) should help identify candidate genes and develop a roadmap for future studies of chimaerism in *C. frondosa* and its potential use as a model organism. Moreover, fusion of allogeneic entities is expected to confer genetic variability, developmental synergism, and immediate increase in size and survivorship (Buss, 1982; Hennige *et al.*, 2014). For instance, an overall gain in fitness was observed in chimaeric ascidians (Rinkevich and Shapira, 1999), corals (Puill-Stephan *et al.*, 2009), and sea anemones (Sun *et al.*, 2012). Therefore, future studies can explore growth and survival rates as well as competitive ability for food uptake between chimaeric and non-chimaeric embryos and juveniles of *C. frondosa* in an aquaculture setup.

As chimaeric embryos of *C. frondosa* are remarkably larger then singletons, it might be interesting to select chimaeric individuals for breeding programs.

The growth rate of *C. frondosa* juveniles is rather slow under ambient (naturally fluctuating) conditions of water temperature and food availability (Hamel and Mercier, 1996a). From current knowledge, *C. frondosa* is expected to require between 12 to 25 years to grow from larva to commercial adult size based on previous field and laboratory studies (Hamel and Mercier, 1996a; So *et al.*, 2010), which is consistent with the growth rates obtained here in Chapter 5 during the early life stages. However, a previous study assessed the isolated effect of water temperature and pH on the development of embryos of *C. frondosa* and showed that an increased in water (5-12°C above ambient) and pH (9) resulted in faster embryonic development up to 128 cells (Hamel and Mercier, 1996a), which suggests that growth rates of juveniles could be manipulated under controlled conditions in the laboratory. A quantitative study of the effects warm water temperature (5-12°C above ambient), and greater food quantity and quality on the growth rates of *C. frondosa* will be required to predict optimal larval and juvenile development. This experiment will determine the time needed to grow from larva to commercial size in the laboratory, which will have direct implications for the aquaculture of this species for the seafood market. Moreover, the body wall of *C. frondosa* contains several compounds such as Frondanol A5 and glycolipids (Al Shemaili *et al*., 2014) used by nutraceutical and pharmaceutical industries, which could be extracted from hatchery-produced juveniles, minimizing the current fishing pressure on the natural populations. While warmer temperatures are not ideal for broodstock conditioning prior to spawning (Chapter 2),

fertilized oocytes will have to be collected from spawning tanks and transferred to incubators where the water temperature can be gradually increased. During this time, developmental kinetics and mortality rates will need to be monitored. Once settlement of juveniles occurs, manipulation of the water temperature and of the composition and abundance of different diets (e.g. bacterial flocs, ciliates, flagellates, copepods) can be undertaken to evaluate the synergistic effects of temperature and food on growth rates of newly-settled juveniles of *C. frondosa* in captivity. These investigations can also lead to studies linking diet composition/energy with body tissues composition, which can help improve our understanding of the mechanisms involved in nutrient transfer and allocation in cold-water sea cucumbers. It would also be interesting to investigate the behaviour, movement, and circadian rhythm of juveniles when exposed to different flow directions combined with various substrate types.

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Appendix

Functional significance and characterization of sexual dimorphism in holothuroids[5]

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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 (Bonduriansky, 2006; Cooper et al., 2011; Shine, 1989; Williams and Carroll, 2009). 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; O'Loughlin 2001; Pawson and Miller 1979). 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

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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 vs 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 A.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 A.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 A.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. A.1A). Two families contained nearly all of the species in the dataset with external dimorphism: Cucumariidae and Psolidae (Fig. A.1B). Approximately 62% and 67% of species belonging to these two families displayed sexual dimorphism of the genital papillae, respectively (Fig. A.1B). Moreover, external sexual dimorphism was only reported for one species of Persiculida (Fig. A.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. A.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. A.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. A.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. A.4). Approximately 50% of brooding and 100% of free-spawning species had males with digitate genital papillae (Fig. A.4). In contrast, extensible papillae were only found among species with internal and external brooding, never in free spawners (Fig. A.4). Approximately 50-60% of brooding species had extensible male papillae (Fig. A.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

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commonly were on the "dorsal" side of the animal (the side typically facing up; Fig. A.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. A.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. A.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 A.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 A.4). In comparison, females had smooth, non-digitate papillae between their oral tentacles (Fig. A.6E-F). Female papillae were ~2 mm long and 2 mm wide (Table A.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. A.7A) and a single string of eggs being released from smooth, non-digitate female papillae (Fig. A.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. A.8A, B). Males collected offshore

(exposed coastal areas) had dark red gonads, very similar in colour to females from both areas (Fig. A.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 A.S.1 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 (Levitan, 1996; McEdward and Chia, 1991; Vance, 1973). 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

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Supplementary Material

Table A.S.1: 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 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	Gerdes and Klages 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 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

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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	Gerdes and Klages 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 2008
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*	No⃞	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).

Tables

Table A.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	Present 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 & 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 (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. com.
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 & 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 A.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 & Stepanov (2005)
Cucumaria djakonovi	5-7	2-4	Levin & Stepanov (2005)
Cucumaria frondosa	1-4	1-3.5	Current Study
Cucumaria frondosa	5-7	2.5-3	Levin & Stepanov (2005)
Cucumaria georgiana	2	1	O'Loughlin (2001)
Cucumaria japonica	5-7	2.5-3	Levin & Stepanov (2005)
Cucumaria okhotensis	5-7	2-4	Levin & 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 & Penchaszadeh (2017)
Overall Mean (±SD)	4.0 (2.0)	2.2 (2.1)	

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

Table A.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 A 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

Figures
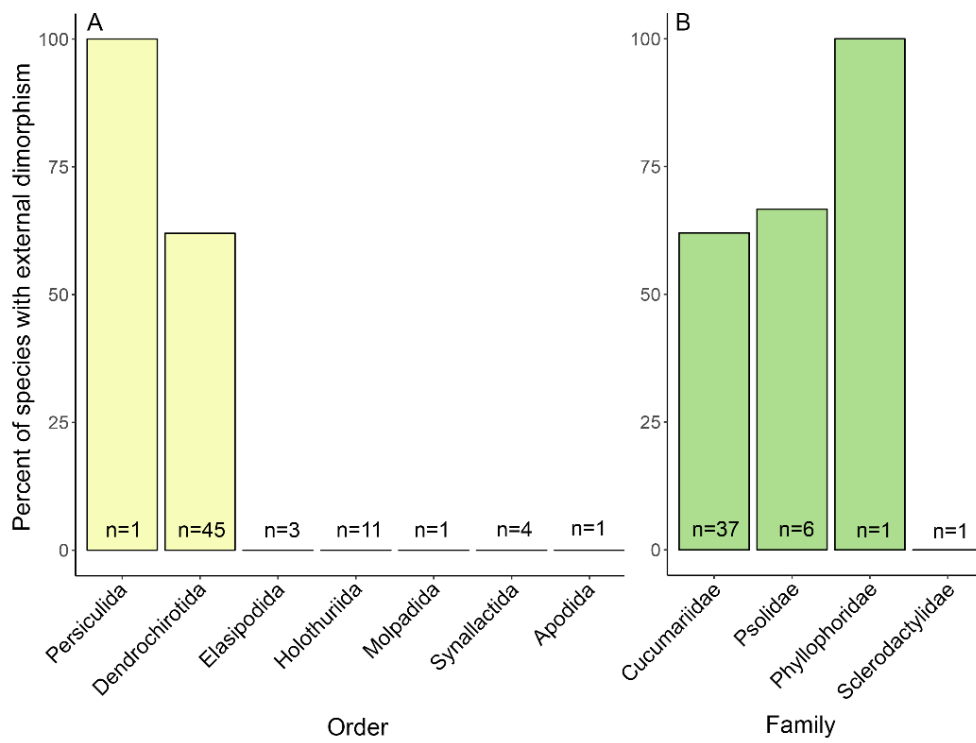
Figure A.1:Percent of sea cucumber species that possess external sexual dimorphism in the length and/or shape of permanent genital papillae relative to taxa. Species were considered sexually-dimorphic if macroscopic variation in papillae enabled reliable sexing of individuals in vivo. A) Dimorphic species 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 A.S.1 for the full dataset.
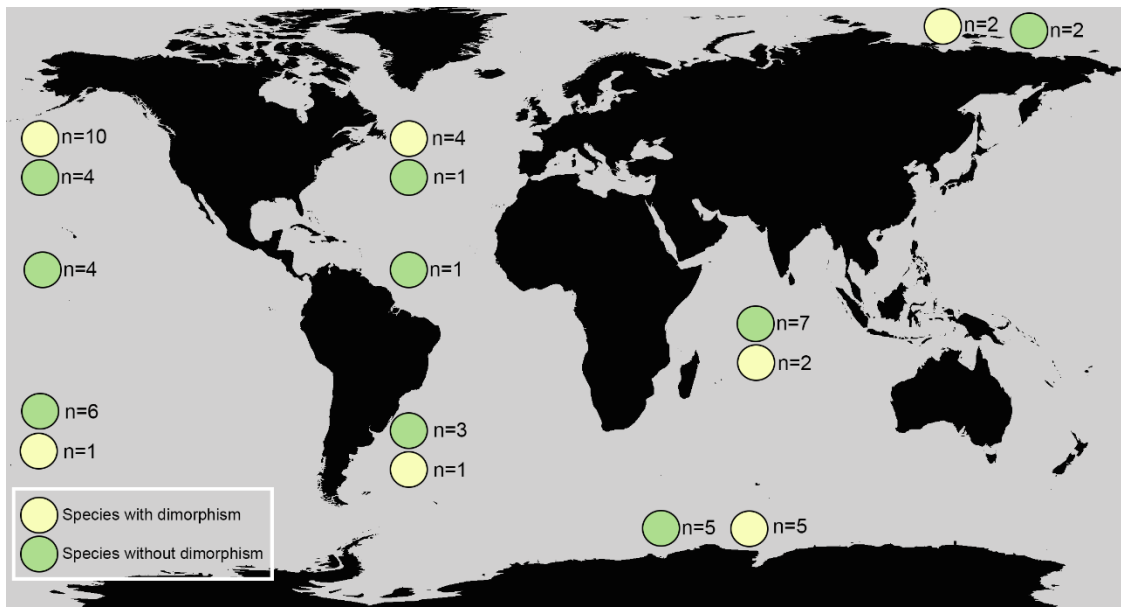
Figure A.2: Global distribution of sea cucumber species reported with (yellow) and without (green) external sexual dimorphism. Species were considered sexually dimorphic if macroscopic variation in papillae enabled reliable sexing of individuals in vivo.
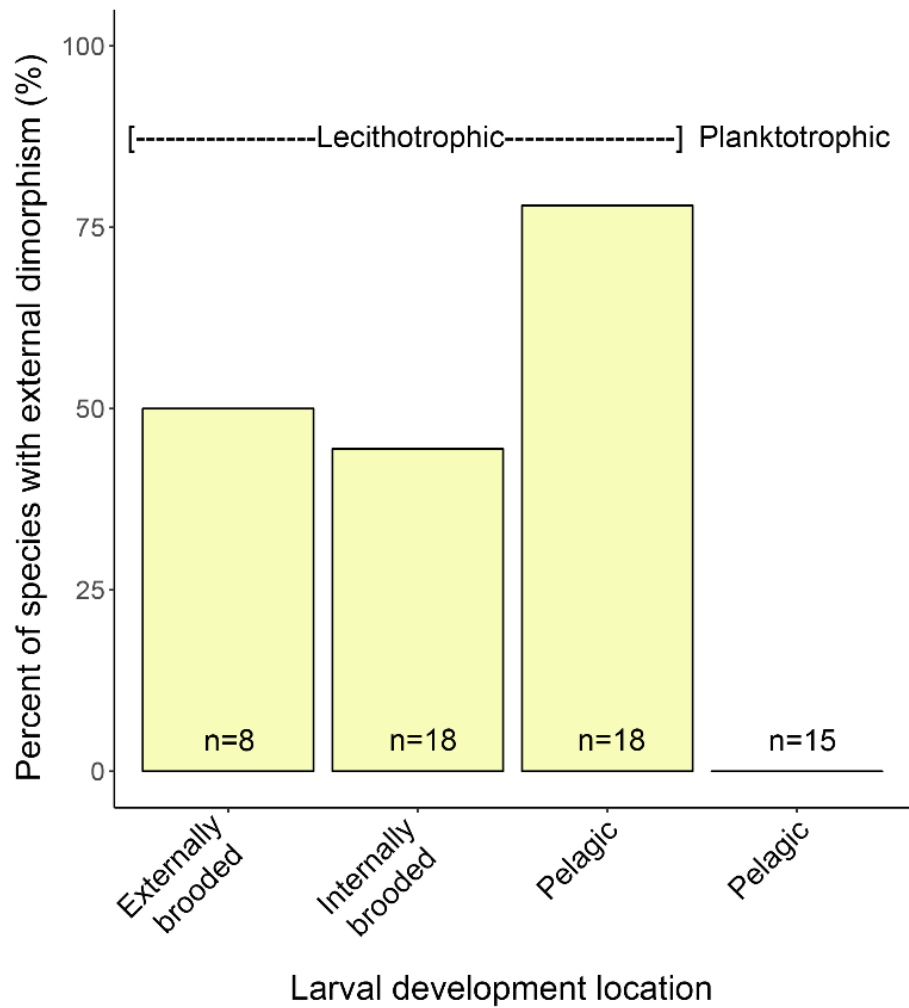
Figure A.3: Percent of sea cucumber species that possess external sexual dimorphism in the length and/or shape of the genital papillae relative to larval development location. Species were considered sexually dimorphic if macroscopic variation in papillae enabled reliable sexing of individuals in vivo. The total number (n) of species examined in each category is indicated on each bar.
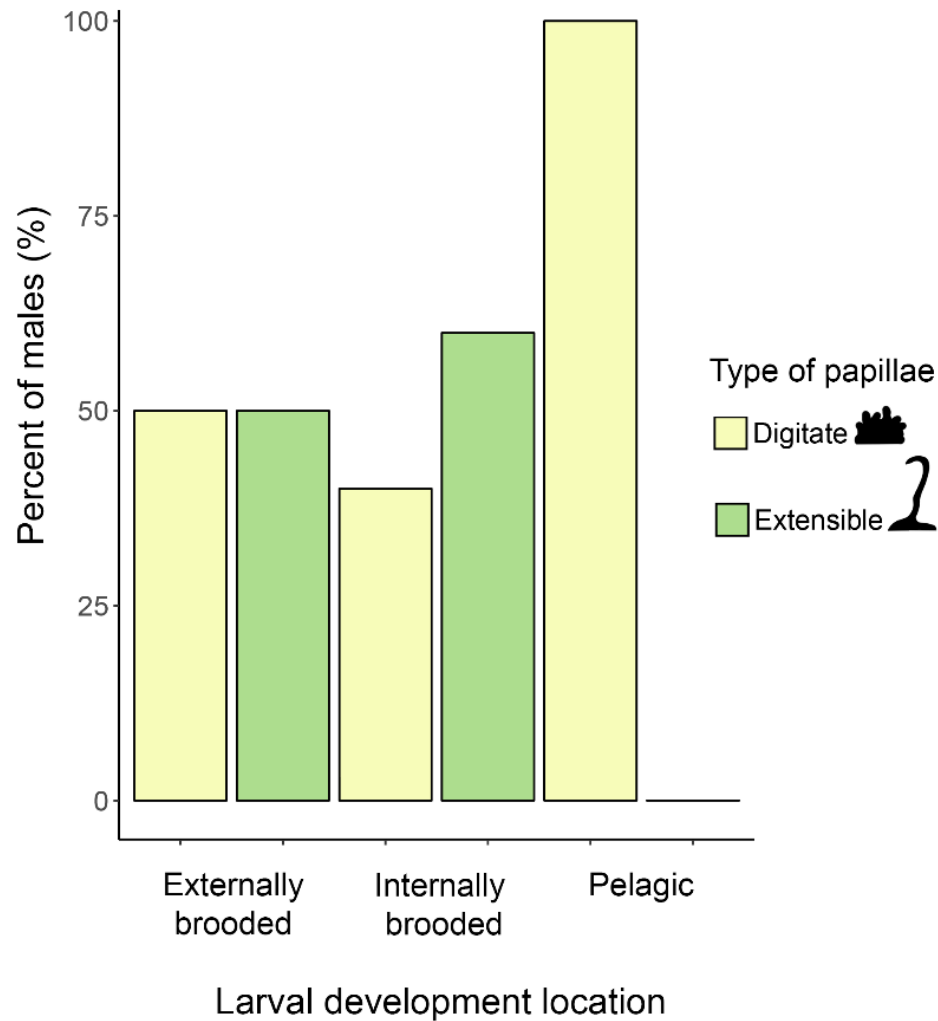
Figure A.4: Percent of male sea cucumbers that possess digitate or extensible genital papillae relative to larval development location. *Digitate* papillae were defined by the presence of branching (>1) of the genital papilla. *Extensible* papillae were defined as long papillae with whip-like characteristics. 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).
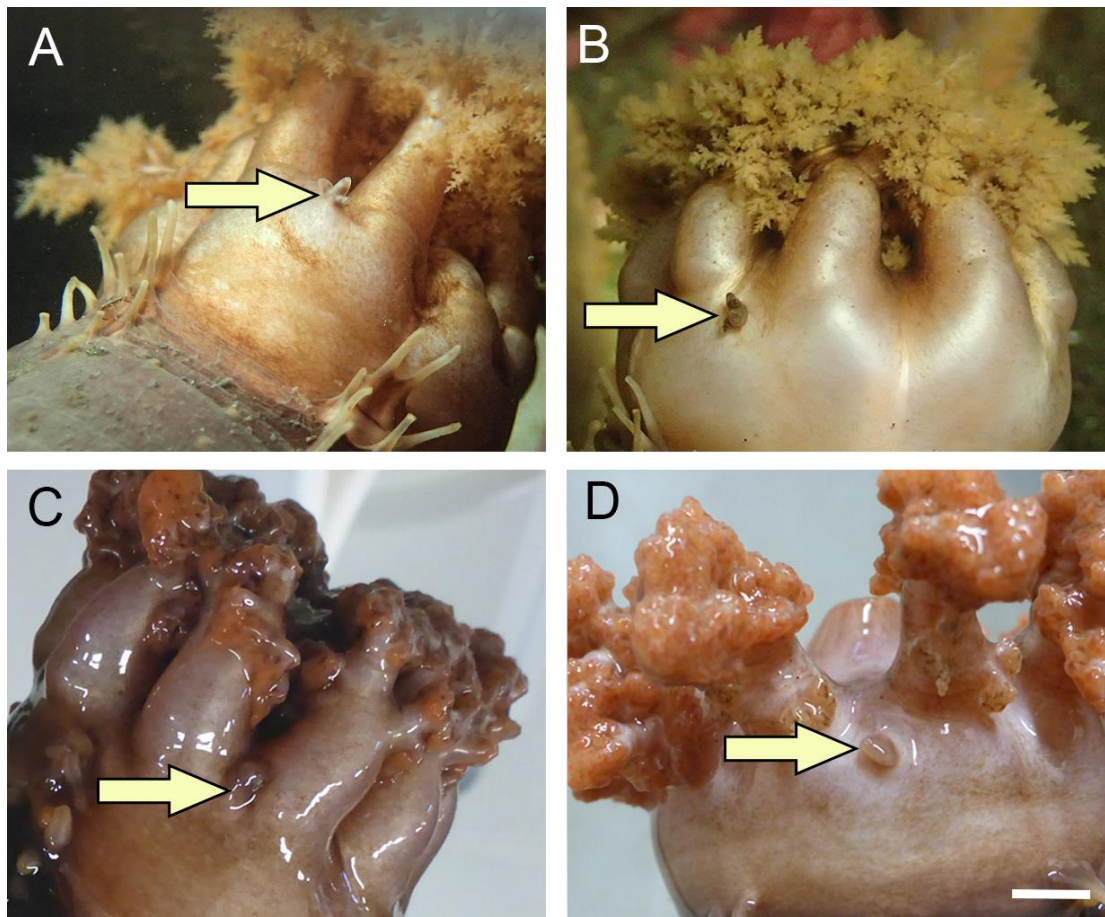
Figure A.5: Location of genital papillae in male (A-B) and female (C-D) *Cucumaria frondosa*. Arrows indicate location of papillae. Note the digitate shape of the male papillae (A-B) versus the smooth, non-digitate, cone-shape papillae of the females (C-D). Scale bar indicates 1 cm.
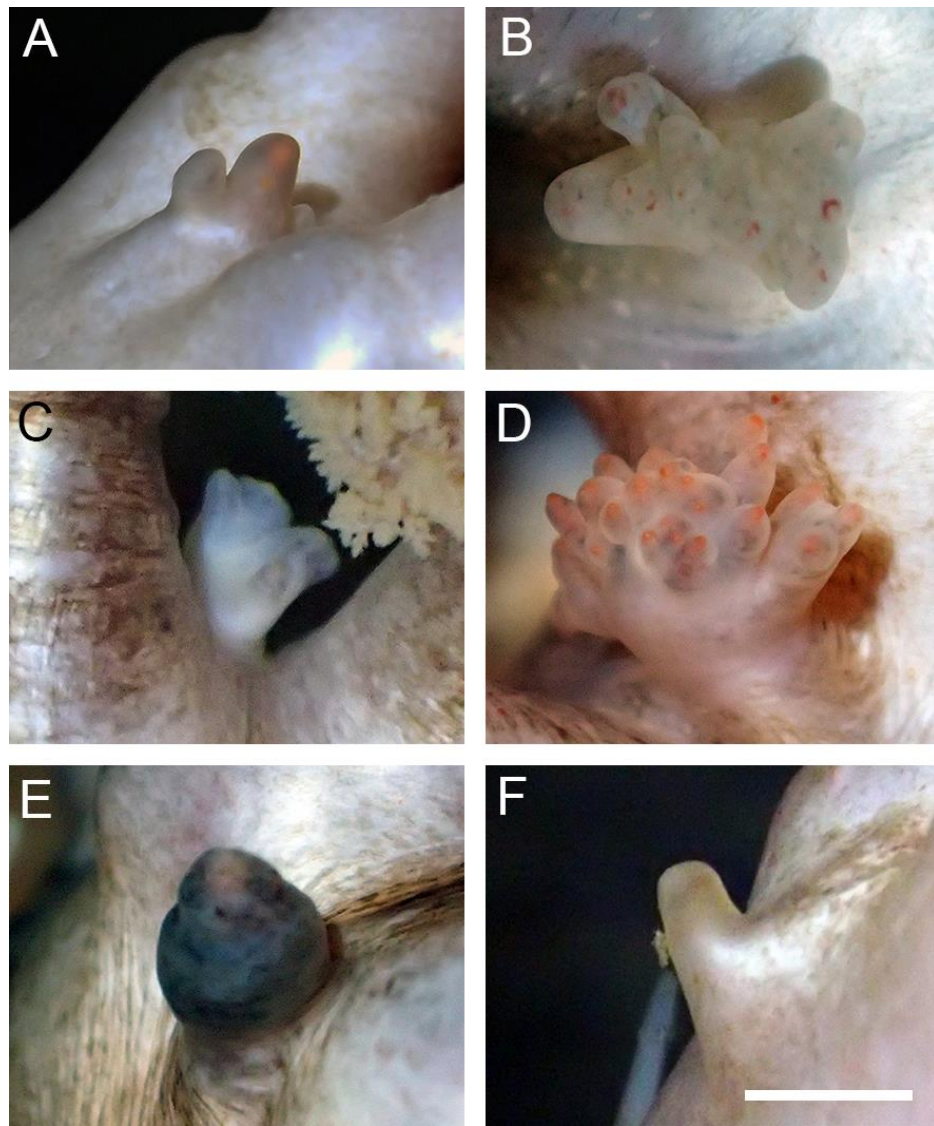
Figure A.6: Diversity of genital papillae in male (A-D) and female (E-F) *Cucumaria frondosa*. Panel A shows trifurcated male papillae. Panels B-D show examples of multifurcated male papillae. Panels E-F show smooth, non-digitate, cone-shape female papillae. Scale bar indicates 0.5 cm.
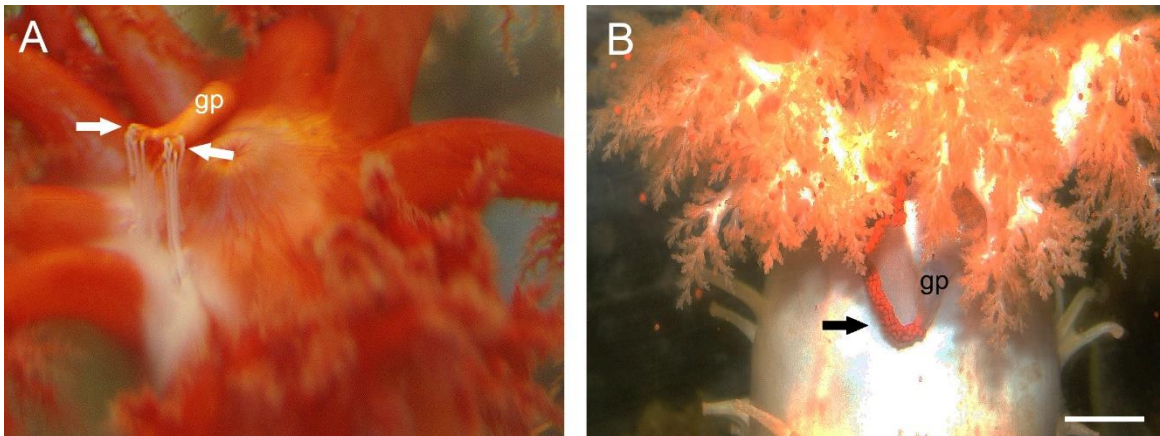
Figure A.7: Spawning in sea cucumbers with pelagic lecithotrophic larvae. Male *Psolus fabricii* (A) show the typical digitate genital papillae (gp) and multiple streams of sperm emerging from numerous gonopores (white arrow). Female *Cucumaria frondosa* (B) release bundled egg "sausages" (black arrow) from a non-digitate genital papilla (gp). Scale bar indicates 2 cm.
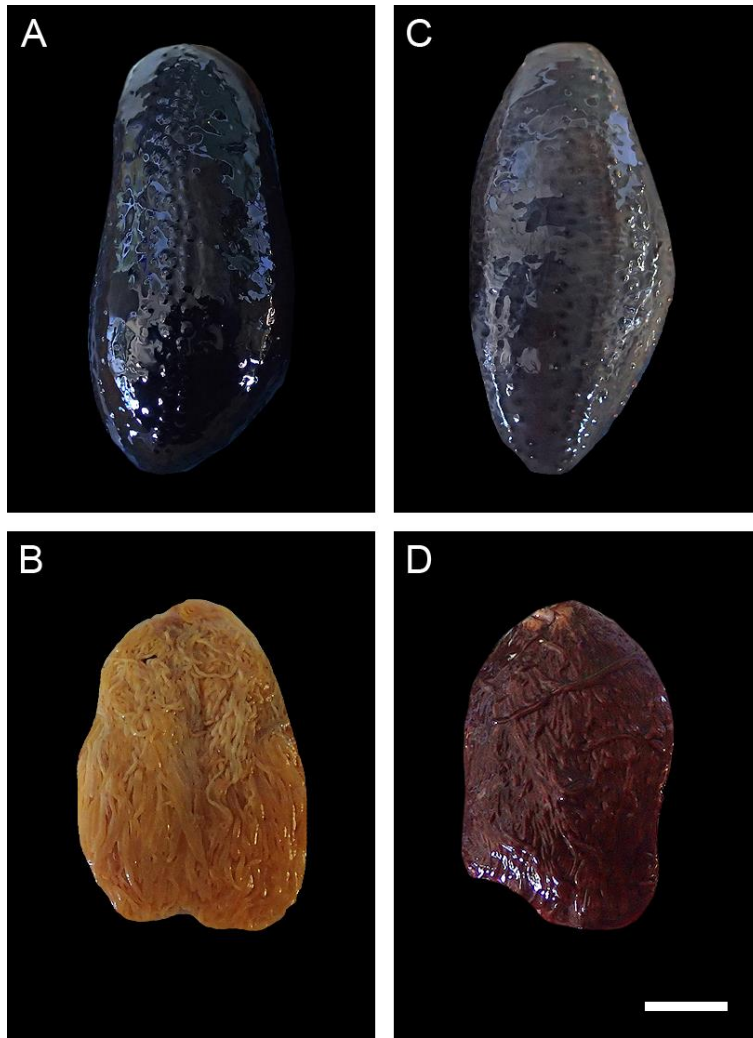
Figure A.8: Comparison of the body wall and gonad of two males of *Cucumaria frondosa* originating from two different geographic locations. A-B) Logy Bay, Newfoundland (male 1). C-D) Grand Banks, Newfoundland (male 2). Individuals were examined in July, a few months after the natural spawning period 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).