Fisheries and Aquaculture Related Biometrics of the Sea Cucumber *Cucumaria frondosa*: Tagging, Resistance to Stress and Influence of Diet on Lipid Composition

By

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Abstract

Cucumaria frondosa is widely distributed in the North Atlantic where it has been increasingly exploited to supplement the growing demand for sea cucumber products in Asian markets. The objectives of this study were based on knowledge gaps identified by the stakeholders of the sea cucumber industry in eastern Canada. The first study investigated marking techniques using passive integrated transponder (PIT) tags to facilitate sea cucumber research. The second study identified the most suitable media for refrigeration during live storage and transport to address concerns with skin and meat integrity prior to processing. The third study focused on principles of aquaculture by examining growth, and lipid class and fatty acid profiles of muscle and gonad tissues of sea cucumbers fed with either diatoms or fish eggs. Implanting PIT tags at the base of the tentacles to reach the aquapharyngeal bulb emerged as one of the most effective techniques ever developed for tagging sea cucumbers reliably and innocuously for long periods. The most suitable transport media for live boreal/temperate sea cucumbers was determined to be iced seawater (cold seawater with freshwater ice). Finally, while sea cucumbers were able to feed on live diatoms (*Chaetoceros muelleri*) as well as commercial fish eggs, the latter diet yielded greater body length increment, specific growth rate and ratio of essential DHA:EPA in gonadal tissues. In contrast, sea cucumbers fed with diatoms exhibited the highest ratio of the essential fatty acids ARA to EPA in muscle tissues. The findings presented here will hopefully assist ecological and conservation studies and the sustainable development of sea cucumber fisheries and aquaculture programs worldwide.

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Co-authorship Statement

The work described in the present thesis was carried out by Bruno Lainetti Gianasi with guidance from Annie Mercier, Jean-Francois Hamel, Cyr Couturier and Christopher C. Parrish. Bruno Lainetti Gianasi was responsible for field and laboratory data collection and analysis with assistance from Annie Mercier. All chapters were written by Bruno Lainetti Gianasi as journal manuscripts with intellectual and editorial input by Annie Mercier and other co-authors as follows:

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Chapter 1: General Introduction

Sea cucumbers have long been an important part of Asian cultures and traditions, particularly in China, where the powerful symbolism and medicinal characteristics of sea cucumber have inspired many legends (Kiew and Don, 2012). As stories often reflect the beliefs of the people who tell them, a deep respect for sea cucumbers has been passed down from generation to generation in many regions of Asia.

Ancient tales of the discovery of sea cucumber as an edible animal, usually involving fishermen lost at sea, date back to approximately 200 BC (Yang *et al.*, 2015). One retelling states that the First Emperor of China sent one of his servants on a boat in search for the everlasting elixirs in Bohai Bay, gulf of the Yellow Sea (northeast China). Everlasting elixirs are considered a type of potion with the power to either never run out or work forever. After a long time sailing and searching for the everlasting elixirs, the servant and his crew ran out of food on the boat; however, they did not want to return, because they had failed to complete the task and the First Emperor was known for his cruelty. They decided to live on an island around which sea cucumbers were abundant. After days spent on a diet of sea cucumbers, the servant felt stronger and younger, so he resolved to eat sea cucumber for the rest of his life. Upon reaching 90 years of age, he still looked very young; he did not have a single white hair, and he was strong and healthy. The servant then understood that the everlasting elixir he was looking for was actually the sea cucumber (Yang *et al.*, 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). The main protein is made up of lysine, arginine and tryptophan and insoluble collagen from the body wall (Wen *et al.*, 2010). The high tryptophan level in the body wall is one of the factors that makes the gelatine of sea cucumber more valuable than other gelatines (Hayes and McKeon, 2014). Studies have shown that organic solvent extracts of sea cucumbers are important sources of high-value compounds with multiple biological benefits, including anticoagulant, anticancer, antioxidant and anti-inflammatory properties (Bordbar *et al.*, 2011; Xia and Wang, 2015). Moreover, sea cucumbers exhibit outstanding self-regeneration properties and tissue repair abilities, which reinforce people's confidence regarding their efficacy in traditional medicine (Kiew and Don, 2012).

East Asian countries have made the sea cucumber one of the most valuable gifts and prized seafood in the world; it is usually served at weddings, banquets and corporate events (Fabinyi, 2012). The most commonly traded product, known as beche-de-mer or trepang, consists of the body wall (skin), generally including the muscle bands, which is dried and sold as a luxury seafood (Purcell, 2014). In North America, muscle bands are sometimes fresh frozen and marketed separately (Hamel and Mercier, 2008a). Liquid extracts, dried aquapharyngeal bulbs (labelled flowers) and viscera (Bechtel *et al.*, 2013) can also be found on the market. The high demand and high market prices for beche-de-mer spurred by cultural and social traditions in China have led to the growth of sea cucumber fisheries and, consequently, to the depletion of wild stocks of high-value

species from Asia and the Indo-Pacific (Anderson *et al.*, 2011; Conand, 2004; Purcell *et al.*, 2013). In recent years, the overexploitation of wild stocks has led to the development of new sea cucumber fisheries targeting under-utilized species around the world (Hamel and Mercier, 2008b).

The sea cucumber *Cucumaria frondosa* is the focus of an emerging fishery in the North Atlantic. It is widely distributed in cold waters, occurring from the Arctic Ocean to Cape Cod as well as along the coasts of northern Europe and Russia (Hamel and Mercier, 2008a). The state of Maine (USA) was the first region to open a commercial fishery for *C. frondosa* in 1980, followed by several Canadian provinces, such as Nova Scotia, New Brunswick and Newfoundland and Labrador (Grant, 2006; Grant *et al.*, 2006; Hamel and Mercier, 2008a; Rowe *et al.*, 2009), as well as Iceland and Russia (Gudimova *et al.*, 2005; Garcia *et al.*, 2006; Therkildsen and Petersen, 2006; Hamel and Mercier, 2008b). Unlike most other fisheries for sea cucumbers, which are traditional (involving hand collection by divers or snorkelers), commercial harvest of *C. frondosa* was initially carried out with scallop gear in Atlantic Canada. Later on, specific drag nets were designed to minimize bycatch and suit local conditions and vessels (Barrett *et al*., 2007). In 2012, landings for this species peaked at almost 6000 metric tonnes in Canada (DFO, 2014). Although growth rates in *C. frondosa* are low in the wild and in captivity (Hamel and Mercier, 1996; So *et al.*, 2010), the species is considered to have potential for aquaculture due to its high marketability for food and pharmaceutical products and because much of its life cycle is well documented (Hamel and Mercier, 1996, 2008a; So *et al.*, 2010). It is currently being explored as an extractive species for integrated multi-trophic aquaculture (Nelson *et al.*, 2012).

Several studies have been conducted on sea cucumber ecology in the context of conservation and management efforts (Conand, 2004; Conand and Byrne, 1993; Hamel and Mercier, 2008a, 2008b; Purcell *et al.*, 2013; Purcell *et al.*, 2014). However, as the sea cucumber industry expands in North and South America and northern Europe, it is important to address issues related to new or emerging species. The present work evolved to fill knowledge gaps identified by the Canadian government and to address priority concerns expressed by primary stakeholders. It also sought to provide significant advances in our general understanding of a dominant benthic species in the Northwest Atlantic.

Although they are typically sedentary and slow moving, sea cucumbers are not always easy to study. The lack of a reliable technique to mark individuals has hindered tracking and capture-recapture studies, which provide key biological information of interest to the fishery and aquaculture sector (e.g. movement and migration patterns, growth, estimates of natural mortality). In an effort to change the situation, Chapter 2 tested whether and how passive integrated transponder (PIT) tags could be used as a reliable and innocuous marking technique in sea cucumbers. The efficacy of PIT tags implanted in various ways into previously unstudied tissues/organs was tested in two size classes of *C. frondosa* by evaluating retention rates, location of implanted PIT tags and post-tagging side effects on the body wall and on feeding and spawning behaviour during a short-term (30 d) and a long-term experiment (300 d).

Storage conditions to which seafood is exposed during transport or at the processing plant are closely linked to the quality of the final product. Sea cucumbers present a particular challenge in this respect because they lack external protection (e.g.

scales, exoskeleton) and can undergo autolysis (self-digestion) under adverse conditions. Chapter 3 investigated the use of different media for refrigeration during live storage of *C. frondosa* based on current techniques and low-cost variants, following the regulation for transport of seafood in Canada. Individuals were classified according to health and body wall condition immediately after storage. Measurement of pH of the meat (body wall and muscle bands) was conducted and water quality in the storage tanks assessed. Finally, individuals were monitored post storage for survival and development of skin damage to identify the least damaging storing method in order to obtain high-quality meat products, susceptible to yield optimal market prices.

Feeding and dietary requirements of cultured species has been a great obstacle for the production of sea cucumber juveniles. There is limited knowledge about the lipid and fatty acid profiles of cold-water sea cucumbers and further information is needed in order to establish knowledge-based protocols. Therefore, Chapter 4 investigated the effect of diets on growth, lipid class and fatty acid composition of muscle and gonadal tissue of *C. frondosa* fed either with live phytoplankton or commercial fish eggs. Results were compared with sea cucumbers feeding on the natural seawater supply (no supplement) and sea cucumbers freshly collected from the field. Specific growth rate and intestinal content were also investigated at the end of the experiment. Finally, essential fatty acid ratios were calculated and compared among treatment groups.

Chapters 2, 3 and 4 are written in a format compatible with the publication guidelines of scientific journals, which explains the repetition of some of the fishery and biological information, as well as the use of first-person plural pronoun (“we”) and possessive determiner (“our”). Chapter 5 summarizes the main findings and their

contribution to advancing the fishery and aquaculture of *C. frondosa*. It also discusses directions for future research in this area.

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Chapter 2: Novel Use of PIT Tags in Sea Cucumbers: Promising Results in the Commercial Species *Cucumaria frondosa*[1]

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2.1 Abstract

The lack of a reliable and innocuous mark-recapture method has limited studies that would provide essential information for the management of commercial sea cucumbers. Tagging sea cucumbers is notoriously difficult because of their plastic nature and autolysis (self-digestion) capacities. Markers previously tested, mainly on or through the body wall, were either lost rapidly or had major drawbacks (e.g. suitable only for batch identification, requiring complex analysis, causing infections, necrosis, behavioural changes and mortality). The present study explored the efficacy of passive integrated transponder (PIT) tags for individually marking sea cucumbers by assessing retention rates and long-term side effects of tags inserted in previously unstudied tissues/organs. Individuals of the species *C. frondosa* were tagged in the body wall, aquapharyngeal bulb and at the base of the oral tentacles and were monitored closely for evidence of stress, infection, change in feeding and spawning behaviour and tag retention rate. Implanting the tag in an oral tentacle to reach the hydrovascular system of the aquapharyngeal bulb achieved the best retention rates in full-size individuals: from a maximum of 92% after 30 days to 68% at the end of the experimental period (300 days). Efficacy was lower in smaller individuals (84% after 30 d and 42% after 300 d). Following a slight increase in cloacal movements for 15 h post tagging, no side effect was noted in sea cucumbers tagged in the aquapharyngeal bulb via the tentacles. Feeding and spawning behaviours were not affected and no signs of infections or abnormal cell development in the vicinity of the tags were observed. Tags implanted in the body wall showed retention of 100% in the first 15 d, dropping considerably thereafter. This study indicates that marking sea

cucumbers with 8.2 mm long PIT tags is an effective technique, particularly when implanted via the oral tentacle, where they are retained rates over long periods without any detectable physiological or behavioural effects.

2.2 Introduction

The high demand and high market prices for beche-de-mer (dry body wall) spurred by cultural and social traditions in China have led to the growth of sea cucumber fisheries and, consequently, to the depletion of wild stocks of high-value species all over the world (Anderson *et al.*, 2011; Conand, 2004; Purcell, 2014; Purcell *et al.*, 2013; Purcell *et al.*, 2014; Conand and Byrne, 1993).

Cucumaria frondosa is the focus of an emerging fishery in the Northwest Atlantic, and it has already become one of the predominant commercial sea cucumber species in terms of landed weight (Hamel and Mercier, 2008). Although growth rates in *C. frondosa* are very low in the wild and in captivity (Hamel and Mercier, 1996a; So *et al.*, 2010), the species is considered to have potential for aquaculture in the North Atlantic due to its high marketability for food and pharmaceutical products and because much of its life cycle is well documented (Hamel and Mercier, 1996a, 2008; So *et al.*, 2010). It is currently being explored as an extractive species for integrated multi-trophic aquaculture (Nelson *et al.*, 2012). Several studies have been conducted on sea cucumber ecology in the context of conservation and management efforts (Anderson *et al.*, 2011; MacTavish *et al.*, 2012; Mercier and Hamel, 2009, 2013; So *et al.*, 2010; So *et al.*, 2011; Hamel and Mercier, 1996a, 1996b). However, the lack of an easy and reliable technique to mark individuals has hindered tracking and capture-recapture studies, which provide key

biological information (e.g. movement and migration patterns, growth, estimates of natural mortality). The development of an effective tagging technique that minimizes tissue damage, stress and infections, while maximising retention rates will be of great value in years to come, as this fishery expands and aquaculture develops. Such a tool will benefit the sea cucumber industry worldwide by allowing fishery-oriented and ecological studies using mark-recapture to examine temporal changes in growth, survival and mortality rates, as well as daily and seasonal migrations, localization of breeding populations and determination of habitat preferences in the field (Cieciel *et al.*, 2009; Shiell, 2006).

The difficulties in tagging sea cucumbers are attributed to the plasticity of the body wall, lack of hard tissue, high likelihood of expelling foreign materials and the common occurrence of infections and necrosis around the tagged area (Conand, 1991; Shiell, 2006). Most of the techniques tested so far (summarized in Table 2.1) have yielded limited success and considerable drawbacks (Slater and Carton, 2010; Uthicke *et al.*, 2004). External tags such as T-bars or anchor tags, which are inserted through the body wall using a tagging gun, have shown relatively high retention rates in the first month (Conand, 1991). However, side effects included damage to internal organs, localized necrosis, influx of seawater into the coelomic cavity through the injection hole, evisceration, mortality and high shedding rates in the long-term (Conand and Byrne, 1993). Also, the fact that anchor tags hang outside the animal's body and are usually colourful works to increase tag loss and mortality by predation (Primavera and Caballero, 1992; Shiell, 2006). Scratched and branded numbers, as well as tags glued or sewed on the dorsal epidermis have also been used to mark sea cucumbers, but the incidence of

infection following such procedures is very common (Shiell, 2006). In addition, when the lesions caused by scratches and burns do not evolve into necrosis, the marks disappear within weeks as superficial wounds heal (Reichenbach, 1999; Shiell, 2006).

Alternatively, chemical, genetic and internal tags have been developed. Chemical tags such as fluorochromes involve exposure to tetracycline or calcein which are incorporated in the carbonate structure of ossicles (Purcell and Blockmans, 2009; Purcell *et al.*, 2006). Although the technique is inexpensive, simple and lasting, it does not provide unique identifiers. Furthermore, these chemicals can be toxic, especially for juveniles, the amount of stained ossicles declines over time, and microscopy is required for reading, which makes it unsuitable for a number of applications (Purcell, 2012; Purcell *et al.*, 2006). Importantly, this technique may not be suitable for cold-temperate and polar species, because the uptake of fluorochromes is temperature-dependent (Purcell and Blockmans, 2009). Genetic markers are effective; however, they are impractical for short-term studies, generally expensive, time-consuming, unsuitable for field monitoring and they require extensive analytical skills (Uthicke and Benzie, 2002; Uthicke *et al.*, 2004; Uthicke and Purcell, 2004). As for internal tags such as coded wire tags (CWTS) injected in the body wall or in the coelomic cavity, they must be excised for identification, precluding repeated readings, because the individuals are usually sacrificed (Cieciel *et al.*, 2009).

Passive integrated transponder (PIT) tags have been used successfully in several vertebrates and were recently expanded to marine invertebrates (Hagen, 1996; Purcell *et al.*, 2008; Wilson *et al.*, 2011). PIT tags are tiny inert microchips with an electromagnetic coil encapsulated in glass and a unique code identifier (Wilson *et al.*, 2011). However, the

presence of PIT tags in the coelomic cavity of green sea urchins, *Strongylocentrotus droebachiensis*, was associated with lower growth, gonad index and survival rates compared to controls (Lauzon-Guay and Scheibling, 2008). To our knowledge, only one study has tested PIT tags in sea cucumbers; the tags were injected through the body wall into the coelomic cavity and resulted in low retention rates (Purcell *et al.*, 2008). The possibility of placing PIT tags in other locations and the long-term effects of these tags on sea cucumber health and behaviour remain untested (Cieciel *et al.*, 2009; Shiell, 2006).

Tagging techniques tested on *C. frondosa* so far include various T-bar tags and dyes in the form of visible implant elastomers (VIE), with maximum retention rates of 65% and 80%, respectively, after 140 days (Kirshenbaum *et al.*, 2006). Like fluorochromes, VIE have limited use for individual identification, which can only be achieved by varying the number of implants and their colour combinations. Moreover, long-term applicability was not examined and side effects were not investigated in depth; they are suspected to be consistent with previous studies of similar methods (Table 2.1).

The aim of the present study was to determine if and how PIT tags could be used as a reliable and innocuous marking technique in sea cucumbers. The efficacy of PIT tags implanted in various ways into previously unstudied tissues/organs was tested in two size classes of *C. frondosa* by evaluating retention rates, location of implanted PIT tags and post-tagging side effects on the body wall and on feeding and spawning behaviour during a short (30 d) and long-term experiment (300 d).

2.3 Materials and Methods

2.3.1 Sea cucumber collections

Large adult sea cucumbers with average (± sd; n=120) immersed weight (So *et al.*, 2010) of 11.7 ± 1.5 g, measuring 14.8 ± 1.3 cm contracted body length were trawl-collected by a fishing vessel (commercially licensed by Fisheries and Oceans Canada; DFO) on the southwest Grand Banks of Newfoundland (46°20'43.5" N: 56°23'0.28" W), eastern Canada, at depths between 20 and 30 m. Smaller individuals with average immersed weight of 2.6 ± 1.1 g and measuring 9.6 ± 2.8 cm (n=60) contracted body length were hand collected by divers in Logy Bay (Avalon Peninsula, 47°37'39.6" N: 52°39'51.4" W), at depths between 5 and 10 m. Dive collections were performed by the Field Services of the Department of Ocean Sciences with the required DFO permits. Large and small sea cucumbers were kept in separate 500 L flow-through tanks in ambient seawater (temperature ~2°C and salinity of 35) for over a month before using them in any trial. Only healthy undamaged individuals exhibiting unblemished tegument, firm attachment to the substrate and regular tentacle deployment and retraction (i.e. normal feeding activity) were selected for tagging trials. Natural planktonic food present in ambient seawater was available to sea cucumbers during the study.

2.3.2 Tagging procedures and experimental conditions

For all experimental trials described below, sea cucumbers were tagged while half submerged in a tray filled with seawater at the same temperature as that measured in the experimental tanks. The latter consisted of 24 L containers supplied with ambient running seawater, at a flow of 10 L h^{-1}. Over the course of the study, the water temperature ranged

between 2.5 and 12.5 °C, following seasonal fluctuations in the field. Light was provided through large windows and complemented by fluorescent lights to a maximum intensity of 200 lux following natural photoperiod which ranged from 8L/16D in winter to 15L/9D in summer. The various treatments and controls (detailed for each trial below) were randomly distributed in the experimental tank system.

Certified passive integrated transponder (PIT) tags measuring 8.21 ± 0.05 mm long, 1.29 ± 0.01 mm wide and weighing 29 ± 0.3 mg (AB10320) were purchased from Loligo Systems (Denmark) together with implanters (AB10490), a handheld reader and an external waterproof antenna. Implanters and PIT tags were sterilized with 100% ethanol before the procedure and all tags were tested for readability, both before and immediately after being implanted in the sea cucumbers.

2.3.3 Experimental designs and data analysis

2.3.3.1 Preliminary experiment

A preliminary experiment was conducted in order to test three basic implanting locations as well as tag readability immediately after the procedure. Five large individuals were tagged into the coelomic cavity as per the only previous study using PIT tags in sea cucumbers (Purcell *et al.*, 2008), 5 individuals were tagged dorsally directly in the body wall just underneath the tube feet row, and 5 individuals were tagged through the body wall directly into the aquapharyngeal bulb. Tags in the coelomic cavity were implanted dorsally at mid-body length and released as soon as the implanter had punctured the body wall and the muscle band. However, these tags were not easily read (as they could move freely inside the body cavity), which made them unsuitable for routine post-tagging

identification, and they were generally expelled within 48 h. The coelomic cavity treatment was therefore not retained in subsequent trials. Based on this preliminary experiment, a short-term experiment was devised using variations of the two most promising tagging locations (body wall and aquapharyngeal bulb).

2.3.3.2 Short-term experiment (30 days)

The short-term experiment consisted of 5 treatments (n=12 sea cucumbers each) monitored for 30 days. The whole tagging procedure (for all individuals and treatments) was completed inside 2 h (taking an average of 2 min per sea cucumber). Only large sea cucumbers (size range provided above) were used. They were distributed in 30 tanks, using 6 tanks per treatment and 2 individuals per tank, for a total of 60 individuals. Treatments consisted of: (1) tagging in body wall, (2) tagging in aquapharyngeal bulb, (3) control for tagging procedure in body wall, (4) control for tagging procedure in aquapharyngeal bulb and (5) handling control. In the first treatment, sea cucumbers were tagged dorsally in the body wall (TBW) underneath the row of tube feet between the epidermis and the longitudinal muscle band at mid body length. Care was taken not to release the tag in the coelomic cavity or to implant it too superficially in the tegument where it could tear the epidermis and be lost or rejected rapidly, although the tissue layer in which the tags were implanted was not confirmed until the sea cucumbers were later dissected. In the second treatment, sea cucumbers were tagged directly in the aquapharyngeal bulb (TAB), by inserting the implanter 1 cm posterior to the oral cavity, at an angle of ~45°. For this treatment, the tag was released immediately after the implanter had punctured the body wall and a second puncture was felt, suggesting that the

aquapharyngeal bulb had been reached (however there was no immediate confirmation that the tag was in the aquapharyngeal bulb rather than in the digestive tract or coelomic cavity). Treatments 3 to 5 were devised to control for the effects of puncturing or handling the sea cucumbers. The third treatment consisted of sea cucumbers punctured in the body wall (PBW), as in treatment one, without any tag being released. The fourth treatment comprised sea cucumbers punctured in the aquapharyngeal bulb (PAB), as in treatment two, but not tagged. Finally, the fifth treatment (Control) consisted of an overall handling control as sea cucumbers were neither tagged nor punctured but simply handled as the implanted sea cucumbers (placed in the surgical tray for 2 min and transferred to the experimental tank).

Apart from tag retention, side effects such as contraction of the entire body and appearance of unusual tension such as ripples in specific areas of the body wall were noted, together with the duration of such effects. Evisceration and lesions, as well as other morphological, physiological and behavioural responses that can provide an indication of stress or suboptimal health in sea cucumbers were monitored. Healthy sea cucumbers were expected to anchor firmly to the substrate, to respond to food by deploying their tentacles, to display the typical escape response to their main predator, and to release gametes during the spawning season. The main indicators that were routinely recorded are described in Table 2.2.

Sea cucumbers were monitored every hour for the first eight hours post tagging, four times a day during the first week, three times a day in the third week and twice a day in the fourth week. On each occasion, still-implanted and shed tags were read, and side effects (if any) noted (Table 2.2). At the end of the 30 days, a natural predator of *C.*

frondosa, the sea star *Solaster endeca* (So *et al.*, 2010), was placed over each sea cucumber in order to assess and time its escape response (Legault and Himmelman, 1993; So *et al.*, 2010), such as contraction, elongation and swelling, for 10 min (Table 2.2). Finally, at the end of the trial, a microscopic investigation was conducted on those individuals that had retained their tags. Tags in the body wall were located with the reader and the surrounding tissue was isolated. Dissections were conducted by slicing thin layers of the tissue until the tags were revealed. Tags in the aquapharyngeal bulb were localized by removing and dissecting the aquapharyngeal bulb. All dissections were conducted under a stereomicroscope (Nikon SMZ1500) coupled to a digital camera (Nikon DXM1200F). Pictures were taken and the exact position of the tag in the tissue layers was determined.

Logrank survival analysis ($\alpha = 0.05$) was used to evaluate differences in PIT tag retention rates among treatments at intervals of 15 days (Jason *et al.*, 2010). The proportion of tags retained was estimated with the Kalpan-Meier estimator followed by multiple comparisons using the Holm-Sidak test (Cieciel *et al.*, 2009). In order to test the hypothesis that any post-tagging swelling was caused by the implanted tag itself instead of the tagging procedure, the total number of observations in which sea cucumbers displayed post-tagging swelling was compared among treatments after 30 d. Data on swelling response violated the assumptions for use of parametric statistics even after transformation. For this reason, Kruskal-Wallis one-way ANOVA on ranks ($\alpha = 0.05$) was used to compare differences in these responses among treatments, followed by Tukey tests as appropriate. To test whether or not the implanted tag or the tagging procedure influenced the time needed by sea cucumbers to display an escape response toward the

presence of a predator, one-way ANOVA was used to compare treatments. Reaction such as elongation was Log_{10} transformed to achieve normality (determined by Shapiro-Wilk test). Contraction of the body was not statistically analyzed, because all sea cucumbers immediately contracted their body when the predator was placed over them. In addition, swelling of the body, as a response to the predator, was also not analyzed, because it occurred in all treatments after the observation period of 10 min, when the sea stars were removed from the tanks.

2.3.3.3 Long-term experiment (300 days)

Based on the findings of the preliminary and short-term experiments, a long-term experiment (300 days) was conducted using and refining the most promising techniques and locations. Two treatments were devised, each with 30 large and 30 small sea cucumbers (size ranges previously outlined). The first treatment involved tagging 30 large and 30 small sea cucumbers dorsally in the body wall (LBW and SBW, respectively). The other treatment consisted of tagging 30 large and 30 small sea cucumbers in the aquapharyngeal bulb but via a deployed oral tentacle (LT and ST, respectively). The latter technique was developed to refine the aquapharyngeal bulb tagging method previously tested in the short-term experiment. Limited retention rates had been obtained due to improper tag placement; a new implantation method was developed to ensure that the tag found its way into the aquapharyngeal bulb via the oral tentacles. The deployment of feeding tentacles was elicited (within ~12 min) by adding live phytoplankton (*Chaetoceros calcitrans*) at a concentration of 5×10^5 cell ml^{-1} to holding tanks. One tentacle was gently held with a flat edge tweezer and the PIT tag implanted at its base,

helping the tag to find its way towards the aquapharyngeal bulb via the hydrovascular system (minimizing the possibility of implantation into the coelomic cavity or digestive tract).

Individuals were monitored for tag retention and side effects once a month for a total of ten months (300 days) using the same criteria as in the short-term experiment (Table 2.2). However, the occurrence of mortalities, skin lesions and evisceration was monitored daily. Since the long-term experiment encompassed the spawning season of *C. frondosa*, gamete release or the presence of either oocytes or sperm in the tanks was also noted.

X-ray photographs were taken of a subset of sea cucumbers that were still tagged after 240 days. Three individuals tagged in the body wall and three individuals tagged in the tentacles were photographed with a Lixi X-ray (Model PS-500 OS) to visualize the location of the PIT tags. The sea cucumbers were placed in trays filled with seawater from their holding tanks and positioned inside the x-ray machine. The photographs took about 2-3 min per sea cucumber. Using these images as guides, all other individuals that were still tagged at the end of the long-term trial (300 days) were dissected for a finer tag location analysis. Individuals tagged in the body wall were dissected as described in the previous section. Sea cucumbers tagged in the aquapharyngeal bulb via the tentacles were dissected by removing the aquapharyngeal bulb, taking care not to damage any structure. A visual evaluation of the aquapharyngeal bulb was carried out to detect any abnormality that might be associated with the implanted tags. Longitudinal cuts were then made until the tag was found.

PIT tag retention rates in the long-term experiment were compared among treatments at two intervals: day 30 (to link with the short-term experiment) and day 300 (at the end of the experiment) by using logrank survival analysis (Jason *et al.*, 2010). The proportion of tags retained was estimated with the Kalpan-Meier estimator followed by multiple comparisons using the Holm-Sidak test.

2.3.3.4 Monitoring cloacal openings and feeding behaviour

After selecting the methods that yielded the highest tag retention rates, this trial was designed specifically to measure the time needed for the sea cucumbers to recover normal rates of water change in the respiratory tree after the tagging procedure. Large sea cucumbers were either tagged or punctured in the body wall (TBW and PBW, respectively) and in the tentacles (TT and PT, respectively) using the techniques applied in the long-term experiment described above. They were compared with control sea cucumbers that were just handled without being tagged (Control). This experiment used 12 individuals per treatment (4 per tank). They were acclimated in the tanks 5 days prior to the onset of the experiment, and monitored for 3 days post tagging.

Cloacal movement was monitored as an indicator respiration rate, i.e. water circulation in the respiratory tree where oxygenation occurs (Doyle and Mcniell, 1964), by counting the number of cloacal openings within 2 min every 5 h for 3 consecutive days. Monitoring started 10 h before tag implantation to determine baseline respiration rates in all individuals immediately before the trial.

The potential effects of PIT tags on the feeding behaviour was evaluated by adding phytoplankton to all treatments at the end of the 3-day trial and the number of sea

cucumbers exhibiting tentacle deployment and insertion into the mouth (Table 2.2) was monitored 30 min and 1 h after stimulation. Live phytoplankton (*Chaetoceros calcitrans*) identified as a food source in *C. frondosa* (Hamel and Mercier, 1998) was used at a concentration of 5 x 10^5 cell ml^{-1}. The water flow was interrupted during this brief procedure to keep the concentration of algae high.

In order to test the hypothesis that the increase in respiration rates caused by the tagging procedure is short-lived, the number of cloacal opening per minute (respiration rate) of each sea cucumber was compared among treatments. Data violated the assumptions for use of parametric statistics even after transformation. For this reason, Kruskal-Wallis one-way ANOVA on ranks ($\alpha = 0.05$) was used to compare differences in this response among treatments, followed by Tukey tests, at intervals of 5 h. Also, to determine whether or not the implanted PIT tag affects the tentacle deployment of sea cucumbers, the total number of sea cucumbers deploying their tentacles as a positive feeding response was compared among treatments 30 min and 1 h after the addition of the phytoplankton to the tanks using one-way repeated measures ANOVA.

2.4 Results

2.4.1 Tag retention rates

In the short-term experiment, sea cucumbers tagged in the body wall had a retention rate of 100% in the first 15 days and 41% after 30 days (Fig. 2.1a). Individuals tagged directly in the aquapharyngeal bulb started to shed tags in the day following implantation. Retention rate for this group dropped rapidly to 26% after 15 days (Fig. 2.1a). There was no shedding over the following 9 days but only 8% of the sea cucumbers

remained tagged for the full 30 days. Retention rates in the body wall were significantly higher than in the aquapharyngeal bulb after 15 days, although no difference between the two locations was noted 30 days post tagging (Table 2.3).

Retention rates in the long-term experiment varied among treatments and were generally higher than during the short-term trial after the first 30 days (Fig. 2.1b). Large sea cucumbers tagged via tentacles into the aquapharyngeal bulb exhibited the highest retention rate throughout the trial, i.e. 92% after 30 days and 68% at the end of the trial. For small sea cucumbers tagged in the tentacles the retention rate was 84% after 30 days and 42% at the end of the experiment (Fig. 2.1b). In both large and small individuals, tag loss occurred only in the first 150 days whereas tag retention remained unchanged over the next 150 days. Retention rates for large sea cucumbers tagged in the body wall were 76% in the first 30 days and 33% at the end of the experiment. The retention rate of small sea cucumbers tagged in the body wall was the lowest measured in all treatments; it decreased quickly, reaching 77% at the end of the first month and dropping further to 10% after 300 days (Fig. 2.1b). During the first 30 days, retention rates in large individuals tagged in the tentacles were significantly higher than in large and small individuals tagged in the body wall, but did not differ statistically from those in small individuals tagged in the tentacles. From day 31 until day 300, large sea cucumbers tagged in the tentacles had significantly higher retention rates than all other treatment groups (Table 2.3).

2.4.2 Tag location

X-ray photographs showed clearly that the tags implanted at the base of an oral tentacle were all lodged in the vesicle of the tentacle, inside the aquapharyngeal bulb (Fig. 2.2). However, x-ray photographs of PIT tags implanted in the body wall were inconclusive given the absence of visually recognizable organs around them (all soft tissues).

Dissections confirmed that the tags implanted in the aquapharyngeal bulb via a tentacle were always located in the tentacle vesicle (Fig. 2.3a). The tags were free in the vesicle, unattached to any tissue (Fig. 2.3b). Tags that were retained in the body wall were implanted 1.3 ± 0.3 mm (n=5) from the surface of the external epithelium, inside connective tissue (Figs. 2.3d and 2.3e).

2.4.3 Side effects of tagging

The tentacle vesicles where the PIT tags were found in the aquapharyngeal bulb were similar in terms of color and shape to the vesicles without tags. Brown bodies could also be seen in vesicles with and without tags (Figs. 2.3a and 2.3b). No scars could be seen in the epidermis or in the connective tissue layers of individuals tagged in the body wall or in the aquapharyngeal bulb via the tentacles. No sign of either infection or abnormal cell development (e.g. proliferation of fibrous cells) was observed in the tissue surrounding the tags (Figs. 2.3c and 2.3f).

Physiological and behavioural side effects were few; those that were noted appeared immediately after the tagging procedure and were short lived. The first observed side effect was a contraction of the entire body immediately after the puncture of the

implanter, irrespective of whether or not a tag was inserted during the procedure. Individuals in the handling control group also showed the same contraction after being handled for measurements. Another nearly immediate side effect took the form of ripples along the body wall in 42 ± 9% of the sea cucumbers in the short-term experiment and 17 ± 6% during the respiration rate experiment (Fig. 2.4a). This effect was limited to individuals tagged in the body wall. Sea cucumbers in all treatments managed to anchor themselves on the tank bottom and regain a normal posture 16.0 ± 1.2 min post procedure. About 30 min post tagging, swelling of the whole body occurred in 42 ± 3% of tagged and punctured individuals, but did not persist for more than 20 h and was only observed in the short-term experiment. In addition, there was no statistical difference among any of the treatments ($H= 7.939$, $df=4$, $p=0.094$).

Another common post-tagging behaviour was related to rates of cloacal movement, as a proxy of respiration rate (Fig. 2.5). Acclimated sea cucumbers showed rates between 0.9 - 1.0 cloacal opening min^{-1} before the tagging procedure (time -10 h) and they did not differ statistically among treatments (Table 2.4). When sea cucumbers were either tagged or punctured, rates immediately increased to 1.6 ± 0.2 cloacal opening min^{-1} in individuals tagged in the body wall and to 1.5 ± 0.3 cloacal opening min^{-1} in individuals punctured in the body wall, whereas values in the control group remained lower at 1.1 ± 0.3 cloacal opening min^{-1} (Fig. 2.5a). The increase was similar for tagged and punctured sea cucumbers in the body wall, both showing faster rates than the control group (Table 2.4). Similar results were found with individuals tagged and punctured in the tentacles, with post tagging increases to 1.5 ± 0.3 and 1.6 ± 0.2 cloacal opening min^{-1}, respectively (Fig. 2.5b; Table 2.4). Five hours post tagging, rates of cloacal movement

exhibited by individuals tagged in the body wall were no longer different from the control group (Fig. 2.5a). Cloacal movements of sea cucumbers punctured in the body wall stabilised to control levels within 15 h post tagging (Table 2.4). Values for individuals tagged and punctured in the tentacles levelled back to control levels 5 h earlier than sea cucumbers tagged or punctured in the body wall (Fig. 2.5b). Fluctuations in cloacal opening rates were thereafter similar in all treatment groups (Table 2.4).

Addition of phytoplankton provoked an increase in tentacle deployment in sea cucumbers tagged in the body wall and tentacles, as well as in the control and punctured sea cucumbers (Fig. 2.4b). The number of sea cucumbers with tentacles deployed did not vary significantly among treatments ($F_{4,49}$=1.702, p=0.242).

Measurements of reaction time in the presence of a natural predator at the end of the short-term experiment revealed that punctured and tagged sea cucumbers behaved similarly. When the sea star was placed on their dorsal surface, the first response was the contraction of the body, thereby increasing mid-body circumference and decreasing total length. The second response was the elongation of the body. The ANOVA analyses did not reveal any statistical difference among treatments in the time needed to initiate the escape response ($F_{4,55}$=0.265, p=0.899), which was on average 1.8 ± 0.3 min in all individuals and treatments. The final behaviour observed was the swelling of the body and production of mucus in all individuals from all treatments; this occurred as soon as the predator was removed. The effect lasted 30 to 40 min before sea cucumbers regained their original size in all treatments.

The long-term experiment covered the spawning season of *C. frondosa*. Sea cucumbers started to spawn 40 days after the tagging procedure. Fertilized oocytes could

be observed in 60% of the tanks hosting sea cucumbers tagged in the tentacles (Fig. 2.4c) and in 70% of the tanks with individuals tagged in the body wall (Fig. 2.4d). Oocytes and sperm were seen in tanks hosting both small and large tagged sea cucumbers as well as in control tanks.

2.5 Discussion

2.5.1 PIT tag location, retention rate and readability

Tags implanted at the base of the tentacles (to reach the aquapharyngeal bulb) were most effective, with retention rates of 84-92% in the first 30 days, and 42-68% after 10 months. The calcareous ring and a series of valves that mediate the movements of the tentacles appear to trap the tag in the vesicle; individuals properly tagged in the aquapharyngeal bulb showed very stable tag retention, complete functionality of the tentacles during extension and retraction, and normal feeding. This stability presumably results from the fact that the well implanted PIT tags cannot escape from the vesicles. Conversely, tags lost during the first 150 days were likely not injected inside the tentacles and instead found their way into the coelomic cavity where preliminary experiments and other studies (Purcell *et al.*, 2008) showed poor retention rates (discussed below). Some tags may also have come out through the injecting hole (Hamel and Mercier, 1996c, 1999). The stable retention rates measured after 150 days suggest that this method should ensure tagging for several months or years, in addition to making the tags easy to read. This marking method is also innocuous (discussed below). Why retention rates were slightly lower in smaller individuals remains unconfirmed; it may simply be a matter of scaling. PIT tags used in the present experiment (~8 mm long) were the smallest available

on the market at the start of the trials (advertised as 7 x 1.35 mm); however, they are likely too large to fit in the tentacle vesicles of some of the small sea cucumbers (~10 cm contracted body length), based on observations during dissections. This suggests that the eventual availability of smaller tags on the market should improve retention rates in smaller sea cucumbers. The efficacy of other physical (internal and external) tags has only explicitly been tested in full-size adults. Overall, PIT tagging at the base of the tentacles straight into the aquapharyngeal bulb emerges as a promising technique for conducting mark-recapture studies in *C. frondosa*, and possibly other sea cucumbers.

It should be noted that *C. frondosa* belongs to the order Dendrochirotida; it possesses 10 oral tentacles, which are fully extended in the water column during suspension feeding (Hamel and Mercier, 1998). Most other commercial species of sea cucumber belong to the order Aspidochirotida and are deposit feeders with tentacles oriented towards the substrate (e.g. *Holothuria scabra*, *Isostichopus fuscus*, *Apostichopus japonicus*). While the latter generally have shorter oral tentacles, injecting tags at the base of the tentacles or in the hydrovascular system around the mouth should still be feasible and will be investigated in the near future. The size of tentacle vesicles will likely be the most important variable in determining the success (persistence) of PIT tags in various species and sizes of sea cucumbers.

Despite not being the most efficient in the long term, tags implanted in the connective tissue of the body wall exhibited retention rates of 100% over the first 15 days, making them very reliable for short-term studies. This technique is also among the easiest to use, and results in excellent tag readability. Several species of sea cucumbers possess thicker body walls than *C. frondosa*, which would make the procedure very

simple. However, whether body wall thickness would necessarily improve tag retention remains uncertain. The tropical species *Holothuria scabra*, which possesses a very thick body wall, expelled T-bar tags within a month (Purcell *et al.*, 2006), although it should be noted that the latter emerge externally, whereas PIT tags are fully buried in the tissues. Incidentally, placement of the PIT tags inside the body wall proved to be important. Microscopic examination of the persistent tags indicated that they were inserted superficially in the connective tissue and never in the longitudinal muscle bands, presumably preventing them from moving into the coelomic cavity, from which they can be expelled more readily.

Indeed, the least successful tag location in the present study was the coelomic cavity, where PIT tags were both difficult to read and expelled more rapidly. A previous investigation of PIT tag efficacy in two tropical sea cucumbers had only examined injection into the coelomic cavity, with similarly poor results (Purcell *et al.*, 2008).

2.5.2 Side effects of tagging

Overall, the present study did not find any of the major disturbances reported with several of the marking techniques tested to date (summarized in Table 2.1). Dermal sores, skin sloughing, evisceration and death have been documented in sea cucumbers tagged with T-bars, scratches and brands (Cieciel *et al.*, 2009; Primavera and Caballero, 1992; Shiell, 2006). The sores appear to be caused by T-bar tags that slip in and out of the body wall, causing stress and internal damage (Cieciel *et al.*, 2009). Fifty percent of *H. whitmaei* individuals tagged with T-bars developed infected wounds, whereas PIT tags implanted in the coelomic cavity did not elicit any detectable lesions (Purcell *et al.*,

2008). On the other hand, a study with the green sea urchin *Strongylocentrotus droebachiensis* showed that PIT tags in the coelomic cavity resulted in lower rates of feeding, growth, movement, gonadal production and survival (Hagen, 1996; Lauzon-Guay and Scheibling, 2008).

The rare side effects of PIT tags evidenced here in *C. frondosa* were short-lived (< 24 h), similar to minor effects reported when numbers were scratched on the dorsal body wall of *H. whitmaei* (Shiell, 2006). The contraction of the body observed in all treatments was akin to the natural reaction following handling, and was therefore seen in the control group. Ripples around the tagging area were only observed in individuals tagged within the body wall, presumably the result of the recognition of foreign material, and disappeared within a few minutes. Tagging sea cucumbers in the aquapharyngeal bulb via the tentacles did not elicit this response, suggesting a less stressful implanting technique.

The most consistent side effect of PIT tag implantation in *C. frondosa* was the brief increase in rates of cloacal movement (as a proxy of respiration rate) until ~15 h post-tagging. Sea cucumbers tagged within the body wall and in the aquapharyngeal bulb through the tentacles showed very similar patterns. However, tagging sea cucumbers in the tentacles again seemed slightly less stressful, based on a more rapid return to baseline rates. Because punctured and tagged sea cucumbers showed similarly limited side effects, we assumed that the increase in cloacal movement (i.e. stress) was due to the puncture rather than the presence of the tag. Indications of increased metabolic activity after tagging have been documented: *H. whitmaei* with scratched numbers on the body wall, *T. ananas* with T-bar tags and *P. californicus* with 6 different tags showed increased mobility in the field during the first 78 h following marking, compared to sea cucumbers

that were just handled (Cieciel *et al.*, 2009; Conand, 1991; Shiell, 2006). Similarly, *H. scabra* marked with fluorescent dye increased their burying frequency in the first days after the procedure (Purcell and Blockmans, 2009). The need for more frequent renewal of water in the respiratory tree recorded here in *C. frondosa* and higher activity rates observed in other sea cucumbers suggest that stress can be induced by any physical disturbance akin to the attack of a predator, which was shown to increase respiration and movements of sea cucumbers in the field (Shiell, 2006). Increased activity was also reported in *C. frondosa* and other sea cucumbers exposed to predators in the laboratory (Kropp, 1982; Legault and Himmelman, 1993). However, in the case of PIT tagging, the reaction was shorter lived and dissipated quickly.

Another indication of the innocuity of PIT tags in the aquapharyngeal bulb and body wall of *C. frondosa* is the similar escape response displayed by tagged, punctured and control individuals in the presence of their natural predator, *Solaster endeca*. This is important information for mark-recapture and restocking studies, because tagged sea cucumbers should not be more vulnerable to a natural predator as a result of stress caused by the implanted microchip. Also, internal tags like PIT tags do not attract predators like T-bar tags may (Primavera and Caballero, 1992; Shiell, 2006).

The presence of PIT tags in the aquapharyngeal bulb (more precisely in the tentacle vesicles) did not affect the feeding behaviour of *C. frondosa*; tagged and punctured individuals deployed their tentacles and moved them toward the mouth as consistently as the control groups. This is interpreted as a very positive sign since contraction/retraction of the tentacles is known to occur under stress (So *et al.*, 2010). Also, tagged individuals spawned during the same period as undisturbed sea cucumbers.

Stress of capture, tagging and exposure to a new environment have previously been reported to inhibit the ability of sea cucumbers to spawn during the first few weeks in captivity (Morgan, 2000). However, the present study indicates that the presence of PIT tags does not have any major effect on feeding or gamete release, under the conditions and for the durations tested.

2.6 Conclusion

PIT tags present a number of advantages over other marking techniques tested so far; chiefly, they are unique identifiers that can be repeatedly read with minimum disturbance (including underwater). While they are more expensive than some of the other tags, they are also reusable. The sea cucumber *Cucumaria frondosa* responded well, with either minor or no side effects, to the presence of PIT tags in most anatomical structures tested. While retention rates varied with the size of the individuals and the technique/location used to implant the tags, most previous studies have not explicitly investigated the effect of body size on tag efficacy, making it impossible to determine whether PIT tags present any advantage/disadvantage in this regard. Overall, implanting PIT tags at the base of the tentacles to reach the aquapharyngeal bulb emerges as one of the most effective techniques ever developed for tagging sea cucumbers reliably and innocuously for long periods, allowing individual marking and repeated identification without requiring emersion, elaborate analyses or lethal manipulations. Embedding PIT tags in the body wall also yields good results, especially over short periods. In both cases, side effects were rare, minor and of short duration, and the presence of PIT tags did not affect feeding, spawning or escape responses to a natural predator. In addition, there were no

records of developing wounds, necrosis or death as a result of tagging. Arguably, further investigations need to be carried out to confirm the suitability of the techniques outlined here in other holothuroids, including aspidochirotes. But taken together, the study provides promising data on the contextual efficacy of PIT tags and identifies means of minimizing side effects of tagging procedures in sea cucumbers. It will hopefully assist fishery, ecological and conservation studies and the sustainable development of sea cucumber aquaculture worldwide.

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2.9 Tables

Table 2.1: Summary of markers tested in sea cucumbers with retention rates and drawbacks of each tagging technique.

Type	Technique	Species tested	Maximum retention rate	Drawbacks	References
External tags	T-bars (through body wall)	*Actinopyga echinites*	5% after 1 year	Evisceration, mortality	(Conand, 1991)
		Actinopyga mauritiana	5% after 1 year	Evisceration, mortality	(Conand, 1991)
		Actinopyga miliaris	60% after 8 days	Necrosis, infection	(Purcell *et al.*, 2008)
		Cucumaria frondosa	65% after 140 days	Damaged internal organs	(Kirshenbaum *et al.*, 2006)
		Holothuria nobilis	5% after 1 year	Evisceration, mortality, reduced growth, increased mobility in the field	(Conand, 1991)
		Holothuria scabra	5% after 1 year	Evisceration, mortality, reduced growth, increased mobility in the field	(Conand, 1991)
		Holothuria scabra	0% after 30 days	Evisceration	(Purcell *et al.*, 2006)
		Holothuria lessoni (as *H. scabra versicolor*)	5% after 1 year	Evisceration, mortality, reduced growth, increased mobility in the field	(Conand, 1991)
		Holothuria whitmaei	50% after 8 days	Necrosis, infection	(Purcell *et al.*, 2008)
		Parastichopus californicus	40% after 224 days	Skin sloughing, open sores, mortality, increased mobility in the field	(Cieciel *et al.*, 2009)
		Stichopus herrmanni (as *S. variegatus*)	5% after 1 year	Evisceration, mortality, reduced growth, increased mobility in the field	(Conand, 1991)

		Thelenota ananas	5% after 1 year	Evisceration, mortality, reduced growth, increased mobility in the field	(Conand, 1991)
	Scratches/brands (on body wall)	*Actinopyga mauritiana*	100% up to 60 days	Reduced growth	(Ramofafia *et al.*, 1997)
		Holothuria fuscogilva	100% up to 30 days	Necrosis	(Reichenbach, 1999)
		Holothuria scabra	100% up to 10 days	Side effects not detected or not studied	(Mercier *et al.*, 2000)
		Holothuria whitmaei	100% up to 21 days	Necrosis, mark disappears, increased mobility in the field	(Shiell, 2006)
Internal tags	Coded wires (in coelomic cavity and body wall)	*Actinopyga echinites*	100% after 63 days	Side effects not detected or not studied, difficult to use in the field, individuals need to be sacrificed	(Lokani, 1992)
		Holothuria fuscogilva	60% after 63 days	Side effects not detected or not studied, difficult to use in the field, individuals need to be sacrificed	(Lokani, 1992)
		Holothuria scabra	33-53% after 1 year	Mortality, difficult to use in the field, individuals need to be sacrificed	(Purcell *et al.*, 2006)
		Parastichopus californicus	37% after 224 days	Increased mobility in the field, difficult to use in the field, individuals need to be sacrificed	(Cieciel *et al.*, 2009)
		Thelenota ananas	100% after 63 days	Side effects not detected or not studied, difficult to use in the field, individuals need to be sacrificed	(Lokani, 1992)
	PIT tags in coelomic cavity	*Actinopyga miliaris*	0% after 8 days	Side effects not detected or not studied	(Purcell *et al.*, 2008)
		Holothuria whitmaei	25% after 8 days	Side effects not detected or not studied	(Purcell *et al.*, 2008)
	PIT tags in tentacles	*Cucumaria frondosa*	92% after 30 day and 68% after 300 days	See text	Present study
	PIT tags in body wall	*Cucumaria frondosa*	41% after 30 days and 33% after 300 days	See text	Present study

Chemical tags	Visible implant elastomers	*Cucumaria frondosa*	80% after 140 days	Side effects not detected or not studied, difficult to use in the field, no unique identifier	(Kirshenbaum *et al.*, 2006)
	Fluorochrome in ossicles	*Holothuria scabra*	100% after 1 year	Mortality, reduced growth, increased burying behaviour, toxicity, no unique identifier, unstable in sunlight and cold water	(Purcell *et al.*, 2008; Purcell and Blockmans, 2009; Purcell *et al.*, 2006)
Genetic tags	DNA	*Holothuria whitmaei* (as *H. nobilis*)	No retention rate	Difficult to use in the field, expensive	(Uthicke and Benzie, 2002; Uthicke and Purcell, 2004; Uthicke *et al.*, 2004)

Table 2.2: Morphological, physiological and behavioural indicators of sea cucumber health monitored during the present study.

Indicator	Description	Tested in
Ripple	Small undulation visible on the sea cucumber body at the site of implantation or puncture (Fig. 2.4a).	Short and long-term experiments
Skin lesion	Occurrence of tissue damage usually visible as different coloration than the surrounding tissue; may be the result of infection or immune reaction.	Short and long-term experiments
Anchorage	Firm attachment to bottom or side of the tank with the ventral podia as determined when individual cannot be dislodged with gentle poking.	Short-term experiment
Swelling	Abnormal enlargement of the sea cucumber body into a balloon shape.	Short and long-term experiments
Contraction	Contraction of the entire body through the action of longitudinal muscle bands.	Short and long-term experiments
Elongation	Lengthening of the body caused by extension of the muscle bands.	Short and long-term experiments
Evisceration	Total or partial extrusion of internal organs such as intestine, gonads and/or respiratory tree.	Short and long-term experiments
Cloacal opening	Number of times cloaca opens and closes in given interval of time, as water circulates through the respiratory tree.	Cloacal opening and feeding behaviour experiments
Tentacle deployment (feeding)	When all ten oral tentacles are fully extended in order to capture food in the water and one tentacle is introduced into the mouth (Fig. 2.4b).	Cloacal opening and feeding behaviour experiments
Spawning event	Presence of oocytes and/or spermatozoa in the tanks (Figs. 2.4c and 2.4d).	Long-term experiment
Escape response	Initiation of reactions such as contraction, elongation and swelling in the presence of a predator.	Short-term experiment

Table 2.3: Statistical comparison of PIT tag retention rates among treatments in the short-term and long-term experiments. Results of Logrank survival analysis followed by Holm-Sidak test after 15 and 30 days (short-term) and after 30 and 300 days (long-term). TBW, tagged in body wall; TAB, tagged in aquapharyngeal bulb (directly); LT large individuals tagged in tentacle (to aquapharyngeal bulb), ST, small individuals tagged in tentacle (to aquapharyngeal bulb); LBW, large individuals tagged in body wall; SBW, small individuals tagged in body wall. Significant results are shown in bold.

Experiment	**Time**	**Retention rates among treatments**	χ^2	**F**	**df**	**p**
Short term	15 d	**TBW > TAB**	**8.000**		**1**	**0.005**
	30 d	TBW = TAB	1.648		1	0.199
Long term	30 d	**LT × ST × LBW × SBW**	**11.257**		**3**	**0.001**
		LT = ST		0.808		0.146
		LT > LBW		**9.468**		**<0.001**
		LT > SBW		**10.743**		**<0.001**
		ST = SBW		0.549		0.212
		LBW = SBW		0.208		0.648
		ST = LBW		0.513		0.276
	300 d	**LT × ST × LBW × SBW**	**233.137**		**3**	**<0.001**
		LT > ST		**53.364**		**<0.001**
		LT > LBW		**63.280**		**<0.001**
		LT > SBW		**225.767**		**<0.001**
		ST > SBW		**64.693**		**<0.001**
		LBW > SBW		**56.408**		**<0.001**
		ST =LBW		0.372		0.542

Table 2.4: Statistical comparison of cloacal movements (respiration rates) among treatments. Results of one-way repeated measures ANOVA on ranks, followed by Tukey tests. The increase in cloacal opening rates of tagged and punctured sea cucumbers in the body wall (TBW, PBW) and in the tentacles (TT, PBW) was compared to the control group (C) at various intervals post tagging. Sea cucumbers tagged at the base of oral tentacles recovered normal rates 5 h earlier than individuals tagged in the body wall. Significant results are shown in bold.

Experiment	**Time (h)**	**Cloacal opening rates among treatments**	**H**	**df**	**p**
Body wall	0	**TBW x PBW x C**	**17.382**	**2**	**<0.001**
		TBW > C			**0.006**
		PBW > C			**<0.001**
		TBW = PBW			0.857
	5	**TBW x PBW x C**	**22.565**	**2**	**<0.001**
		TBW = C			0.109
		PBW > C			**<0.001**
		TBW = PBW			0.104
	10	**TBW x PBW x C**	**26.42**	**2**	**<0.001**
		TBW = C			0.053
		PBW > C			**<0.001**
		TBW = PBW			0.077
Tentacles	0	**TT x PT x C**	**15.192**	**2**	**<0.001**
		TT > C			**<0.001**
		PT > C			**<0.001**
		TT = PT			0.887
	5	**TT x PT x C**	**10.107**	**2**	**0.006**
		TT > C			**<0.001**
		PT > C			**<0.001**
		TT = PT			0.834

2.10 Figures

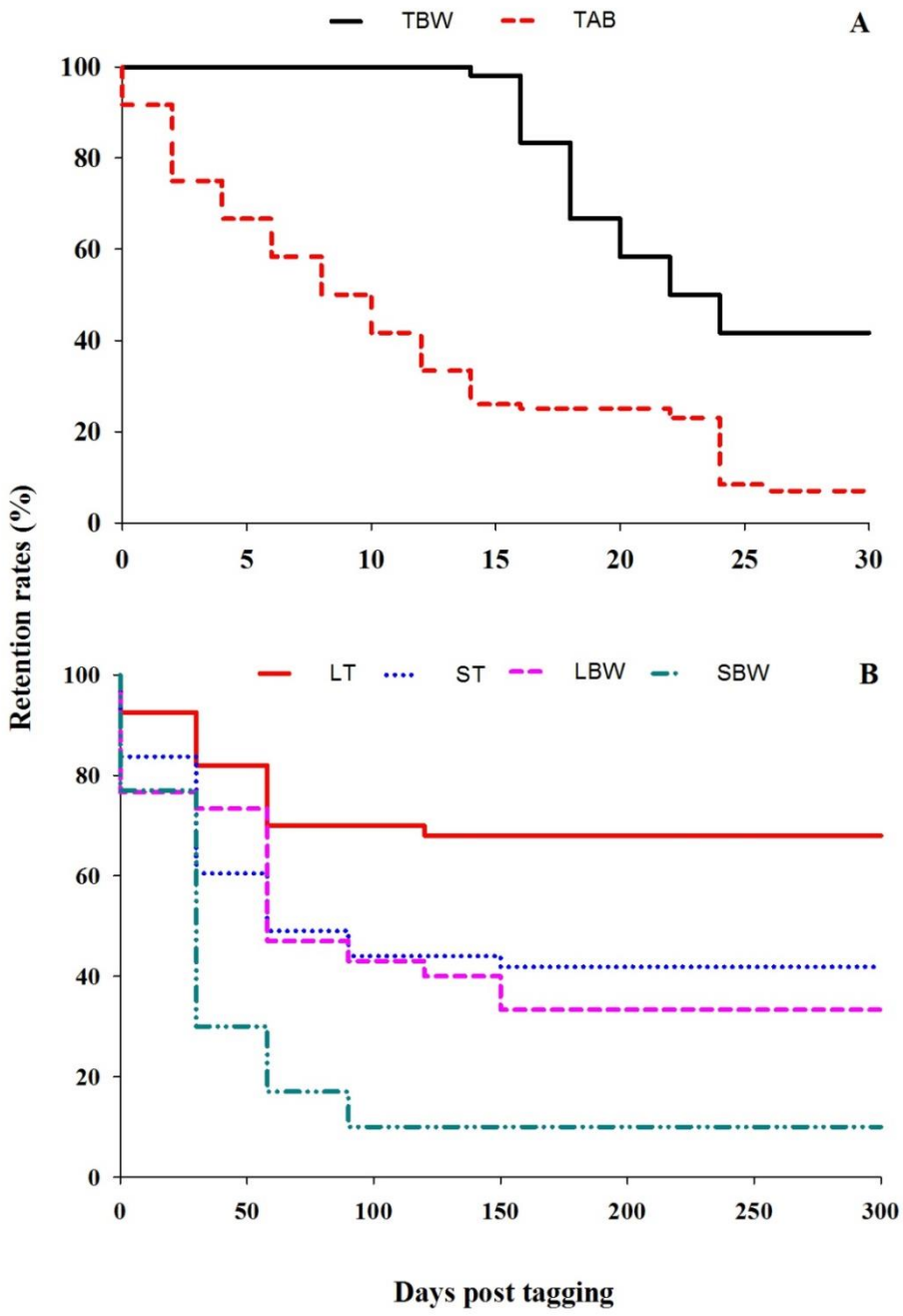
TBW
TAB
A
100
80
60
40
20
0
0
5
10
15
20
25
30
Retention rates (%)
LT
ST
LBW
SBW
B
100
80
60
40
20
0
0
50
100
150
200
250
300
Days post tagging

Figure 2.1 (previous page): PIT tag retention rates in sea cucumbers. (A) Retention rates of tags implanted in the body wall and aquapharyngeal bulb during the short-term experiment. (B) Retention rates of tags implanted in the tentacles and body wall during the long-term experiment. TBW, tagged in body wall; TAB, tagged in aquapharyngeal bulb (directly); LT, large individuals tagged in tentacle (to aquapharyngeal bulb); ST, small individuals tagged in tentacle (to aquapharyngeal bulb); LBW, large individuals tagged in body wall; SBW, small individuals tagged in body wall.

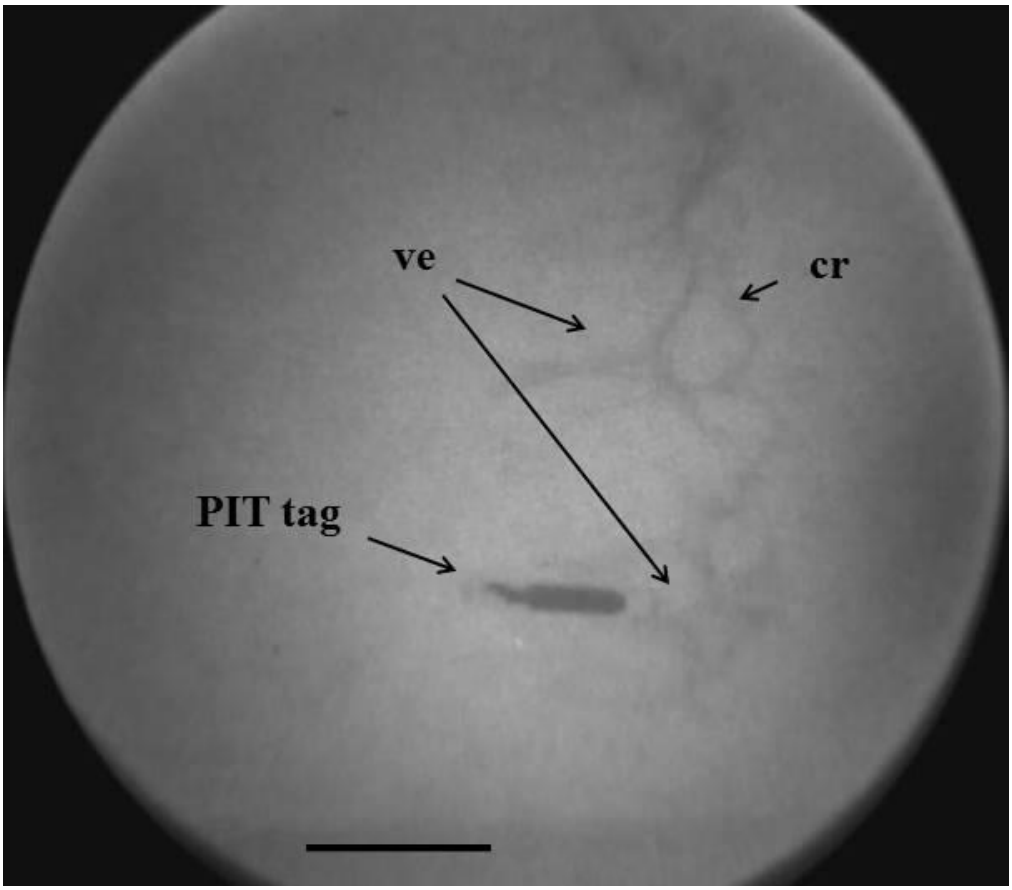

Figure 2.2: X-ray photograph of sea cucumber tagged in the aquapharyngeal bulb. PIT tags successfully implanted in the aquapharyngeal bulb through a deployed tentacle lodged themselves in one of the tentacle vesicles (ve) close to the calcareous ring (cr). Scale bars represent 2 cm.

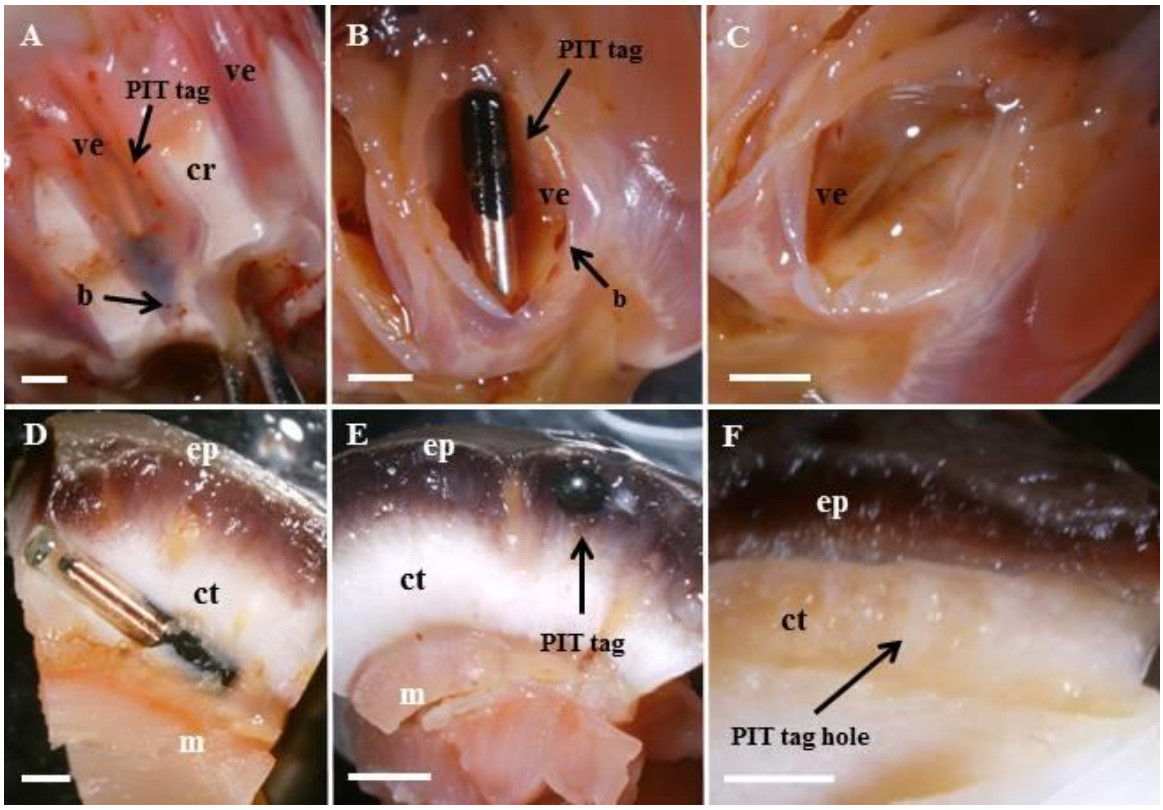

Figure 2.3: Localisation of retained PIT tags. (A-C) Tags retained in the aquapharyngeal bulb were found in the vesicles of the tentacles (ve). The calcareous ring (cr) and brown bodies (b) are identified. (D-F) Tags retained in the body wall were implanted in the connective tissue (ct) between the epidermis (ep) and the longitudinal muscle bands (m) below the ambulacral podia. Scale bars represent 2 mm.

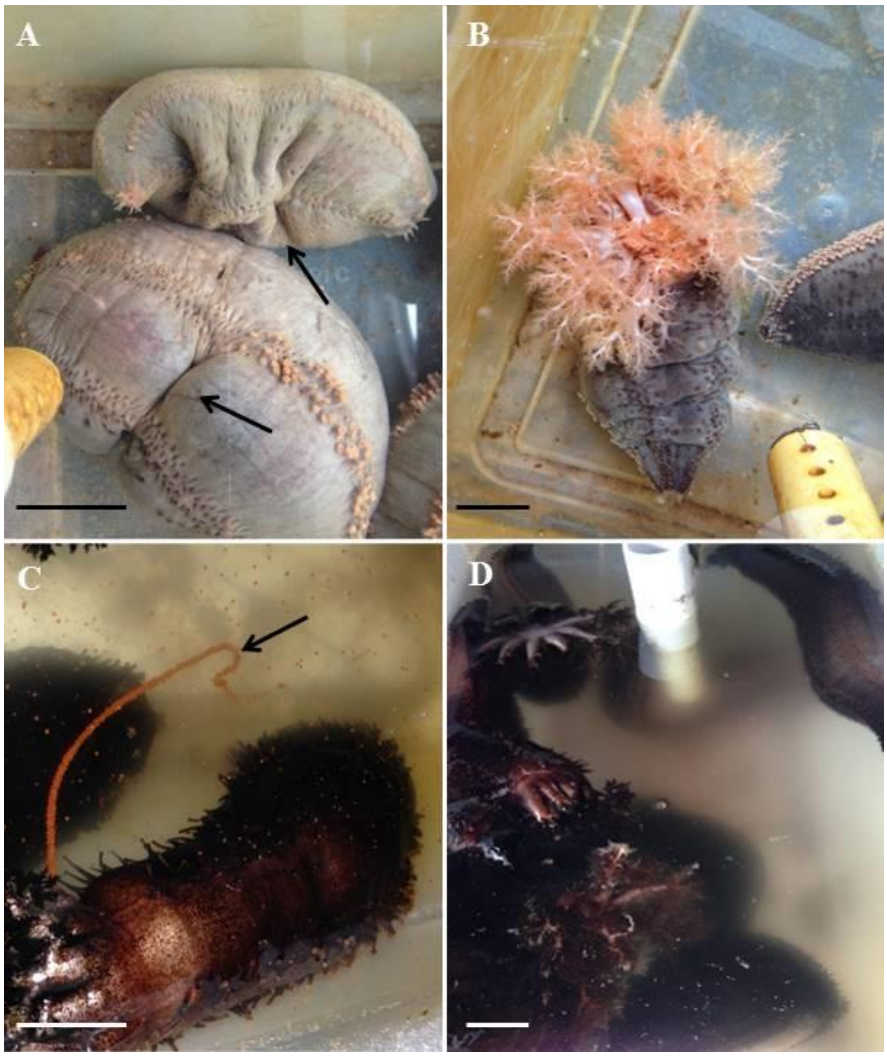

Figure 2.4: Minor side effects and normal behaviours recorded in tagged sea cucumbers. (A) Sea cucumbers tagged in the body wall showing ripples (arrows) around the implantation area immediately after tagging. (B) Large sea cucumbers tagged in the tentacles showed normal feeding, extending their tentacles fully and alternatively inserting them into the mouth. (C) A female sea cucumber tagged in the tentacles is releasing oocytes, visible as a reddish string (arrow), 40 days post tagging. (D) Water clouded with sperm in a tank holding sea cucumbers tagged in the body wall. Scale bars represent 3 cm.

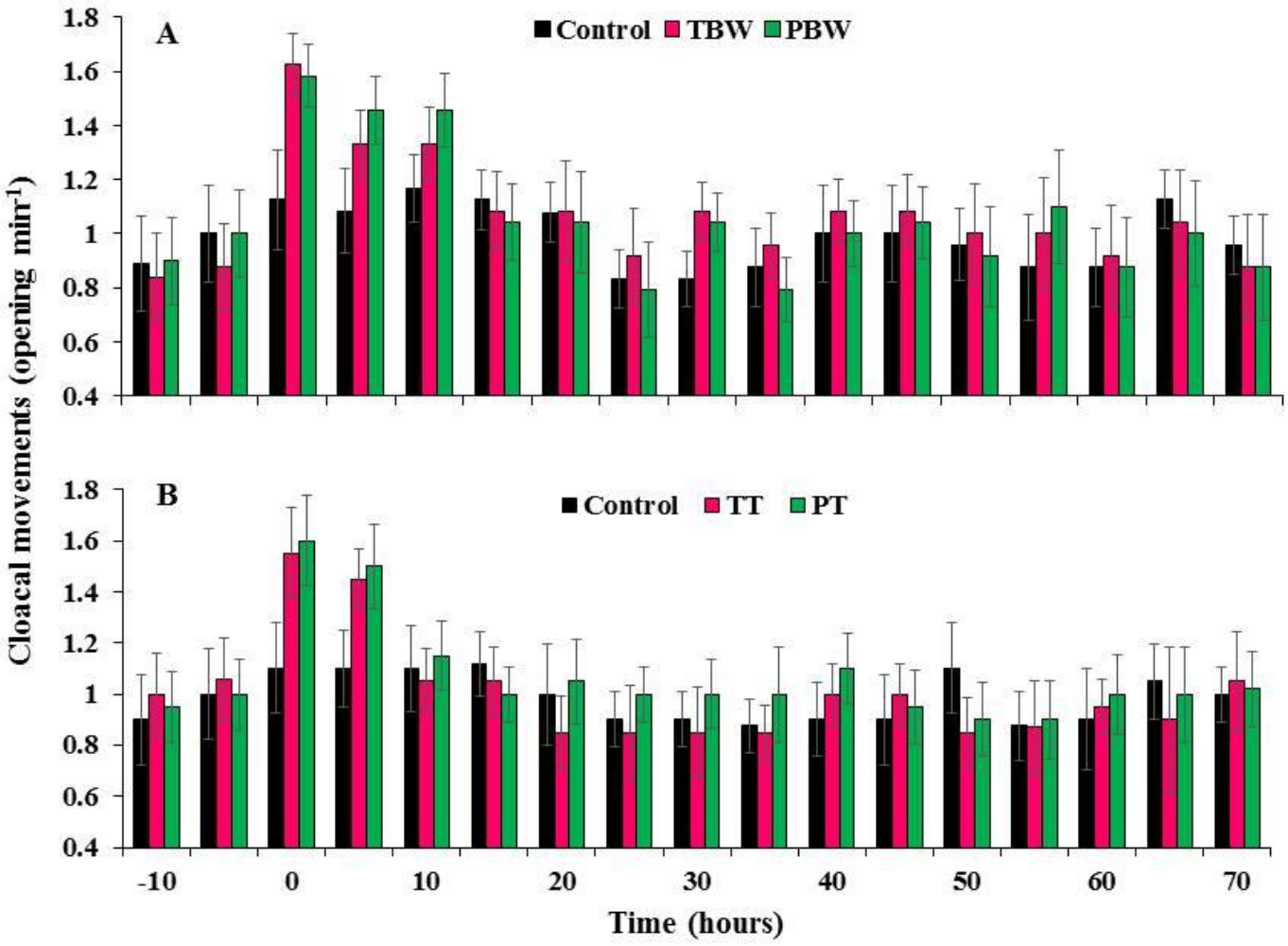
A
Control
TBW
PBW
B
Control
TT
PT
Cloacal movements (opening min-1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
-10
0
10
20
30
40
50
60
70
Time (hours)

Figure 2.5 (previous page): Cloacal movements of tagged, punctured and handled sea cucumbers. (A) Response of sea cucumbers tagged in the body wall. (B) Response of sea cucumbers tagged in the tentacles. Table 2.4 shows statistical results. TBW, tagged in body wall; PBW, punctured in body wall; TT, tagged in tentacle; PT, punctured in tentacle; Control, handled but not tagged or punctured.

Chapter 3: Experimental Test of Optimal Holding Conditions for Live Transport of Temperate Sea Cucumbers[2]

[2] A version of this chapter was submitted to Fisheries Research

3.1 Abstract

Sea cucumber is one of the top five luxury seafoods in Asia and its commercialization primarily revolves around the processed body wall. Hence, live transport and storage of sea cucumbers prior to processing must preserve the condition of the body wall and underlying muscles. Unlike most commercial shellfish on which industry standards are chiefly based, sea cucumbers lack a protective exoskeleton and have the ability to autolyze. Here, we tested the efficacy of different live storage methods on *Cucumaria frondosa*, a commercial species that is widely distributed in the North Atlantic and the Arctic. Current technologies and low-cost variants were experimentally tested under conditions prescribed for the transport of seafood in Canada. Markers of post-storage health, body wall condition and muscle integrity were compared among treatments. The most common method currently in use (icing and salting) yielded the highest rates of mortality and skin necrosis, whereas iced seawater emerged as the best storage condition. The mix of cold seawater with freshwater ice served to maintain conditions similar to those found in the habitat of *C. frondosa*, resulting in healthy appearance of sea cucumbers post-storage and high survival rates. All other media yielded skin necrosis, evisceration of internal organs and mortalities post-storage. These findings should help stakeholders adapt their methodologies to optimize the exploitation of temperate and cold-water sea cucumber resources.

3.2 Introduction

Sea cucumbers have been consumed and used in traditional medicine for centuries in Asia; over the past 50 years they have become one of the most prized seafoods in the

world (Fabinyi, 2012; Purcell, 2014; Yang *et al.*, 2015). The most commonly traded product, known as beche-de-mer or trepang, consists of the sea cucumber body wall (skin), generally including the muscle bands, which is dried and sold as a luxury seafood (Purcell, 2014). In North America, muscle bands are sometimes fresh frozen and marketed separately (Grant *et al.*, 2006; Hamel and Mercier, 2008a). Dried aquapharyngeal bulbs (labelled flowers), liquid or gel extracts and various supplements can also be found on the market. It is believed that the consumption of sea cucumber has significant health benefits (Cheung and Wu, 2012; Bechtel *et al.*, 2013). Studies have shown that the body wall and muscles of sea cucumbers are important sources of high-value compounds exhibiting anticoagulant, anticancer, antioxidant and anti-inflammatory properties (Wen *et al.*, 2010; Bordbar *et al.*, 2011; Xia and Wang, 2015). Because body wall is the chief commercial product, special attention should be given to the storage and transport of live sea cucumbers following harvest in order to maintain their organoleptic and nutritional properties.

Compared to other shellfish, live sea cucumbers have proven difficult to store and transport, because they lack a protective exoskeleton and have the ability to autolyze when they are stressed or taken out of seawater (Duan *et al.*, 2010; Zang *et al.*, 2012). Autolysis is a physiological response which leads to dermis (body wall) degradation through protein breakdown (Wu *et al.*, 2013). Endogenous proteases have been reported to be responsible for the autolysis process, which is often associated with changes in the organoleptic properties of the meat in various marine species (Sun *et al.*, 2013). The formation of new compounds, following lipid oxidation and protein breakdown, alters the color, odour, flavour and texture of the meat (Rodríguez *et al.*, 2009; Ghaly *et al.*, 2010).

During storage, transport, handling and processing, sea cucumbers are exposed to air and UV and undergo abrupt changes in temperature and salinity (Ji *et al.*, 2008; Zhu *et al.*, 2009). These factors may lead to damage of the body wall, promote the development of strong odour and even cause the animal's death (Zhu *et al.*, 2009). Furthermore, the digestive tract secretes enzymes (e.g. trypsin, chymotrypsin and cathepsin) which act in the hydrolysis of collagen (Yan *et al.*, 2014), the main component of the body wall (~70%). Dead or unhealthy sea cucumbers exhibit deteriorated body walls that decrease the final products' quality and result in economic losses (Saito *et al.*, 2002; Wu *et al.*, 2013; Sun *et al.*, 2013).

Market price for sea cucumber is based on many criteria that include general appearance (e.g. color, shape, texture) and smell (Tuwo, 2005; Kinch *et al.*, 2008; Purcell, 2014), all of which can be affected by handling. Storage and transport methods of live marine organism have been investigated in fishes (Berka, 1986; Froese, 1998; Harmon, 2009), crustaceans (Fotedar and Evans, 2011; Barrento *et al.*, 2012) and molluscs (Buzin *et al.*, 2011; Wyatt *et al.*, 2013). The few studies that have been conducted on sea cucumbers are restricted to the transport of hatchery-produced juveniles for restocking programs (Purcell *et al.*, 2006; Zamora and Jeffs, 2014), and more generally apply to tropical species.

The shortage of data on storage and transport of live sea cucumbers for food processing, and on temperate or cold-water species in particular, may be explained by the chiefly artisanal nature of harvesting and processing techniques used in the Indo-Pacific countries, where harvests outside China have traditionally concentrated. Tropical sea cucumbers are generally handpicked, and immediately eviscerated, boiled and dried on

the shore or a nearby site; processing is essentially manual (Hair *et al.*, 2012; Purcell *et al.*, 2013). In China, hatchery-produced juveniles of the temperate species *Apostichopus japonicus* are transported to the restocking sites either in damp sealed plastic bags without seawater or in buckets inside fiberglass tanks full of aerated seawater (Guo *et al.*, 2014; Tan *et al.*, 2014). Depending on the distance from the hatchery to the restocking site and the accessibility, transport can take more than 10 h (Tan *et al.*, 2014).

In recent years, the overexploitation of high-valued sea cucumbers from Asia and the Indo-Pacific has led to the development of new fisheries for under-utilized species around the world (Therkildsen and Petersen, 2006; So *et al.*, 2010; Anderson *et al.*, 2011). The sea cucumber *Cucumaria frondosa* is the focus of an emerging fishery in the North Atlantic. This species is widely distributed in temperate and cold waters, occurring from the Arctic Ocean to Cape Cod as well as along the coasts of northern Europe and Russia (Hamel and Mercier, 2008a). The state of Maine (USA) was the first region to start a commercial fishery for *C. frondosa* in 1980, followed by several eastern Canadian provinces (Hamel and Mercier, 2008a; Rowe *et al.*, 2009), as well as Iceland and Russia (Gudimova *et al.*, 2005; Garcia *et al.*, 2006; Therkildsen and Petersen, 2006; Hamel and Mercier, 2008b). Commercial harvest of *C. frondosa* was initially carried out with scallop gear; eventually, specific drag nets were designed to minimize bycatch and suit local conditions (Barrett *et al.*, 2007). Storage of *C. frondosa* between the fishing wharfs and the processing plants can range from a few hours in Iceland to almost 2 days on the east coast of Canada (personal observation).

With the expansion of sea cucumber fisheries in North and South America and northern Europe, and developing aquaculture ventures, the need to optimize storage and

transport of cold-water and temperate species of sea cucumber from wharfs or farms to processing plants is increasing. Optimum storage conditions that minimize stress and mortalities are of significant value not only for the emerging industry around *C. frondosa* in the North Atlantic, but also for other commercially important temperate species around the world such as *A. japonicus*, *Parastichopus californicus*, *C. japonica*, and *Australostichopus mollis*.

The present study investigated the use of different media for refrigeration during live storage of the sea cucumber *C. frondosa.* Current methods used by the industry and low-cost variants were investigated, following the general guidelines of the fish inspection regulations of Canada (FIR, 2014). Individuals were classified according to health and body wall condition immediately after storage. Measurement of pH of the meat (body wall and muscle bands) were conducted and water quality in the storage tanks assessed. Finally, individuals were monitored post storage for survival and development of skin damage to identify the optimal storing method in order to obtain high-quality body wall and meat products.

3.3 Material and Methods

3.3.1 Sea cucumber collection

Sea cucumbers weighing 8.1 ± 1.3 g (immersed weight) and measuring 12.2 ± 2.4 cm (contracted body length) were collected by divers in Bay Bulls, Newfoundland (47°17’44.6”N, 52°46’8.9”W), eastern Canada, at depths between 5 and 10 m. Dive collections were performed by the Field Services Unit of the Department of Ocean Sciences with the required permits from the Department of Fisheries and Oceans Canada

(DFO). Sea cucumbers were kept in holding tanks with running seawater at ambient temperature (1.2 – 2.1°C) for over a month before using them in any trial. Only healthy and undamaged individuals displaying normal pigmentation and feeding activity, firm attachment to the substrate and no skin lesion were selected.

3.3.2 Experimental conditions and data collection

3.3.2.1 Testing storage of sea cucumbers

The Canadian fish inspection regulations (FIR, 2014) do not explicitly regulate the transport and storage of sea cucumbers. However, it mentions that fish and shrimp that are being transported or held in a preparatory room prior to entering the processing line must be iced or chilled to maintain their temperature not higher than 3-4°C. The same regulation stipulates that trucks designed to transport seafood should be equipped with appropriate insulated/sealed fish containers in order to avoid the discharge of any fluid and effluent during transport (FIR, 2014).

Sea cucumbers were distributed in plastic tanks (0.40 m long x 0.28 m wide x 0.22 m height, total volume of 24 L) without a drainage system in order to retain any water resulting either from the storage media or from the sea cucumbers, as per regulations (FIR, 2014). The tanks were stored for 48 h, representing the maximum time that sea cucumbers are held in fish vats during transport on the east coast of Canada, in a dark, cold room between 1-3°C (FIR, 2014). Sea cucumbers were distributed in one layer of 12 individuals on the bottom of each tank. This design ensured that every sea cucumber had comparable exposure to any storage medium. Triplicate groups of sea cucumbers were submitted to one of six storage treatments: Seawater ice (T1), freshwater ice (T2),

freshwater ice with fish salt (T3), iced seawater (T4) and bagged freshwater ice (T5). The no-medium group (T6) consisted of sea cucumbers stored damp without any ice or water in the experimental tanks. The various transport media tested (Table 3.1) were based on the technology already available, and on potential improvements that would demand only minimal investments from the fishing industry.

Seawater ice used in treatment 1 was made by freezing seawater with salinity of 35 at -20°C for 48 h prior to the start of the experiment. The seawater ice was then crushed manually until it reached approximately the same texture (granule size) as commercial freshwater ice. The freshwater ice used in treatments 2, 3, 4 and 5 was prepared by an ice machine (ITV IQ300Ca). According to the manufacturer, the ice is granular with pieces ranging from 1 to 2 mm in diameter. For treatments 2 and 3, freshwater ice was removed straight from the ice machine and spread on top of the sea cucumbers. Fish salt (Avalon®) was added over the freshwater ice in treatment 3 in order to replicate the method currently used by many companies to transport shrimp, fish and sea cucumber on the east coast of Canada. The amount of fish salt added followed the proportion used by the industry of 6 g L^{-1} and it was spread uniformly over the freshwater ice. Iced seawater used in treatment 4 was prepared by mixing equal amounts of freshwater ice and seawater with salinity of 35 at ambient temperature (2.1°C). The result was a slushy solution with a temperature of 0°C and salinity of 29 (measured immediately after preparation with a YSI® 556 MPS probe). In treatment 5, freshwater ice was kept in plastic bags and placed on the bottom of the tanks with sea cucumbers packed on top of the bags to avoid applying pressure with the weight of the ice. In preliminary experiments with bagged ice, individuals were crushed when bagged ice was placed over them. The

bags were used to avoid direct contact of the sea cucumbers with the ice and melted water while maintaining a low temperature. The amount of ice used in each treatment followed the proportion used by fishing industries in eastern Canada, which is 2 volumes of sea cucumbers for 1 volume of ice.

3.3.2.2 Evaluation of post storage condition

Following the 48-h storage, sea cucumbers were evaluated and classified according to morphological alterations: Signs of evisceration (expulsion of internal organs) and abnormally extruded tentacles (i.e. limply hanging outside the body), frozen body parts (i.e. difference in skin texture along the body wall that may indicate frostbite), excessive odour and mucus production, blisters, skin necrosis and evidence of a swollen body. Alterations to the skin were scored relative to the total surface area they covered on the body of the sea cucumbers. Conditions recorded after storage were classified according to the rubric in Table 3.2. Pictures from the alterations caused by the storage were taken from all treatments for comparison purposes.

Following the initial evaluation, 3 sea cucumbers were sampled haphazardly from each treatment tank. Their muscle bands and body wall were removed and first inspected for colour and texture. Another group of 9 individuals was collected haphazardly from holding tanks and were labelled live non-exposed controls (NE). Following visual inspection, the muscle bands and the body wall from each sample (3 individuals per storage tank and 9 non-exposed controls) were blended using a dilution of 1 g of tissue in 10 ml of deionized water until the solution was smooth and uniform (Benjakul *et al.*, 1997). The final solution was sieved (1 mm mesh size) to remove any remaining particles

and triplicate pH measurements performed at 18°C (air temperature in laboratory) using a YSI® 556 MPS probe.

The remaining sea cucumbers from each treatment were transferred into 24 L flow-through tanks (9 sea cucumbers per tank, 3 tanks per treatment) for a recovery/monitoring period of 30 days. Conditions included a flow of ~10 L h^{-1}, natural photoperiod of 14L/10D, water temperature between 1 and 3°C and ambient salinity of 35. Each tank was monitored daily for survival rates, number of sea cucumbers with tentacles deployed and signs of skin necrosis. Tentacle deployment was used in this study as an indicator of health, because healthy sea cucumbers tend to fully extend their oral tentacles in order to capture food in the water more often than stressed individuals (Hamel and Mercier, 1998). Skin necrosis was used as an indicator of damage caused by the storage condition; it included sloughing of the outer layer of the dermis resulting in the exposure of the white connective tissue. Evisceration of internal organs such as intestine, gonads and/or respiratory tree led to death of the sea cucumbers and eviscerated individuals were thus removed promptly.

3.3.2.3 Water quality in the storage tanks

Water quality was assessed in each storage tank as soon as sea cucumbers had been sampled and transferred to recovery tanks. The water in the storage tank, which resulted either from the storage media and/or from the sea cucumbers, was mixed with a glass rod. Dissolved oxygen, salinity and pH were measured with an YSI® 556 MPS probe. In addition, total ammonia nitrogen was quantified with La Motte® Smart 3 Colorimeter. A measurement was taken in each tank from all 6 treatments, at a water temperature of 3°C.

3.3.3 Data Analysis

The proportion of sea cucumbers under each health category (Table 3.2) immediately after storage (48 h) violated the assumptions for parametric statistics even after transformations. For this reason, Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks ($\alpha = 0.05$) followed by Tukey test was used to compared condition of sea cucumbers among treatments. One-way ANOVA was used to compare pH in the muscle and body wall of sea cucumbers among all treatments, followed by Holm-Sidak tests for pairwise comparisons.

One-way ANOVA was used to compare the proportion of sea cucumbers with tentacles fully extended among treatments (integrated over the period of 30 days). Pairwise comparisons were made using the Holm-Sidak test. Data on the proportion of sea cucumbers that had developed skin necrosis at the end of the recovery period (30 d) violated the assumptions for parametric statistics. Hence, one-way ANOVA on ranks was carried out to compare the proportion of sea cucumbers with skin lesion among treatments, followed by Tukey tests as appropriate. Ammonia nitrogen, dissolved oxygen, water pH and salinity in the tanks after storage (48 h) were compared among treatments using one-way ANOVA followed by Holm-Sidak tests for pairwise comparisons.

Logrank survival analysis ($\alpha = 0.05$) was used to compare survival rates among treatments. Survival rates were estimated with the Kalpan-Meier estimator (followed by multiple comparison Holm-Sidak tests) at the end of storage, as well as on days 10, 20 and 30 of the subsequent recovery period.

Tentacle deployment, skin lesions and survival rates were not assessed in treatment 3 (T3), because all sea cucumbers had died after the storage period of 48 h.

Data in the text are expressed as mean ± standard error. Statistical analyses were conducted with Statistica®.

3.4 Results

3.4.1 Immediate post storage condition

3.4.1.1 Health condition of sea cucumbers in the storage tanks

Treatment 4 (iced seawater) resulted in the highest proportion of sea cucumbers (92 ± 4%) scored as exhibiting very good health (VG) after storage in the cold room for 48 h (Figure 3.1a). No visible skin lesions could be observed in these individuals (Figures 3.2a and 3.2b). They looked like freshly collected individuals, responding immediately to handling by contraction of the body and presenting a very faint fish smell. A single sea cucumber (8 ± 4%) in treatment 4 was scored lower (G), because of a small blister on the dorsal anterior side of its body wall (Figure 3.1b).

Treatments 1 (seawater ice) and 6 (no storage medium) also yielded relatively high proportions (75 ± 4%) of sea cucumbers scored as VG (Figures 3.1a and 3.2a). These individuals had no visible damage on the body wall and a slight fish smell. Few sea cucumbers (25 ± 4%) were classified as G (Figure 3.1b) as small blisters could be detected on their dorsal tegument, although it covered <20% of the total body wall surface.

Treatment 5 (bagged ice) resulted in a high proportion (75 ± 4%) of sea cucumbers in VG condition (Figures 3.1a and 3.2a). Few individuals were classified as G condition (17 ± 5%), because of small blisters were detected over the dorsal tegument covering less than 20% of the total body surface (Figure 3.1b). However, treatment 5 also

yielded sea cucumbers under slightly deteriorated (SD) condition (8 ± 5%; Figure 3.1c). Individuals categorized under SD showed blisters covering more than 20% of their body (Figure 3.2c) and a more pronounced odour, as detailed in Table 3.2. In addition, abundant mucus on the body wall made the sea cucumbers very slippery. Normally, healthy sea cucumbers contract their body immediately when handled; however, these individuals contracted their body ~5 times slower than other sea cucumbers under VG condition.

Treatment 2 (freshwater ice) resulted in 58 ± 5% in VG condition (Figures 3.1a and 3.2a). One quarter of sea cucumbers exposed to this treatment were classified under G after storage (Figure 3.1b), because of small blisters on the dorsal tegument. Sea cucumbers scored as VG and G responded well to handling and did not exude any strong fish smell. Treatment 2 showed the highest proportion of sea cucumbers scored as SD (25 ± 4%; Figure 3.1c) due to blisters covering more than 30% of their body (Figure 3.2c), abundant mucus on the body wall, fishy odour and reaction to handling ~5 times slower than individuals under G condition.

In treatment 3 (freshwater ice with sea salt), all sea cucumbers (100%) were classified as deteriorated (D) after 48 h in the cold room (Figures 3.1d). They were all eviscerated (Figure 3.2d), did not respond to handling and exhibited multiple skin lesions covering more than 50% of their body wall (Figure 3.2e). In addition, abundant sticky mucus was noticed on the surface of the water in the storage tanks.

One-way ANOVA on ranks revealed that the proportion of sea cucumbers in VG condition was significantly higher in treatment 4 when compared to the other treatments (Table 3.3). Also, treatments 1, 5 and 6 had similar proportion of sea cucumbers under

VG condition and did not differ statistically (Table 3.3). Treatment 3 had the lowest proportion of sea cucumbers under VG condition and the highest proportion under D condition and both were significantly different than in all the other treatments (Table 3.3).

3.4.1.2 Visual assessment and pH of muscle bands and body wall

Non-exposed control sea cucumbers displayed firm and pink coloured muscle bands and their body wall was firm and elastic. The pH for non-exposed individuals was 7.5 ± 0.1 in the muscle bands and 7.2 ± 0.1 in the body wall (Figure 3.3).

The muscle bands (longitudinal and circular) and body wall of sea cucumbers exposed to treatment 4 exhibited a colour and texture comparable to those of non-exposed sea cucumbers. Muscle bands and body wall were firm and easily separated from each other (Figure 3.2b). The pH of muscle bands and body wall of sea cucumbers exposed to treatment 4 was 7.6 ± 0.1 and 7.3 ± 0.0 respectively (Figure 3.3).

Individuals exposed to treatments 1, 2, 5 and 6 also exhibited standard organ colour and texture, similar to freshly collected live sea cucumbers. The pH of muscle bands for these treatments ranged from 7.4 ± 0.1 in treatment 1 to 7.5 ± 0.1 in treatment 5. The pH of body wall ranged from 7.1 ± 0.1 in treatment 1 to 7.2 ± 0.0 in treatment 6 (Figure 3.3).

Sea cucumbers exposed to treatment 3 also exhibited pinkish muscle bands, comparable to non-exposed sea cucumbers; however, red spots could be observed all over the longitudinal muscles (Figure 3.2f). In addition, tissues were extremely flaccid and were very easily torn during the dissection procedure so that separation of the muscle bands and body wall was hard to achieve. The pH measurements in the muscle bands and

body wall of individuals exposed to treatment 3 were the lowest among all treatments, at 6.8 ± 0.1 for the muscle bands and 6.6 ± 0.1 for the body wall (Figure 3.3).

Results of one-way ANOVAs showed that pH of the muscle bands did not differ among treatments or between treatments and non-exposed live controls, except for treatment 3 where values were significantly lower than in the controls and other treatments when compare to non-exposed sea cucumbers (Table 3.4). Measurements of pH in emulsions of the body wall varied from 6.6 ± 0.1 in treatment 3 to 7.4 ± 0.1 in treatment 4. The statistical analyses showed that the pH of body wall was significantly higher in treatment 4 than in the other treatments and in non-exposed sea cucumbers. In contrast, measurements of body wall pH in individuals from treatments 1, 2, 5 and 6 and non-exposed controls did not differ statistically. Finally, the pH of body wall from sea cucumbers exposed to treatment 3 was significant lower than in all other treatments (Table 3.4).

3.4.2 Water quality in the storage tanks

At the end of the trials, the water in the tanks from treatment 4 was clear, did not present any marked odour and the freshwater ice had melted completely. Also, there was no evidence of abundant froth/mucus on the surface of the water. Tanks from treatments 4 had ammonia nitrogen levels of 2.7 ± 0.2 ppm, dissolved oxygen levels of 6.5 ± 0.3 mg L^{-1}, pH values of 7.2 ± 0.0 and salinity levels of 30.7 ± 0.3 (Figure 3.4).

Treatments 5 and 6 also showed clear water at the end of the trial and no fish smell could be noticed. Big chunks of ice could still be found in the bags (60% melted) from treatment 5. The ammonia nitrogen level in theses tanks was the highest among all

treatments at 4.0 ± 0.7 ppm in treatment 5 and 3.6 ± 0.8 ppm in treatment 6. Levels of dissolved oxygen were the second lowest among treatments at 2.2 ± 0.2 mg L^{-1} in treatment 5 and 2.5 ± 0.6 mg L^{-1} in treatment 6. Water pH values of 7.3 ± 0.1 in both treatments were the highest among all treatments. Salinity in these tanks ranged from 34.0 ± 2.8 in treatment 5 to 35.0 ± 0.7 in treatment 6 (Figure 3.4).

Tanks from treatment 1 exhibited clear water with no strong fishy odour at the end of the storage period. Approximately 90% of the ice had melted. The concentration of ammonia nitrogen in tanks from treatment 1 was the second lowest (1.7 ± 0.1 ppm) among treatments. Dissolved oxygen was 4.4 ± 0.2 mg L^{-1}, pH was 7.1 ± 0.0 and salinity was 26.2 ± 1.0 (Figure 3.4).

The water in the storage tanks from treatment 2 was also clear with no strong fishy odour. Approximately 90% of the ice had melted during storage. The concentration of ammonia nitrogen was 3.0 ± 0.4 ppm. Dissolved oxygen reached the highest concentration among all treatments at 7.0 ± 0.3 mg L^{-1} after storage. Water pH was 7.1 ± 0.0 and salinity 17.1 ± 1.8, which were the lowest values among the treatments (Figure 3.4).

Tanks from treatment 3 exhibited a strong fishy odour and the colour of the water was reddish (Figure 3.2d). Mucus was also noticed on the surface of the water in such high concentration that it could stick to fingers. The freshwater ice had melted completely during storage. The concentration of ammonia nitrogen (2.6 ± 0.4 ppm), dissolved oxygen (2.6 ± 0.4 mg L^{-1}) and the pH (6.8 ± 0.0) in tanks from treatment 3 were the lowest among all treatments. However, salinity (66.3 ± 2.2) measured in the water from the storage tanks was the highest among all treatments (Figure 3.4).

Results of one-way ANOVAs showed that similar concentrations of ammonia nitrogen were observed in treatments 1, 2 and 4. The concentration of ammonia nitrogen was significantly higher in treatments 5 and 6 than in the other treatments. Also, treatment 3 had a lower ammonia nitrogen concentration when compared to the other treatments (Table 3.5). Dissolved oxygen was significantly higher in treatments 2 and 4 than in the other treatments. The lowest concentration of dissolved oxygen could be observed in treatment 3. Water pH was similar in all treatments, except treatment 3 which was significantly lower than in the other ones. Salinity was statistically higher in treatment 3 then in the other treatments. Also, treatment 2 had significantly lower salinity when compared to the other treatments (Table 3.5).

3.4.3 Post storage monitoring

Survival rates immediately after the 48-h storage in the cold room was recorded as 100% for all treatments except for treatment 3, which resulted in massive mortality of sea cucumbers (Figure 3.2d) and was, therefore, excluded from post-storage analysis.

When sea cucumbers were transferred to flow-through recovery tanks, the majority sank to the bottom of the tanks and became firmly attached to the bottom within 1 h. However, a few individuals (17 ± 10%) from treatment 2 remained floating on the water surface and did not get attached to the tank walls or bottom (Figure 3.2g). Attachment of these individuals was finally observed 48 h after they had been transferred to the tanks.

Treatment 4 exhibited the highest survival rate (100%) after the full 30 days. For all other treatments, survival rates decreased over time (Table 3.6). Treatment 4 also

resulted in the lowest proportion of sea cucumbers (11 ± 6%) developing skin necrosis over 30 days (Figure 3.5a). Moreover, this treatment yielded the highest overall proportion of sea cucumbers with tentacles fully deployed (91 ± 3%) during the recovery period of 30 days (Figure 3.5b).

Treatment 6 showed the second highest survival rate during recovery. Survival decreased to 89% after 10 days and to 67% after 30 days (Table 3.6). This treatment also resulted in the second lowest proportion (22 ± 7%) of sea cucumbers with skin lesions (Figure 3.5a) and the second highest proportion (71 ± 7 %) of individuals with tentacles fully deployed during the recovery period (Figure 3.5b).

Treatment 1 showed the lowest survival rate among treatments during the recovery period, at only 44 ± 18% (Table 3.6). The proportion of skin necrosis in individuals exposed to this treatment was high (47 ± 7%; Figure 3.5a); the body wall and muscle bands were both affected and internal organs eviscerated (Figure 3.2h). Treatment 1 also resulted in low proportions (44 ± 6%) of sea cucumbers with tentacles fully deployed during recovery (Figure 3.5b).

Treatments 2 and 5 showed similar results for survival rates, development of skin lesions and tentacle deployment over the recovery phase. Treatment 2 had a survival rate of 67 ± 13% in the first 10 days, dropping to 55 ± 7% after 30 days. Survival for treatment 5 was 89 ± 7% in the first 10 days decreasing to 55 ± 13% after 30 days (Table 3.6). The proportion of sea cucumbers developing skin lesion during recovery was the highest for treatment 2, with 51 ± 6%, followed by 48 ± 8% for treatment 5 (Figure 3.5a). The body wall (comprising epidermis and connective tissues) and the muscle bands were both affected. In those instances, the body wall and muscle bands were torn away, leaving

a hole through which internal organs (e.g. intestinal tract, gonads and respiratory tree) were eviscerated, resulting in the death of the sea cucumbers (Figure 3.2h). Additionally, both treatments displayed the lowest proportion of sea cucumbers with tentacles deployed during recovery, at 33 ± 6% for treatment 2 and 32 ± 7% for treatment 5 (Figure 3.5b).

Mortality of sea cucumbers (Figure 3.2i) was always accompanied by evisceration of internal organs (e.g. gonad, intestine and respiratory tree) and was observed in all treatments except in treatment 4. Also, necrosis usually affected the outer layer of the dermis (epidermis) exposing the white subepidermal connective tissue (Figure 3.2j).

Statistical analyses showed that survival rates were significantly higher in treatment 4 than in all other treatments over the entire recovery period of 30 days (Table 3.7). Treatment 4 also had significantly lower proportions of sea cucumbers with skin necrosis (Table 3.8). In addition, the proportion of sea cucumbers with skin lesions in treatments 1, 2 and 5 was significantly higher than in the other treatments (Table 3.8). The results of one-way ANOVA revealed that individuals exposed to treatment 4 had the highest proportion of sea cucumbers with tentacles fully deployed when compared to the other treatments (Table 3.9). The proportion of sea cucumbers with tentacles deployed was significantly higher in treatment 6 than in treatments 1, 2 and 5, with no significant differences among the latter (Table 3.9).

3.5 Discussion

Sea cucumbers have long been consumed and used in traditional medicine throughout Asia, where they are marketed as a health food and attributed various curative and preventive properties. Over the past few decades, they have become one of the most

prized seafoods in the world. Market prices for dried sea cucumbers vary among species, largely based on morphological criteria such as size, shape, texture, colour and smell (Ferdouse, 2004). Because today's consumers are extremely quality conscious, the market demands high-grade products. However, live sea cucumbers have proven difficult to store and transport, because of their ability to autolyze when they are stressed or taken out of seawater (Duan *et al.*, 2010; Zheng *et al.*, 2012). Unlike fish and shellfish, such as bivalves and crustaceans, sea cucumbers are not protected by any scales or hard exoskeleton that would prevent contact with ice, salt or any other media during storage. As the body wall is the chief commercial product of sea cucumbers, adapting storage and transport methods to preserve the visual and nutritional integrity associated with high-grade products is of great importance.

Among the media tested here, iced seawater was the most effective for live storage of *C. frondosa*, yielding 100% survival and largely intact body wall and muscles. This mix of cold seawater and freshwater ice maintained conditions of salinity, pH, ammonia and dissolved oxygen in the storage tanks within acceptable ranges, consistent with the seawater characteristically found in the natural habitat of *C. frondosa*. The storage temperature was also within the range experienced by this species in nature (Hamel and Mercier, 1996b). The mixing of ice and seawater in this treatment minimized the occurrence of frostbite observed in the treatments where ice was applied directly over the sea cucumbers. Also, iced seawater surrounded the sea cucumbers completely, buffering them against changes in environmental conditions and preventing exposure to air, which is known to cause stress in sea cucumbers (Duan *et al.*, 2010; Zang *et al.*, 2012). This medium offered no jagged or sharp edges, which can inflict injuries to the

body wall. Overall, iced seawater offered a clean, cold and oxygenated storage medium, which decreased stress on the sea cucumbers compared to conditions that favoured contact with air, ice and/or salt.

Salinity is known to be one of the principal factors driving physiological responses in marine organisms. As sea cucumbers lack an osmoregulatory mechanism, their coelomic fluid remains isosmotic with the environment (Yu *et al.*, 2013). Nevertheless, *C. frondosa* can be found in a variety of habitats with a wide range of salinities, from maritime estuaries to oceanic waters (Hamel and Mercier, 1996). The salinity of 29 measured in the storage tanks with iced seawater falls inside the range where extensive populations of *C. frondosa* occur, such as the St. Lawrence Estuary, in eastern Canada (Hamel and Mercier, 1996b).

Another key driver of marine animal physiology is dissolved oxygen (DO). Values of DO measured were relatively high in the water surrounding the sea cucumbers after 48 h of storage in iced seawater. These high values indicate low sea cucumber metabolism and minimal release of ammonia during storage. Sea cucumbers in iced seawater developed limited or no tissue damage and consequently generated very little bacterial activity except that associated with normal excretion. Ammonia is known to be highly toxic for marine organisms (Burgess, 2000; Wang *et al.*, 2014), especially at high pH, where skin ulcers, necrosis and deaths can occur, as observed in fish (El-Shafai *et al.*, 2004). It was also shown that an increase in water temperature resulted in greater oxygen consumption and ammonia excretion rates in the temperate sea cucumber *Apostichopus japonicus* (Yang et al., 2006).

Although treatments with freshwater ice, bagged ice and no medium also yielded high proportions of sea cucumbers exhibiting very good condition immediately after storage, they all resulted in post-storage skin lesions, eviscerations and mortalities, indicating that these storage methods were less ideal (or suboptimal) for the transport of live sea cucumbers when compared to iced seawater. Frequent development of skin necrosis and low survival during the recovery period might be explained by direct skin contact with the frozen media, which induced the development of blisters and frostbite. The dermis of sea cucumbers appears to be much more sensitive to ice than that of fish or shellfish, which is protected by scales and an exoskeleton, respectively. It is more reminiscent of mammal skin, in which response to contact with a low-temperature surface varies from skin necrosis, which can also lead to inflammatory wounds, to tissue destruction as a result of progressive failure of the microcirculation in the affected area (Gage and Baust, 1998). Moreover, the fact that sea cucumbers stored in freshwater ice had more difficulty initially attaching to the substrate during the recovery period might be due to freshwater from the melted ice somehow impeding attachment. This effect was not permanent and eventually dissipated.

Sea cucumbers stored with either bagged ice or without any medium suffered mainly from exposure to low DO levels and high concentrations of ammonia nitrogen measured in the product water found in the tanks after storage. This water emanated from their respiratory trees and intestines; it therefore contained metabolic wastes. Because there was no medium in contact with the sea cucumbers in these treatments, the wastes were not diluted, resulting in low DO levels and high levels of ammonia. As previously discussed, high concentrations of ammonia can elicit skin ulcers and necrosis (El-Shafai

et al., 2004) and might be the reason for the rapid development of skin lesion and mortalities during the recovery period in individuals stored without any medium or with bagged ice.

Of all the treatments, freshwater ice with added fish salt proved to be the most inefficient to store live sea cucumbers. All sea cucumbers were found dead and eviscerated after the storage period of 48 h. The added fish salt increased the salinity in these tanks to very high values that may have triggered evisceration. Unlike certain tropical sea cucumbers, *C. frondosa* does not eviscerate to deter predators and is not known to readily regenerate viscera, making this process a lethal outcome of stress (J.-F. Hamel, personal observation). Due to relatively rapid mass mortality, high salinity and cold temperature, the production of ammonia through metabolism remained low compared to most other treatments. However, the low levels of DO and water pH measured in the resulting water suggest the beginning of bacterial degradation.

Apart from effects on the health and skin condition of sea cucumbers immediately after storage, the type of storage medium also influenced meat quality. Irrespective of external appearance and health, individuals stored in seawater ice, freshwater ice, iced seawater, bagged ice and without any medium all exhibited firm and pinkish muscle bands and an overall firm body wall, comparable to those of non-exposed sea cucumbers. In contrast, sea cucumbers stored in freshwater ice with fish salt exhibited very soft tissues, which were easily torn during sampling, likely a result of autolysis, which is associated with degradation of abdominal tissue and muscle softening in many marine organisms (Ando *et al.*, 1999; Zheng *et al.*, 2012; Sun *et al.*, 2013). The firmness of fresh muscle is directly correlated with the amount of collagen in the tissue; intestinal proteases

can degrade collagen leading to muscle softening (Ando *et al.*, 1999). Sea cucumbers stored with freshwater ice and fish salt presented an unpleasant appearance and a pronounced smell. In addition, the red spots observed in the longitudinal muscle bands of these sea cucumbers can be associated with lipid oxidation and protein breakdown (Fu *et al.*, 2005; Wu *et al.*, 2013).

The lower pH measured in tissue emulsions following exposure to freshwater ice with fish salt can be the result of *post mortem* bacterial and chemical degradation when compared to values measured in individuals stored in iced seawater and in non-exposed sea cucumbers. Although there is no documentation of pH in the muscle bands or body wall of sea cucumbers, it was expected that sea cucumbers classified under deteriorated condition (D) would exhibit lower tissue pH, as acidity is typically associated with softening of tissues in fish (Dunajski, 1980; Martinez and Gildberg, 1988). It was also initially presumed that pH associated with the body wall of *C. frondosa* would be higher than the pH associated with the muscle bands, due to the presence of calcareous ossicles in the body wall. However, the emulsions of muscle had a higher pH than those of body wall. Measurement of pH in fresh sea cucumber tissues (meat) should be investigated further, as it might serve as an indicator of integrity and quality after live storage.

3.6 Conclusion

Live storage of sea cucumbers is a challenge due to their soft unprotected body wall and ability to autolyze once out of water or after experiencing stress. Deterioration of the body wall and underlying muscles, which together constitute the chief marketable sea cucumber products, will likely translate into commercial products of a lower grade. As

fisheries of *C. frondosa* expand in the North Atlantic in order to supply Asian countries, optimal methods for their live storage and transport on the way to the processing line will become crucial to the production of high-quality end products.

Among the conditions tested in the present study, storage in iced seawater is the most promising, generating the best overall health conditions immediately after storage, survival rates during the post-storage monitoring, and meat quality. This means that the method could be used for transporting and storing the animals before processing without any negative impact on the final products. While this method differs from those currently used with *C. frondosa* along the eastern coast of Canada, the modification proposed should not involve any major financial cost. In the end, as the organoleptic properties of the body wall account for most of the commercial value of sea cucumber, this transport technique could help producers obtain higher prices. In addition, it would be greatly beneficial for preserving the quality of the meat, especially since fresh frozen muscle bands are the second most valuable product derived from *C. frondosa*.

The findings presented here provide important information about the live storage of sea cucumbers for transport and holding. They will hopefully help improve this processing step and optimize the quality of sea cucumber products in countries that commercialize *C. frondosa* and other temperate or cold-water sea cucumbers.

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3.9 Tables

Table 3.1: Description of the various media and conditions used in treatments 1 through 6 (T1 to T6) over 48 h.

Code	Storage Medium	Description
T1	**Seawater ice**	Crushed seawater ice prepared by freezing seawater with a salinity of 35.
T2	**Freshwater ice**	Freshwater ice (granule type, ~1 mm) prepared by an ice machine (ITV IQ300Ca) at -2°C.
T3	**Freshwater ice + fish salt**	Freshwater ice spread uniformly over the sea cucumbers with fish salt added on top.
T4	**Iced seawater**	A slush solution made of seawater and freshwater ice, with a temperature of 0°C and salinity of 29.
T5	**Bagged freshwater ice**	Freshwater ice kept in plastic bags and placed on the bottom of the tanks with sea cucumbers positioned above the bags.
T6	**No medium**	No storage medium was added to the storage tanks.

Table 3.2: Classification of external health conditions exhibited by the sea cucumbers immediately after the storage treatments.

Health Condition	Description
Very Good (VG)	No visible alterations on the body wall such as scratches, blisters, frostbite or skin lesions. Individuals respond immediately to handling by contraction, have faint/fresh fish smell and thin layer of mucus over the body wall.
Good (G)	Less than 20% of the total body surface showing presence of blisters, scratches, frostbite or any other skin lesion. Individuals respond immediately to handling by contraction and have faint/fresh fish smell.
Slightly Deteriorated (SD)	Clear signs of blisters, scratches, depigmentation, swelling, frostbite or any other skin lesion that represent 20 to 50% of the total body surface. Individuals covered with sticky mucus, displaying relaxed and flaccid body, slow contraction response and acrid, slightly fishy odour. Sea cucumbers with partially or completely extruded tentacles are included in this category if they do not show signs of skin alterations.
Deteriorated (D)	Multiple skin/body alterations covering more than 50% of the total body surface. Individuals do not response to handling, present strong fish odour and are abundantly covered with sticky mucus. In addition, eviscerated (dead/dying) sea cucumbers are included in this category.

Table 3.3: Comparison of sea cucumber external health conditions among treatments after the storage period of 48 h. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Sea cucumbers were classified as very good (VG), good (G), slightly deteriorated (ST) and deteriorated (D) as per Table 3.2. One-way ANOVA on ranks followed by Tukey test was used to compare the proportion of individuals in each health condition category among treatments. Significant results are shown.

Sea cucumber Condition	**Treatments**	**H**	**df**	**p**
VG	T1 x T2 x T3 x T4 x T5 x T6	13.874	5	0.016
	T1>T3			<0.001
	T1>T2			0.039
	T2>T3			<0.001
	T4>T3			<0.001
	T4>T1			0.038
	T4>T2			0.013
	T4>T6			0.030
	T4>T5			0.030
	T5>T3			<0.001
	T5>T2			0.039
	T6>T2			0.039
	T6>T3			<0.001
G	T1 x T2 x T3 x T4 x T5 x T6	11.204	5	0.014
	T1>T3			0.013
	T2>T3			0.041
	T5>T3			0.041
	T6>T3			0.013
SD	T1 x T2 x T3 x T4 x T5 x T6	14.5044	5	0.012
	T2>T1			<0.001
	T2>T3			<0.001
	T2>T4			<0.001
	T2>T5			0.017
	T2>T6			<0.001
D	T1 x T2 x T3 x T4 x T5 x T6	16.875	5	0.005
	T3>T1			<0.001
	T3>T2			<0.001
	T3>T4			<0.001
	T3>T5			<0.001
	T3>T6			<0.001

Table 3.4: Comparison of pH of muscle bands and body wall tissues after storage of *Cucumaria frondosa* for 48 h under different conditions. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5), and no medium (T6), as per Table 3.1, as well as a control consisting of live non-exposed individuals (NE). One-way ANOVA followed by Holm-Sidak test was used to compare pH values among treatments. Significant results are shown.

Tissue	**Treatments**	**F**	**df**	**p**
Muscle bands	T1 x T2 x T3 x T4 x T5 x T6	39.874	5	<0.001
	T1>T3			<0.001
	T2>T3			<0.001
	T4>T3			<0.001
	T5>T3			<0.001
	T6>T3			<0.001
	NE>T3			<0.001
Body wall	T1 x T2 x T3 x T4 x T5 x T6	36.533	5	<0.001
	T1>T3			<0.001
	T2>T3			<0.001
	T4>T1			0.007
	T4>T2			0.039
	T4>T3			<0.001
	T4>T5			0.038
	T4>T6			0.039
	T4>NE			0.040
	T5>T3			<0.001
	T6>T3			<0.001
	NE>T3			<0.001

Table 3.5: Comparison of water quality in tanks after 48 h storage under different conditions. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5), and no medium (T6), as per Table 3.1. One-way ANOVA followed by Holm-Sidak test was used to compare ammonia nitrogen, water pH, dissolved oxygen and salinity among treatments. Significant results are shown.

Water quality	**Treatments**	**F**	**df**	**p**
Ammonia nitrogen	T1 x T2 x T3 x T4 x T5 x T6	12.063	5	<0.001
	T1>T3			0.034
	T2>T3			0.002
	T4>T3			0.005
	T5>T3			<0.001
	T6>T3			<0.001
	T6>T1			0.001
	T6>T2			0.049
	T6>T4			0.047
	T5>T1			<0.001
	T5>T2			<0.001
	T5>T4			0.036
pH	T1 x T2 x T3 x T4 x T5 x T6	19.846	5	<0.001
	T1>T3			<0.001
	T2>T3			<0.001
	T4>T3			<0.001
	T5>T3			<0.001
	T6>T3			<0.001
Dissolved oxygen	T1 x T2 x T3 x T4 x T5 x T6	40.809	5	<0.001
	T1>T3			<0.001
	T2>T3			<0.001
	T4>T3			<0.001
	T5>T3			<0.001
	T6>T3			<0.001
	T2>T1			<0.001
	T2>T5			<0.001
	T2>T6			<0.001
	T4>T1			<0.001
	T4>T5			<0.001
	T4>T6			0.003

Salinity	T1 x T2 x T3 x T4 x T5 x T6	22.194	5	<0.001
	T3>T1			<0.001
	T3>T2			<0.001
	T3>T4			<0.001
	T3>T5			<0.001
	T3>T6			<0.001
	T1>T2			<0.001
	T4>T2			<0.001
	T5>T2			<0.001
	T6>T2			<0.001

Table 3.6: Survival rates (mean percent ± se) of sea cucumbers exposed to different storage treatments for 48 h. Treatments consisted of seawater ice (T1), freshwater ice (T2), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6), as per Table 3.1. Survival rate was not assessed in T3 (freshwater iced with fish salt), because all sea cucumbers died after the initial storage period of 48 h. See Table 3.7 for statistical analyses.

Days	**Treatments**				
	T1	**T2**	**T4**	**T5**	**T6**
0	100	100	100	100	100
10	78 ± 7	67 ± 13	100	89 ± 7	89 ± 7
20	67 ± 13	55 ± 18	100	67 ± 6	78 ± 5
30	44 ± 18	55 ± 7	100	55 ± 13	67 ± 18

Table 3.7: Comparison of survival rates of sea cucumbers among storage treatments. Treatments consisted of seawater ice (T1), freshwater ice (T2), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Treatment 3 (freshwater ice with fish salt) was not assessed, because all sea cucumbers died after 48-h storage. Significant results of a Logrank survival analysis followed by Holm-Sidak test at intervals of 10 days post storage are shown.

Time (d)	**Treatments**	χ^2	**F**	**df**	**p**
10	T1 x T2 x T4 x T5 x T6	11.486		5	<0.001
	T4>T1		9.652		0.011
	T4>T2		11.562		<0.001
	T4>T5		8.325		0.034
	T4>T6		8.358		0.035
20	T1 x T2 x T4 x T5 x T6	11.645		5	<0.001
	T4>T1		9.613		0.012
	T4>T2		11.248		<0.001
	T4>T5		9.612		0.012
	T4>T6		9.241		0.021
30	T1 x T2 x T4 x T5 x T6	11.955		5	<0.001
	T4>T1		11.828		<0.001
	T4>T2		11.165		<0.001
	T4>T5		11.154		<0.001
	T4>T6		10.325		<0.001

Table 3.8: Comparison of sea cucumbers with skin necrosis after recovery period of 30 d following exposure to various storage treatments. Treatments consisted of seawater ice (T1), freshwater ice (T2), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Treatment 3 (freshwater ice with fish salt) was not assessed, because all sea cucumbers died after 48-h storage. Significant results of one-way ANOVA on ranks followed by pairwise Tukey test are shown.

Treatments	**H**	**df**	**p**
T1 x T2 x T4 x T5 x T6	18.329	4	<0.001
T1>T4			0.009
T1>T6			0.044
T2>T4			0.036
T2>T6			0.046
T5>T4			0.003
T5>T6			0.045
T6>T4			0.048

Table 3.9: Comparison of sea cucumbers with tentacles deployed after recovery period of 30 d following exposure to various storage treatments. Treatments consisted of seawater ice (T1), freshwater ice (T2), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6). Treatment 3 (freshwater ice with fish salt) was not assessed, because all sea cucumbers died after 48-h storage. Significant results of one-way ANOVA followed by pairwise Holm-Sidak tests are shown.

Treatments	**F**	**df**	**p**
T1 x T2 x T4 x T5 x T6	133.044	4	<0.001
T4>T1			0.017
T4>T2			0.004
T4>T5			0.002
T4>T6			0.045
T6>T1			0.044
T6>T2			0.045
T6>T5			0.037

3.10 Figures

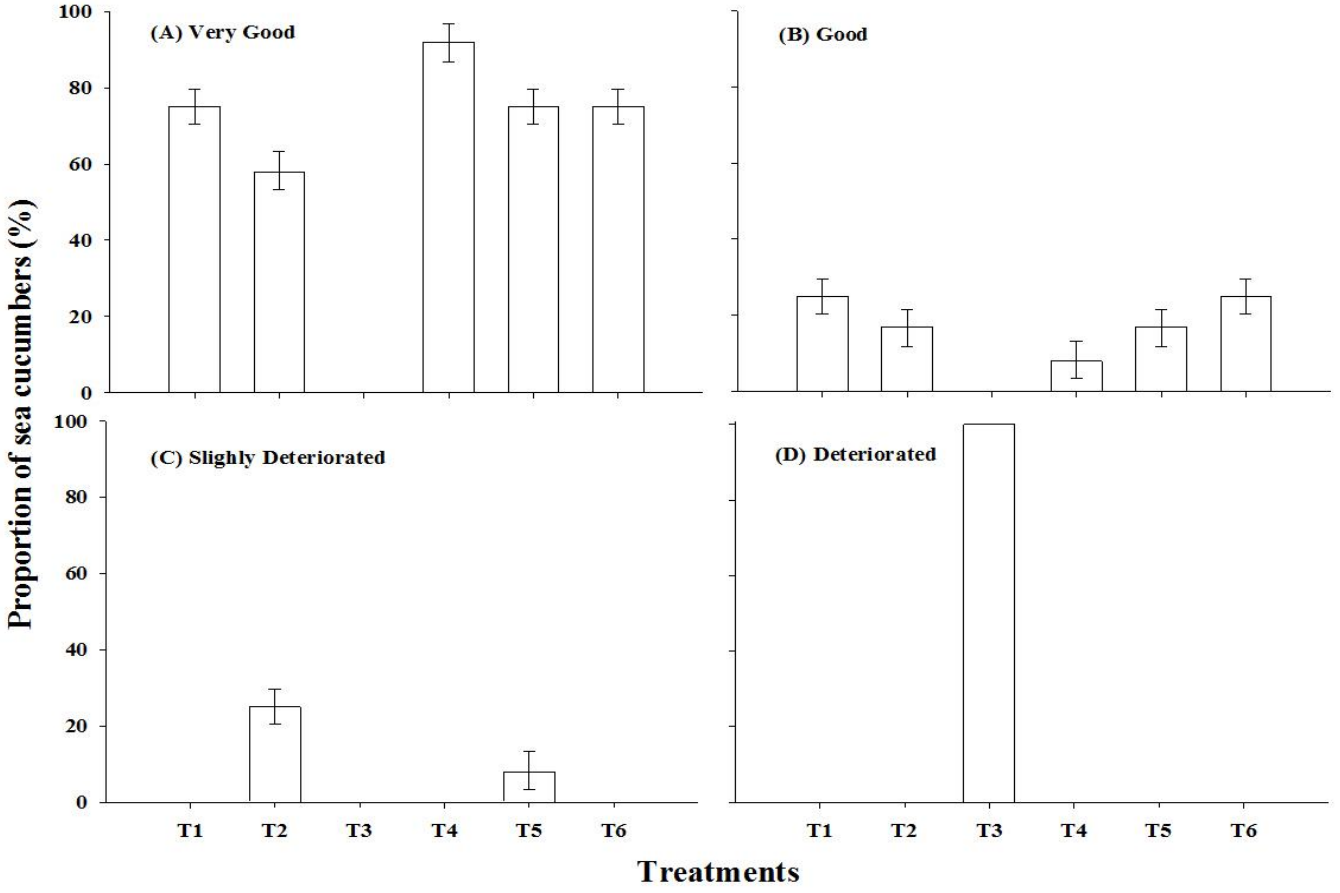
(A) Very Good
(B) Good
(C) Slighly Deteriorated
(D) Deteriorated
Proportion of sea cucumbers (%)
100
80
60
40
20
0
T1
T2
T3
T4
T5
T6
Treatments

Figure 3.1 (previous page): Status of sea cucumbers after exposure to various storage treatments for 48 h. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Sea cucumbers were scored as very good (VG), good (G), slightly deteriorated (SD) and deteriorated (D) as per Table 3.2. Data shown as mean ± se (n=3). See Table 3.3 for statistical results.

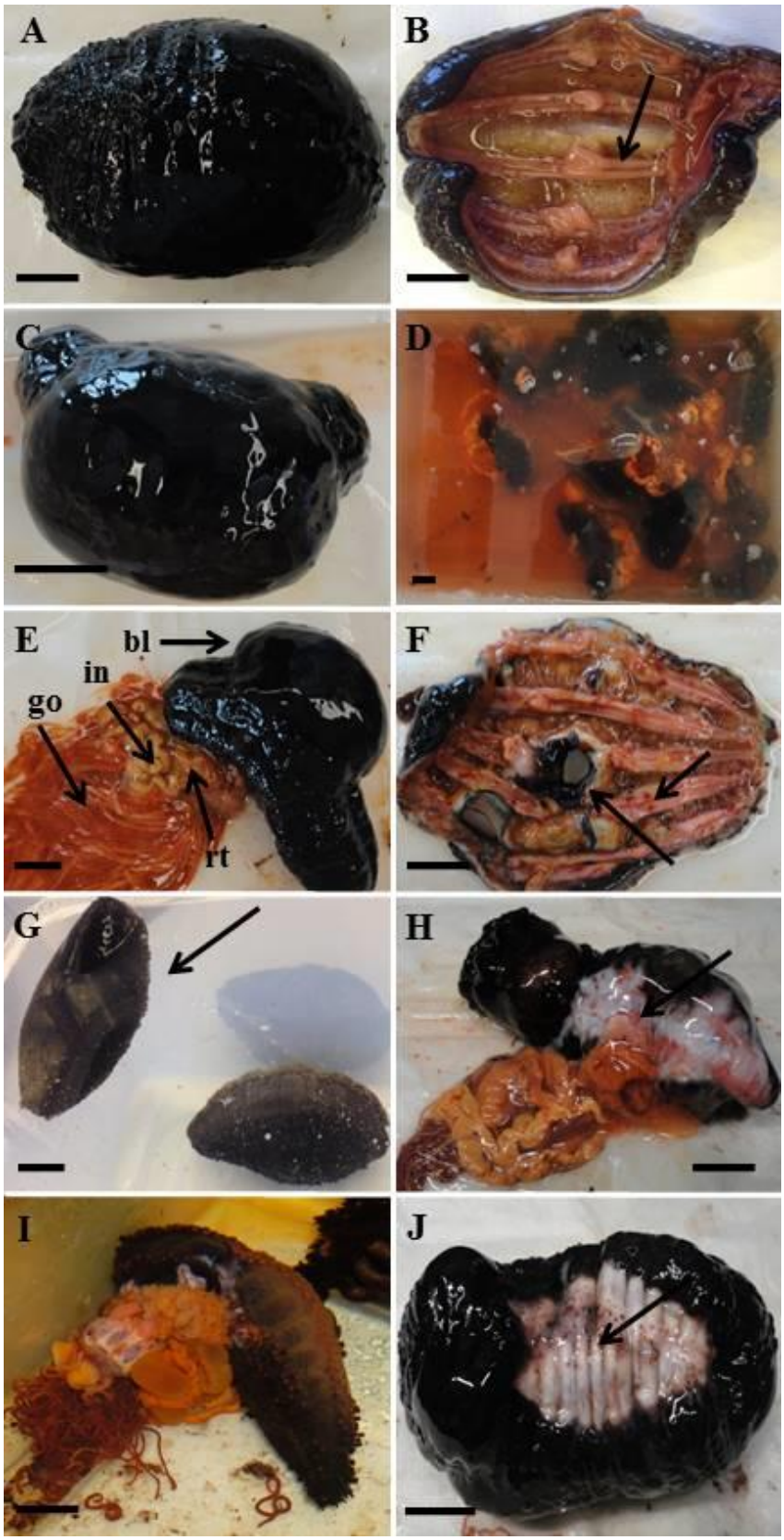
A
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Figure 3.2 (previous page): Post storage condition of sea cucumbers stored in various media. (A) External aspect of a sea cucumber in very good condition, showing firm body wall and no visible skin damage. (B) Internal view of sea cucumber in very good condition, showing healthy pinkish longitudinal muscle bands (arrow). (C) Slightly deteriorated sea cucumber showing blisters covering 20-50% of the total body surface. (D) Worst example of sea cucumbers scored as deteriorated (treatment 3). All individuals were eviscerated and dead/moribund (not responding to handling). The water was reddish, had a strong fish smell and was covered with mucus. (E) Example of a deteriorated sea cucumber, with a blister on the body wall (bl) and eviscerated gonad (go), intestine (in) and respiratory tree (rt). (F) Internal view of a deteriorated sea cucumber, showing tears in body wall and red spots on longitudinal muscle bands (arrows). (G) Some sea cucumbers stored with freshwater ice were floating (arrow) when transferred to the recovery tanks. (H) Skin necrosis and hole (arrow) in the sea cucumber's body wall resulting in evisceration of internal organs. (I) Dead and eviscerated sea cucumbers were recorded in treatments 1, 2, 5, and 6 during the recovery period. (J). Sea cucumber showing signs of skin necrosis (arrow). The outer dermis layer (epidermis) is shedding off, exposing the connective white tissues (arrow). Scale bars represent 2 cm. Treatments are defined in Table 3.1 and sea cucumber health conditions are defined in Table 3.2.

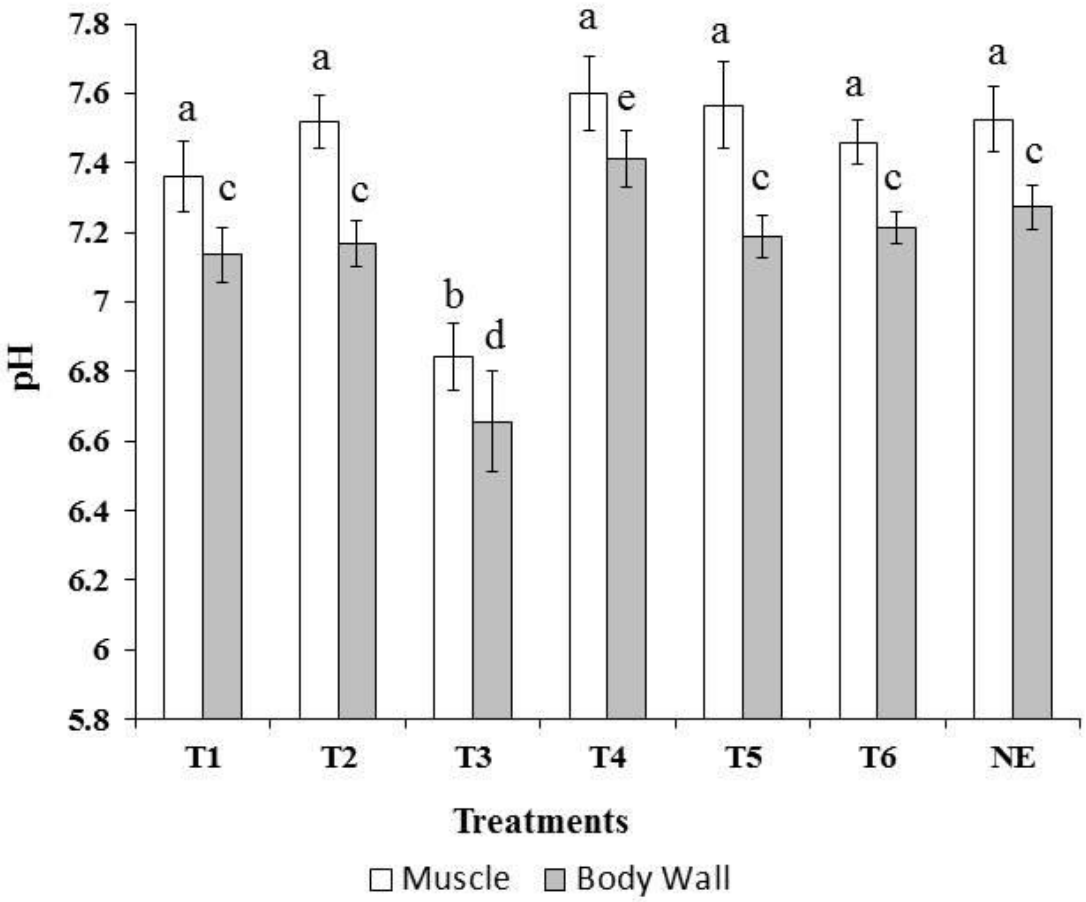

Figure 3.3: Post-storage pH in muscle and body wall of sea cucumbers. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Measurements were conducted immediately after 48-h storage. Data shown as mean ± se (n=3). Means with different letters are significantly different. See Table 3.4 for statistical results.

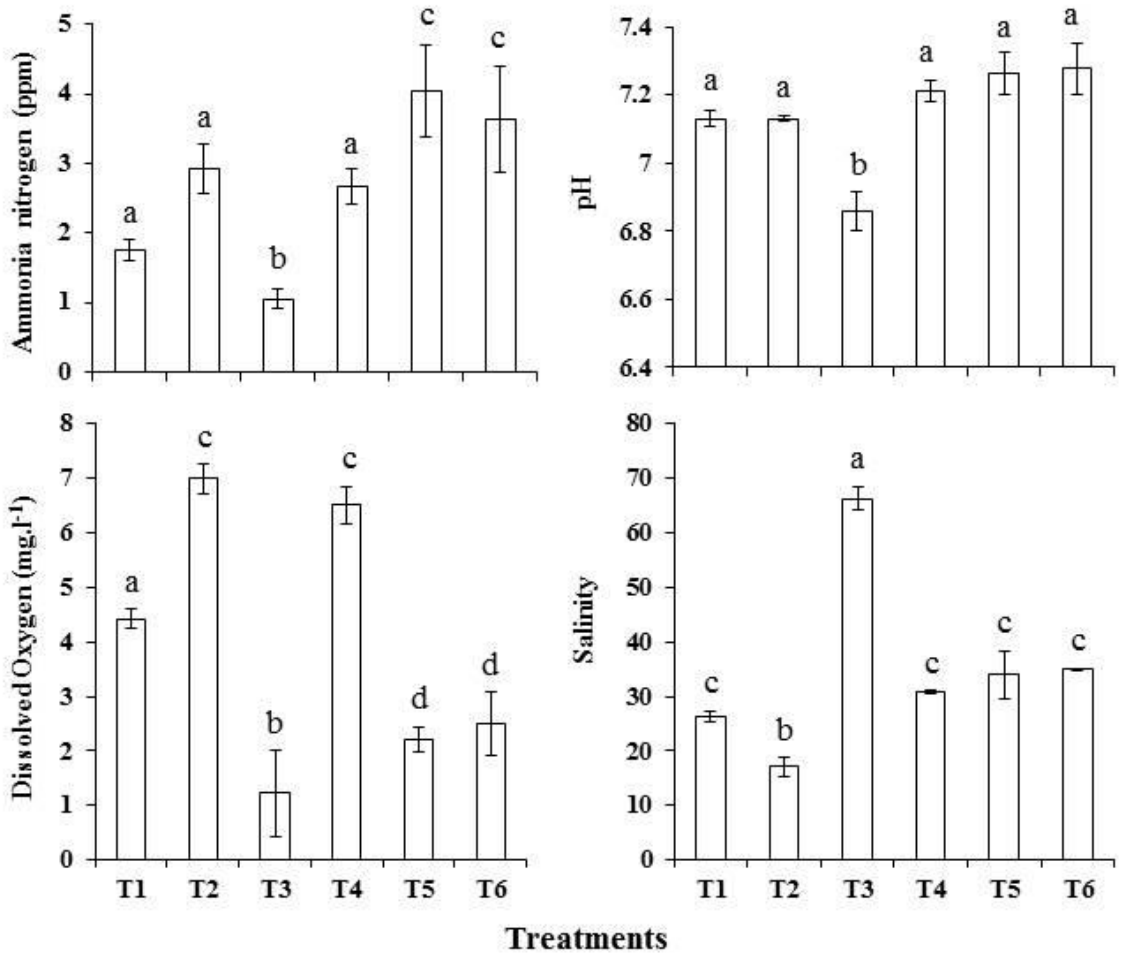

Figure 3.4: Water quality in the tanks immediately after storage. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Ammonia nitrogen, dissolved oxygen, pH and salinity were measured in water resulting either from the melted ice and/or water expelled from the respiratory tree of sea cucumbers in the storage tanks after 48 h. Data shown as mean ± se (n=3). Means with different letters are significantly different. See table 3.5 for statistical results.

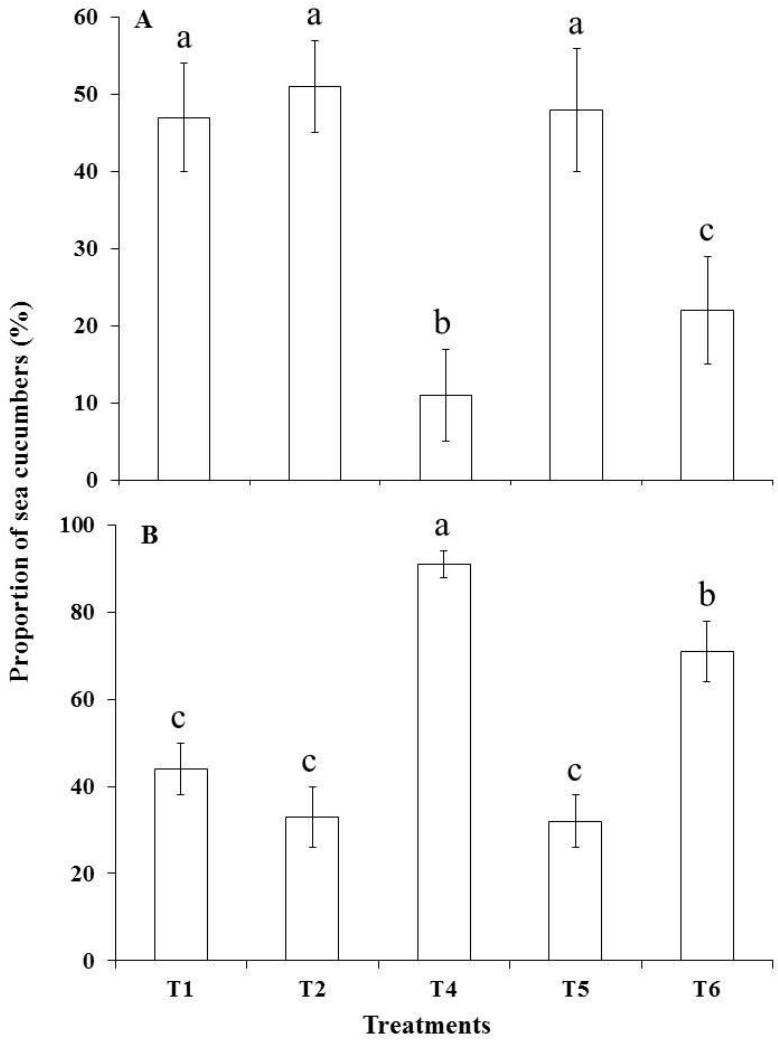

Figure 3.5: Proportion of sea cucumbers with skin necrosis and tentacle deployment during recovery period of 30 days. (A) Development of skin necrosis and (B) tentacle deployment. Treatments consisted of seawater ice (T1), freshwater ice (T2), freshwater ice with fish salt (T3), iced seawater (T4), bagged freshwater ice (T5) and no medium (T6) as per Table 3.1. Data shown as mean ± se (n=3). Means with different letters are significantly different. See Tables 3.8 and 3.9 for statistical results.

Chapter 4: Influence of Diet on Growth, Lipid Class and Fatty Acid Composition of the Sea Cucumber *Cucumaria frondosa*

4.1 Abstract

The sea cucumber *Cucumaria frondosa* is widely distributed in cold waters and is commercially exploited in the North Atlantic. It is also considered to have potential for aquaculture due to its high marketability for food and nutraceutical/pharmaceutical products. Here, the effects of food sources on growth, lipid class and fatty acid composition of muscle and gonadal tissues were studied in *C. frondosa*. Individuals were either left to feed on natural micro/nanoplankton present in ambient seawater, or supplemented with either live diatoms (*Chaetoceros muelleri*) or with a commercial preparation of fish eggs for period of 84 d. Specific growth rate, lipid class and fatty acid profiles of body wall muscles and gonads were investigated and compared among experimental groups and with sea cucumbers freshly collected from the field. Intestinal contents confirmed capture and ingestion of both diatoms and fish eggs. Individuals fed with fish eggs had the highest body length increment (1.60 ± 0.50 cm), total specific growth rate (0.05 ± 0.03% d^{-1}) and the highest ratio of the essential fatty acids DHA:EPA (1.90 ± 0.01) in gonadal tissues. In contrast, sea cucumbers fed with diatoms had the highest ratio of the essential fatty acids ARA:EPA (0.40 ± 0.10) in muscle tissues. Un-supplemented sea cucumbers showed a reduction in body length (by 0.30 ± 0.30 cm) yielding a specific growth rate of –0.24 ± 0.07% d^{-1}. This study confirmed that manipulating the diet of *C. frondosa* has the potential to modulate its growth rate and suggests that captive rearing on live and commercial diets is possible. The nutritional value of sea cucumbers meat does not appear to be compromised and could even be enhanced with diet formulations for market-specific qualities.

4.2 Introduction

The ever increasing demand for beche-de-mer has led to the overexploitation of high-valued sea cucumbers and stimulated the development of new fisheries for underutilized species (Anderson *et al.*, 2011; Purcell *et al.*, 2013). Meanwhile, declines in natural populations have encouraged the development of aquaculture programs around the world (Lovatelli *et al.*, 2004; So *et al.*, 2010; Purcell *et al.*, 2013). Processed whole sea cucumbers and various derived products such as gonads and intestines are in demand and command high market prices (Conand and Byrne, 1993; Purcell, 2014; Zhong *et al.*, 2007). Efforts are therefore being made to optimize culture systems and improve production, which is often tied to the feeding ecology of target species.

In Indo-Pacific countries, aspidochirotid sea cucumbers are hatchery produced and juveniles are transferred to ponds (i.e. land-based tanks), sea pens or sea ranching sites for grow-out (Chen, 2003; Hair *et al.*, 2012). Growth of sea cucumber juveniles is sensitive to a variety of environmental factors, in particular stocking densities and the quality and quantity of food available. Several commercial feeds have been tested in juveniles of deposit-feeding sea cucumbers including prepared diets of dried powdered diatoms (e.g. *Navicula*, *Amphora*, *Achnanthes* and *Nitzschia* species), macroalgae, such as *Undaria pinnatifida, Ulva lactuca*, *Sargassum* spp. and *Saccharina longissima* (Battaglene *et al.*, 1999; Kang *et al.*, 2003; Yuan *et al.*, 2006; Xia *et al.*, 2012), and soybean meals as an alternative source of protein (Seo and Lee, 2011). High specific growth rates were recorded in *Apostichopus japonicus* when juvenile and adults were fed with a prepared diet made with fish, soybean and seaweed powders with 3% fat content and protein levels

between 28.8 to 35.5% (Yang *et al.*, 2015). However, inadequate supply of essential amino acids and fatty acids for juveniles during the grow-out phase may result in a number of deficiency symptoms such as impaired collagen formation in the body wall, retarded growth and depressed immunity (Okorie *et al.*, 2008).

Lipids play an important role in providing energy and essential fatty acids for growth and survival of sea cucumbers. Generally, the fatty acid composition of diets is reflected in the lipid deposited in their tissue, which can be traceable (Drazen *et al.*, 2008). By manipulating the quality and quantity of fatty acids in the diets, it is possible to elevate the nutritional value of sea cucumbers. However, not all products benefit from exhibiting appreciable quantities of fatty acids such as omega-3 polyunsaturated fatty acids since they can oxidize and generate distinct and unpleasant fishy odors and flavors that can affect acceptability (Kolanowski *et al.*, 2001; Turner *et al.*, 2011).

The sea cucumber *Cucumaria frondosa* is widely distributed in cold waters, occurring from the Arctic Ocean to Cape Cod as well as along the coast of northern Europe and Russia; it is the focus of a commercial fishery in the North Atlantic (Grant, 2006; Grant *et al.*, 2006; Hamel and Mercier, 2008; Rowe *et al.*, 2009). This species is also considered to have potential for aquaculture due to its high marketability for food and nutraceutical and pharmaceutical products (Bordbar *et al.*, 2011). 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 high-valued compounds with multiple biological benefits, including anticoagulant, anticancer, antioxidant, anti-inflammatory and antibacterial properties (Haug *et al.*, 2002; Mamelona *et al.*, 2007).

Previous studies investigating the fatty acid composition of freshly collected *C. frondosa* identified eicosapentaenoic acid (EPA) as the major fatty acid comprising ~50% of total fatty acids. Other fatty acids such as 16:1ω7, 18:0 and docosahexaenoic acid (DHA) were also detected in the body wall but in proportions <5% (Zhong *et al.*, 2007). The balance between docosahexaenoic (DHA), eicosapentaenoic (EPA) and arachidonic (ARA) acids is very important for egg synthesis and embryonic development in many marine fish and bivalves (Fearman *et al.*, 2009). High levels of EPA compared to ARA may inhibit the production of eicosanoids derived from ARA, causing deficiencies in fecundity, egg quality and hatching success (Moksness *et al.*, 2008). A more complex combination of fatty acids may also reflect positively on growth rates. When *A. japonicus* was fed with a diet that had fatty acid ratios of 10:5:6:1 for 18:2ω6:18:3ω6:DHA:EPA, juveniles exhibited the fastest growth (Yang *et al.*, 2015).

To date, studies on the feeding and lipid composition of sea cucumbers have largely focused on hatchery-produced deposit-feeding species in the order Aspidochirotida, such as *A. japonicus* and *Holothuria scabra* (Knauer, 2011; Yang *et al.*, 2015). In contrast, *C. frondosa* is a suspension-feeding sea cucumber belonging to the order Dendrochirotida (Lawrence, 1987; Hamel and Mercier, 1998). Several aspects of the biology of *C. frondosa* have been documented over the past decades such as population distribution, reproductive cycle and larval development and growth (Hamel and Mercier, 1996a, 1996b, 1996c, 1998; Singh *et al.*, 1998; So *et al.*, 2010) although few studies have investigated its feeding ecology (Hamel and Mercier, 1998; Singh *et al.*, 1998). To our knowledge, there is no study correlating diets, lipid classes and fatty acid composition in tissues of *C. frondosa* from an aquaculture perspective.

There is limited knowledge about the lipid and fatty acid profiles of cold-water sea cucumbers in general, and further information is needed in order to establish rearing protocols. With the expansion of sea cucumber aquaculture in North and South America and northern Europe, developing knowledge with respect to nutritional composition of valuable sea cucumber products will be of great interest. The present study investigated the acceptability of live and commercial feeds, and their influence on growth rates, lipid class and fatty acid profiles of muscles and gonads of *C. frondosa*. Treatments consisted of natural nano/microplankton found in ambient seawater alone, and supplements of either live diatoms or commercial fish eggs. Results were compared among treatments and with sea cucumbers freshly collected from the field.

4.3 Material and Methods

4.3.1 Sea cucumber collection

Small sea cucumbers weighing 2.3 ± 1.0 g immersed weight (So *et al.*, 2010) and measuring 10.5 ± 2.3 cm contracted body length were hand collected by divers in the Avalon Peninsula, Newfoundland (47°17'44.6"N, 52°46'8.9"W), eastern Canada, at depths between 5 and 15 m. They were kept in holding tanks with running seawater at ambient temperature (1.2 – 2.1°C) for over a month. Plankton present in natural seawater was available to sea cucumbers during this period. Only healthy and undamaged individuals displaying normal pigmentation and feeding activity, firm attachment to the substrate and no skin lesion were selected for the feeding trials. In order to compare total lipid and fatty acid contents in tissues of *C. frondosa* fed in the laboratory with those of sea cucumbers from the field, another group of 5 individuals was collected immediately

after the feeding experiment. They weighed 5.2 ± 1.1 g immersed weight and measured 11.4 ± 1.2 cm contracted body length.

4.3.2 Experimental Design

4.3.2.1 Experimental tanks

There were 3 tanks for each treatment, containing 12 sea cucumbers each (36 sea cucumbers per treatment). Treatment tanks were randomly distributed in the experimental design. Sea cucumbers were acclimated in the tanks 5 days prior to the start of the experiment; no additional food was provided during this period. Tanks consisted of 150 L flow-through (15 L h^{-1}) units which were moderately aerated with air bubbling in order to maintain the provided food (diatoms and fish eggs) in suspension. Water temperature was kept at 7 ± 1°C and salinity at 35 (both factors were monitored daily with a YSI 556 MPS® probe). Light was provided by fluorescent bulbs at a maximum intensity of 150 Lux measured with a light meter (Traceable®) at the water surface. The photoperiod was adjusted once a week in order to match the natural environment, from 8.5L/15.5D to 12L/12D over the experimental period. The water flow was interrupted for 2 h during feeding times to insure that all sea cucumbers had enough time to react to the feeding cues as the deployment of *C. frondosa* tentacles occurs within 12 min after the addition of food in the tanks (Gianasi *et al.*, 2015). The filtration system which supplied water for the experimental tanks consisted of sandbed filters, heaters, ultraviolet, degassing media and foam fractionation filters which allow the removal of suspended and dissolved organic matters. Particles over 50 μm were removed by the filtration system before reaching the

tanks, leaving a portion of the micro/nanoplankton (including small ciliates, flagellates, diatoms and copepod nauplii.

4.3.2.2 Diets and feeding procedure

The experimental diets were chosen based on a literature review of the feeding ecology of *C. frondosa*, which showed that they feed on planktonic particles typically ranging from 4 to 350 μm in diameter, and that eggs may also be ingested (Hamel and Mercier, 1998; Singh *et al.*, 1998). The diatom *Chaetoceros muelleri* (~4 – 9 μm diameter) was cultivated in 400 L polyethylene bags illuminated 24 h daily by a set of fluorescent bulbs (daylight and cool light). Nutrients A and B and sodium metasilica (Kent Marine Pro-Culture®) were added to the culture weekly. Fish eggs consisted of the preparation "Real Oceanic Eggs" commercialized by Reed Mariculture Inc. (Reef Nutrition®, Campbell, CA, USA). According to the manufacturer, eggs were 0.7-1.5 mm in diameter and consisted mostly of Atlantic cod eggs preserved in seawater with citric, ascorbic, sorbic and proprionic acids and sodium alginate. They were kept refrigerated at 4°C at all times. Experimental groups were fed twice daily (at 9:00 and 14:00 h) with either the diatom *C. muelleri* (Fed diatoms) or fish eggs (Fed fish eggs) for a total of 84 days (12 weeks), whereas a control group did not receive any additional food (Un-supplemented). In order to provide the same biomass of both diets (~2 g dry weight per feeding session), a concentration of 95 x 10^3 cell ml^{-1} of *C. muelleri* and 0.20 egg ml^{-1} of fish eggs was achieved in the experimental tanks at the onset of each feeding period. Although the same biomass was provided to each supplemented treatment, diets contained different proportions of organic/inorganic matter. Diatom frustules are

composed mainly of silica which can represent 20-40% of the total dry weight in *Chaetoceros* sp. (Renaud *et al.*, 1999) while cod eggs have ~15% mineral content (Finn *et al.*, 1995). Prior to feeding, the diatoms and fish eggs were mixed with 10 L seawater at the same temperature as the experimental tanks. These preparations were then spread out in the tanks to reach the desired concentration. With the assistance of the aeration in the experimental tanks (described below), the feeds were evenly distributed in the tanks inside ~30 s. Only seawater was added to the tanks of the sea cucumbers in the un-supplemented treatment.

4.3.2.3 Sampling and data collection

All sea cucumbers (n=12 per tank) were measured (mouth to anus, contracted) at the beginning of the experiment and again 3 h after the last feeding using a flexible measuring tape. Immersed weights (iw) of sea cucumbers were obtained (So *et al.*, 2010) using an underhook weighing system (OHAUS® V21PW6) to which a net was attached that held the sea cucumber while it was immersed in seawater. Using immersed weight is advantageous because it is less stressful and provides a more accurate measure of weight in sea cucumbers, since wet weight (ww) can vary with the amount of seawater trapped in organs, such as the respiratory tree (Gudimova *et al.*, 2004; Stansbury and Hynick, 2009). For the sake of comparison with other studies, rough equivalences were determined by measuring both the wet weight (Battaglene *et al.*, 1999) and immersed weight of 15 sea cucumbers of various sizes. A linear relationship was found ($iw = 0.0098 * ww$, $R^2 = 0.926$), which allowed us to determine that the wet weight of the experimental sea cucumbers ranged between 51 and 408 g. The specific growth rate (SGR) was calculated

for each experimental tank as: SGR (% body weight gain day^{-1}) = ($\ln W_2$-$\ln W_1$)/T * 100, where, W_1 and W_2 are the initial and final weights of sea cucumbers and T is the duration of the experiment in days.

In order to confirm that sea cucumbers captured and ingested the food offered during the experiment, 2 sea cucumbers from each tank (n=6 per treatment) were selected haphazardly for investigation of intestinal contents 3 h after the last feeding time and final measurements. The intestine was removed, placed in a glass petri dish half filled with filtered seawater and examined for contents under a stereomicroscope (Nikon SMZ1500) coupled to a digital camera (Nikon DXM1200F). For comparison purposes, the same procedure was conducted using 3 individuals freshly collected from the field.

Finally, 4 sea cucumbers from each tank (n=12 per treatment) were haphazardly selected and samples of gonad and body wall muscle bands were collected for total lipid and fatty acid analysis. The gonad was removed from its point of attachment to the gonoduct, lightly blotted on a paper towel and weighed. A total of 3 g wet weight of gonadal tissue was collected from the largest tubules and preserved in lipid-clean vials (see below). The muscle bands were removed from the body wall using a metal spatula, lightly blotted on a paper towel and weighed. A total of 4 g wet weight of longitudinal and circular muscle tissue was collected from the center of the muscle and preserved in lipid-clean vials. The same procedure was carried out for sea cucumbers collected in the field.

Three samples (10.5 g) of fish egg diet (~21 x 10^3 eggs) were collected from a previously shaken bottle and preserved in lipid-clean vials. Three 50-ml samples of the diatom diet (3.6 x 10^6 cells ml^{-1}) were vacuum filtered on microfiber glass filters with

pore size of 0.70 μm (Whatman GF/F) that had previously been burnt in a muffle furnace at 450°C for 24 h. All samples for lipid analysis were preserved in lipid-clean 50-ml centrifuge tubes with 8 ml of chloroform ($CHCl_3$) topped up with nitrogen gas (N_2). The tubes were then sealed with Teflon® tape and placed in a freezer at -20°C until extraction (see below).

4.3.3 Total lipid and fatty acid analysis

Lipid and fatty acid analyses were performed on the gonad and muscle bands (longitudinal and circular) of sea cucumbers from the feeding trial and sea cucumbers collected in the field, as well as on each of the two supplement diets. A modified Folch procedure was used to extract lipids (Folch *et al.*, 1957; Parrish, 1999). All samples were homogenized in a 2:1 chloroform:methanol solution using a Polytron PCU-2-100 homogenizer (Brinkmann Instruments®, Rexdale, Ontario, Canada). Chloroform extracted water was used to bring the sample to a methanol:chloroform:water ratio of 1:2:1. Then samples were sonicated for 5 min in an ice bath and centrifuged at 3000 rpm for two min. The bottom, organic layer was then removed using a double pipetting technique to avoid disturbing the aqueous, top layer. Chloroform was then added back to the sample and the procedure was repeated a total of three times. All organic layers were pooled and concentrated using a flash-evaporator (Buchler Instruments®, Fort Lee, New Jersey, USA). Final samples were blown down to volume using nitrogen, sealed with Teflon® tape and stored at -20°C until measurements of fatty acids and total lipid classes were undertaken. For fatty acid analysis, 20 μL of lipid extract were transferred to lipid clean vials. Fatty acids were transesterified using the Hilditch reagent ($1.5H_2SO_4$:98.5

anhydrous MeOH) for 1 h at 100°C. FAMEs were analysed on a HP 6890 Series GC system and run for 30 min. Chromatograms were compared to a prepared standard and analyzed using Varian Galaxie® Chromatography Data System, version 1.93.2 (Agilent Technologies, Colarada, USA).

Total lipid was determined using a series of developing and conditioning sequences routinely used for the separation of aquatic lipid classes on Chromarods, quartz rods covered in silica (Parrish, 1987). Only gonad samples needed to be diluted (10 µl of sample extract in 50 µl of chloroform) before being spotted on Chromarods. The amount spotted on the rods was 0.5 µl directly from the samples or from the dilution (gonad samples) and this was focused in acetone, then developed twice in hexane:diethyl ether:formic acid (98.95:1:0.05). After drying for five min at constant humidity, the rods were scanned using an Iatroscan MK-6 to quantify non-polar lipids. Next, to develop more polar lipids two development sequences were used. First, samples were developed in hexane:diethyl ether:formic acid (79.9:20:0.1), dried and scanned. For the last sequence, samples were developed twice in 100% acetone, then developed twice in chloroform:methanol:chloroform-extracted-water (5:4:1) and scanned a final time. Chromatograms were compared to a prepared standard and analyzed using PeakSimple® Chromatography Software, version 2.38 (SRI Instruments, California, USA).

4.3.4 Data analysis

The presence of tank effects on the dataset was tested with one-way analysis of variance (ANOVA) for final contracted body length and one-way ANOVA on ranks for specific growth rates, lipid class and fatty acid ratios of DHA:EPA and ARA:EPA in

tissues of *C. frondosa*. However, no significant tank effects were found ($p>0.184$), and the tank factor was therefore removed from further analyses.

One-way analysis of variance (ANOVA) followed by Holm-Sidak pairwise comparisons was used to compare contracted body length of sea cucumbers among treatments at the start and at the end of the feeding trial. Specific growth rate (SGR) violated the assumptions for parametric statistics even after transformations. For this reason, ANOVA on ranks followed by Tukey test was used to compared SGR of sea cucumbers among treatments at the end of the study.

Total lipid content and fatty acid ratios of docosahexaenoic:eicosapentaenoic (DHA:EPA) and arachidonic:eicosapentaenoic (ARA:EPA) in muscle and gonadal tissues also violated the assumptions for parametric statistics; one-way ANOVA on ranks was used to compare them among treatments. Because the number of sea cucumbers was unequal among treatments (12 sea cucumbers from each treatment in laboratory and 5 sea cucumbers from the field), pairwise comparisons were done using Dunn's test. The total lipids, the amount of DHA and EPA and ratio of ARA:EPA in the diatom and fish egg diets were compared using Student's *t*-test.

Non-metric multidimensional scaling analysis (nMDS) was performed based on a Bray-Curtis similarity coefficient to visualize how fatty acid profiles varied among tissues (muscle and gonad) and diets. No transformation was applied to the dataset. Permutational multivariate ANOVA (PERMANOVA) analysis was conducted on the fatty acid profiles in order to determine the average similarity among tissues, treatments (un-supplemented, fed sea cucumbers and individuals collected from the field) and diets (diatom and fish egg) and whether treatments differ from each other. One-way similarity

percentage (SIMPER) analysis was used to determine which fatty acids were more influential in each tissue and diet. Finally, multivariate dispersion indices (MVDISP) were used to quantify the variability of fatty acid profiles in each diet, tissue and experimental group.

Fatty acids which represented less than 2% of total fatty acids in all samples were removed from further analyses. Despite removing fatty acids with low contribution, the remaining fatty acids still represented more than 80% of the total fatty acid in each sample. Data in the text are expressed as mean ± standard error. Statistical analyses were conducted with Statistica® and Primer® using $\alpha = 0.05$.

4.4 Results

4.4.1 Growth and intestinal contents

No significant different among treatments was detected in the body length of sea cucumbers at the start of the experiment. However, the body length of sea cucumbers fed with diatoms (11.30 ± 0.30 cm) and fish eggs (11.70 ± 0.30 cm) was statistically higher than that of un-supplemented sea cucumbers (10.01 ± 0.2 cm) at the end of the experiment (Fig. 4.1). No significant difference occurred in the final length of sea cucumbers fed either supplements (diatoms or fish eggs). Overall, the increase in contracted body length was highest in sea cucumbers fed with fish eggs (1.60 ± 0.50 cm increment), followed by individuals fed with live diatoms (0.90 ± 0.50 cm), whereas un-supplemented sea cucumbers exhibited a reduction in mean body length by an average of 0.30 cm (Table 4.1).

Specific growth rates (SGR) were significantly higher for sea cucumbers fed with fish eggs (0.05 ± 0.04% d^{-1}) and diatoms (0.04 ± 0.03% d^{-1}) than for sea cucumbers that did not receive any supplement (–0.24 ± 0.07% d^{-1}). In addition, the SGR did not differ between individuals supplemented with fish eggs or diatoms (Fig. 4.2, Table 4.2).

Investigation of the intestinal contents revealed that sea cucumbers fed with diatoms exhibited a greenish mass of phytoplankton inside the intestinal cavity (Fig. 4.3a), while individuals fed with fish eggs exhibited white/yellow spots which corresponded to the ingested eggs (Fig. 4.3b). There was a visible accumulation of food, especially ~5 cm from the stomach, in fed sea cucumbers. The intestine of un-supplemented sea cucumbers and individuals collected from the field did not present any evidence of recently ingested organic material.

4.4.2 Total lipid content

The commercial fish egg diet had significantly higher concentration of lipids (3.40 ± 0.28 mg g^{-1} ww) than the diatom diet (0.17 ± 0.02 mg ml^{-1} ww; Fig. 4.4a; Table 4.3). Free fatty acids, phospholipids, sterols and ethyl ketones contributed ~80% of the total lipid classes of fish eggs, whereas acetone mobile polar lipids (which include chlorophyll), phospholipids and triacylglycerols represented ~70% of the total lipid classes in the diatoms (Table 4.4).

The concentration of lipids was statistically higher in gonadal tissue than in muscle tissue across treatments (Fig. 4.4b; Table 4.3). The majority of lipids found in the gonad comprised triacylglycerols, free fatty acids and phospholipids, which together represented ~80% of the total lipid content (Table 4.4). Sea cucumbers freshly collected

from the field exhibited the highest lipid content in the gonad (88.23 ± 8.87 mg g^{-1} ww), followed by un-supplemented sea cucumbers (80.63 ± 16.31 mg g^{-1} ww) and sea cucumbers fed with fish eggs (77.01 ± 14.36 mg g^{-1} ww). The lowest lipid content in gonadal tissue was found in individuals fed with diatoms (46.96 ± 8.19 mg g^{-1} ww, Fig. 4.4b). However, no statistical differences were detected except for significantly lower concentrations of lipid in gonadal tissue of sea cucumbers fed with diatoms relative to all other treatments (Table 4.3).

Muscle bands chiefly contained phospholipids and acetone mobile polar lipids, which represented ~80% of the total lipids (Table 4.4). Sea cucumbers fed with diatoms exhibited the highest lipid content in muscles (13.00 ± 0.63 mg g^{-1} ww), followed by individuals collected in the field (12.76 ± 0.84 mg g^{-1} ww) and un-supplemented sea cucumbers (9.95 ± 0.4 mg g^{-1} ww). The lowest concentration of lipid in muscle tissue was found in sea cucumbers fed with fish eggs (9.34 ± 0.80 mg g^{-1} ww, Fig. 4.4b). Similar amounts of lipid occurred in muscle tissue of sea cucumbers fed with diatoms and individuals collected from the field; those were significantly higher than in individuals receiving no supplement or fed with fish eggs (Table 4.3).

4.4.3 Fatty acid profiles

The non-metric multidimensional scaling analysis (nMDS) revealed that the difference in fatty acid profiles in muscle and gonadal tissues of *C. frondosa* is great enough to distinguish the two (Fig. 4.5). The highest variation occurred in gonad samples. Also, the profile of diatoms grouped closer to that of sea cucumber gonad (Fig. 4.5).

Permutational multivariate ANOVA (PERMANOVA) analysis revealed low similarity (~40%) of fatty acids and consequently statistically different profiles between diets (diatoms and fish eggs) and when diets were compared to tissues (~32-54%) of sea cucumbers in all treatments (Table 4.5). Fatty acid profiles of tissues of sea cucumbers freshly collected from the field showed intermediate levels of similarities (55-71%) when compared to un-supplemented sea cucumbers and sea cucumbers fed with diatoms or fish eggs, although the fatty acid profile in this group was statistically different from other treatments (Table 4.5). On the other hand, high similarity (60-78%) was found in either muscle or gonadal tissues of sea cucumbers from any of the treatments in laboratory and it did not differ statistically among them (Table 4.5). Similarity percentage (SIMPER) analysis determined that eicosapentaenoic acid (EPA; 20:5ω3), adrenic acid (ADA; 22:4ω6) and arachidonic acid (ARA; 20:4ω6) dominated muscle tissue, whereas palmitoleic acid (16:1ω7), eicosapentaenoic acid (EPA; 20:5ω3) and methyltetradecanoic acid (*ai*15:0) were dominant in gonadal tissue (Table 4.6 and Fig. 4.5). The diatoms were rich in palmitoleic acid (16:1ω7), eicosapentaenoic acid (EPA; 20:5ω3) and palmitic acid (16:0), whereas fish eggs were rich in docosahexaenoic acid (DHA, 22:6ω3), palmitic acid (16:0) and eicosapentaenoic acid (EPA; 20:5ω3; Table 4.6; A1). The multivariate dispersion index indicated low variability in the fatty acid profiles across both diets and in muscle tissues across all treatments (Table 4.7). Conversely, gonadal tissue had the highest variability among fatty acid profiles across treatments (Table 4.7).

The fish egg diet had significantly higher DHA (29.2 ± 0.04%) than the diatom diet (Fig. 4.6a), because docosahexaenoic acid (DHA, 22:6ω3) was bellow detection in the latter, whereas it represented ~30% of the total fatty acids in the former (Table 4.8).

The diatom diet showed significantly higher level of EPA (17.9 ± 0.7%) than the fish egg (15.0 ± 0.09%; Table 4.8). Overall, gonads showed significantly higher DHA:EPA than muscles (Fig. 4.6b, Table 4.8). The gonad of sea cucumbers fed with fish eggs had significantly higher DHA:EPA (0.42 ± 0.03) than all other gonad samples (Fig. 4.6b). Gonad samples of un-supplemented sea cucumbers (0.10 ± 0.03), sea cucumbers fed with diatoms (0.16 ± 0.02) and individuals collected from the field (0.17 ± 0.05) showed similar ratios of DHA:EPA (Table 4.8). Muscle tissue of sea cucumbers fed with diatoms (0.04 ± 0.00) and fish eggs (0.05 ± 0.00) showed similar ratios of DHA:EPA. Those were significantly higher than un-supplemented sea cucumbers (0.01 ± 0.00) and individuals collected in the field (0.01 ± 0.00). Also, the later had similar DHA:EPA to un-supplemented sea cucumbers (Fig. 4.6b, Table 4.8).

The ratio ARA:EPA was similar for both diatoms (0.14 ± 0.00) and fish eggs (0.14 ± 0.00; Fig. 4.7a, Table 4.9). Sea cucumbers fed with diatoms had the highest ARA:EPA (0.40 ± 0.10) in muscle tissue among all treatments, followed by individuals fed with fish eggs (0.27 ± 0.06) and un-supplemented individuals (0.17 ± 0.01). The lowest ARA:EPA in muscle tissue was found in sea cucumbers freshly collected from the field (0.09 ± 0.25, Fig. 4.7b). A similar ratio was observed in muscle tissues of sea cucumbers fed with diatoms and fish eggs, which were significantly higher than ratios in un-supplemented sea cucumbers and individuals collected from the field (Table 4.9). Sea cucumbers fed with fish eggs had the highest ARA:EPA in gonadal tissue (0.27 ± 0.02) among all treatments, followed by field sea cucumbers (0.25 ± 0.00) and individuals fed with diatoms (0.10 ± 0.03, Fig. 4.7b). Sea cucumbers fed with fish eggs and individuals collected from the field had similar ARA:EPA in gonad samples (Table 4.9), which was

significantly higher than that of un-supplemented sea cucumbers and individuals fed with diatoms (Table 4.9).

4.5 Discussion

A growing interest in sea cucumber aquaculture around the world has led to the exploration of new species with captive-breeding potential. Compared to aspidochirotid sea cucumbers, little is known about the diets and feeding habits of dendrochirotid sea cucumbers in captivity. Several factors influence the suspension-feeding activity of dentrochirotids, such as water movement, and size and chemical composition of particles, which can be related to their nutritional content. The combination of all these factors mediates the extension of the oral tentacles for capture, as well as the digestion and assimilation of nutrients for growth and maintenance of basal metabolism. Inadequate food supply may result in retarded growth, loss of weight and low assimilation of essential amino acids and fatty acids.

Live diatoms and the commercial preparation of fish eggs (mostly Atlantic cod eggs) were both well accepted by *C. frondosa* under laboratory conditions. This species is widely distributed in cold waters covering rocky substrates at depths down to 300 m (Singh *et al.*, 1998; Hamel and Mercier, 2008), and is therefore exposed to a wide diversity of food sources including phytoplankton, zooplankton and organic detritus. It was initially assumed that *C. frondosa* could only capture and ingest small particles ranging from 4 to 350 μm in diameter, which included mainly phytoplankton such as *Coscinodiscus centralis*, *C. debilis*, *Skeletomena costatum*, *Thalassiosira gravida* (Hamel and Mercier, 1998) and *Isochrysis galbana* (Singh *et al.*, 1998). However, the results

from the present experiment revealed that *C. frondosa* not only captures and ingests small particles like the diatom *C. muelleri* (~4 – 9 μm diameter), but also larger fish/cod eggs with diameters ranging from ~0.70 to 1.50 mm. The adhesive mucus covering the extended tentacles might be involved in capturing larger suspended particles. However, whether a preference for larger particles over smaller ones or for particles with higher versus lower energetic contents exists in *C. frondosa* still remains to be clarified.

The presence of organic particles of any size in the water column apparently stimulates the feeding response of *C. frondosa*, which supports the non-selective feeding strategy reported in the St. Lawrence estuary for this species (Hamel and Mercier, 1998). Such an opportunistic behaviour may explain the broad, nearly circumpolar, distribution of the species in the Northern Hemisphere. The concentration of food observed in the initial part of the intestine of supplemented sea cucumbers was due to the feeding time which took place 3 h before the dissections. The absence of any evidence of food in the intestinal tract of sea cucumbers collected in the field may be explained by the seasonal feeding rhythm of *C. frondosa* in the North Atlantic, which has been documented to virtually cease in September/October (Singh *et al.*, 1998), the period when sampling took place.

Length increments and specific growth rates of sea cucumbers were affected by the availability of food. Individuals fed with fish eggs and diatoms exhibited a significantly greater size and weight increment than un-supplemented sea cucumbers, indicating that fed individuals can keep growing as long as food is available in the water column. Although *C. frondosa* was fed twice a day and growth could be observed during the 84-d experiment, it was lower than values reported for some temperate aspidochirotid

(deposit-feeding) sea cucumbers maintained at higher temperatures. *A. japonicus* fed with either formulated diets containing macroalgae (*Sargassum* spp.) or a mixed diet of dried bivalve faeces and powered algae had a SGR between 1.31% and 2.13% d^{-1} at water temperatures ranging from 13.2 to 19.8°C (Yuan *et al.*, 2006; Dong *et al.*, 2006). The SGR of *A. japonicus* cultured under sea bream cages ranged from 2.40% to 4.10% d^{-1} at water temperatures ranging from 9 to 24°C (Yokoyama, 2013). On the other hand, small individuals of *Parastichopus californicus* had a SGR of 0.11% d^{-1} under sablefish cages at water temperatures varying between 7 and 14°C (Hannah *et al.*, 2013). Water temperature has a major impact on growth and food intake in marine organisms. Considering that *C. frondosa* is a cold-water sub-Arctic species, slow growth is expected and has been reported previously (Hamel and Mercier, 1996a; So *et al.*, 2010). Un-supplemented sea cucumbers that were held in ambient seawater could not maintain their body weight over 84 days, resulting in negative SGR under the experimental conditions (7°C). Loss of body weight is common in sea cucumbers; it has been reported before in un-supplemented *C. frondosa* (So et al., 2010) as well as in temperate aspidochirotids, including *A. japonicus* during aestivation (Yang *et al.*, 2005) and *Australostichopus mollis* at high stocking density (Slater and Carton, 2007).

Lipid contents and fatty acid profiles of muscle and gonadal tissues either from individuals fed in the laboratory or from individuals collected from the field support the hypothesis that *C. frondosa* has a non-selective suspension feeding strategy. The majority of fatty acids encountered in muscle and gonad (e.g. EPA, ARA, DHA, 16:1ω7 and *ai*15:0) are typical biomarkers of diatoms, red algae, dinoflagellates, protozoa and bacteria (Parrish, 2013) which are the principal components of plankton in local coastal

waters. A previous study detected similar fatty acid profiles in fresh and processed *C. frondosa* (Zhong *et al.*, 2007). High proportions of DHA, EPA and 16:1ω7 have also been identified in the body wall of deposit-feeding sea cucumbers such as *A. japonicus* and *P. californicus* (Bechtel *et al.*, 2013; Lou *et al.*, 2012) since those feed on sediments rich in organic material, phytoplankton and bacteria.

Previous studies have shown that cod eggs collected from wild or farmed fish presented high levels of DHA and EPA (Salze *et al.*, 2005), consistent with results obtained for the commercial fish-egg feed studied here. Although those fatty acids were also predominant in muscle and gonadal tissue, it is unlikely that *C. frondosa* feeds on cod eggs in the wild since cod is a pelagic spawner and oil drops in their eggs provide positive buoyancy that would propel them to the water surface (Jung *et al.*, 2012). In the present experiment, *C. frondosa* was only able to feed on cod eggs because they had been preserved in seawater with citric, ascorbic, sorbic and proprionic acids and sodium alginate, which may have altered their natural buoyancy. In addition, the aeration of the tanks helped maintain the eggs in suspension in the water column, which facilitated their capture by the suspension-feeding sea cucumbers. While unidentified fish eggs were found in the intestinal tract of *C. frondosa* sampled in St. Lawrence Estuary (Hamel and Mercier, 1998), it is likely that they were demersal eggs such as those of capelin or halibut.

As expected, significant differences in fatty acid profiles were found between diets and sea cucumber tissues (muscles and gonads). The fish egg diet showed high levels of DHA (~30% of the total fatty acid), whereas DHA was below detection in the

diatom diet. Comparisons between tissues showed that fatty acids 16:1ω7 and *ai*15:0 dominated in gonads, while EPA and DHA were dominant in muscles.

Although sea cucumbers were fed twice a day with diatoms or fish eggs for 84 d, no significant change in the overall amount of lipids either in muscle or gonadal tissue was detected among treatments. The high levels of DHA (~30%) present in the fish egg diet was not reflected in tissues of sea cucumbers fed with fish eggs; the fatty acid profile of this group was similar to un-supplemented individuals and sea cucumbers fed with diatoms. The low metabolism and slow growth of *C. frondosa* might not have allowed the incorporation of new fatty acids in the tissues over the relatively short duration of the study (~3 months). Fatty acid profiles in muscle and gonad of sea cucumbers collected from the field were however different from those of captive-fed individuals, with the proviso that there was a lower samples size for the field group. Multivariate dispersion indices further showed that there was more variability in the fatty acid profiles of gonadal than muscle tissues. Gonads are known to act as a short-term storage organ for lipids during reproduction in sea cucumbers (Hudson *et al.*, 2004) and the constant transfer of energy might explain the variability among experimental groups. In contrast, muscle tissue displayed low variability among treatments as this tissue is primary used for long-term storage of energy.

Even though the period over which sea cucumbers were fed was not long enough to affect the broad fatty acid profiles, essential fatty acid ratios differed among treatments. The high DHA:EPA measured in muscle and gonadal tissues of *C. frondosa* fed with fish eggs suggests that the latter can maintain better reproductive performance during the year than un-supplemented individuals maintained in ambient conditions. Also, the ARA:EPA

ratio in tissues of individuals supplemented with diatoms or fish eggs was slightly higher than that in un-supplemented sea cucumbers or those freshly collected from the field. This indicates that feed-supplemented sea cucumbers are able to maintain a better balance of ARA and EPA and consequently eicosanoids, with implications for reproductive output. A study of gamete maturity and fecundity in sea cucumbers from the different treatments is under way to explore this hypothesis.

4.6 Conclusion

Understanding the feeding ecology of a species is a pivotal step in the development of adapted aquaculture protocols. The cold-water suspension-feeding sea cucumber *C. frondosa* has been identified as a potential candidate for aquaculture. The present study provides preliminary data on the influence of different diets on growth and lipid composition in muscle and gonadal tissues. The non-selective feeding strategy of *C. frondosa* was confirmed, as individuals were able to capture and ingest both types of food supplement provided, including diatoms *Chaetoceros muelleri* and fish (cod) eggs. The overall lipid and fatty acid profile of muscle and gonad tissues of supplemented versus un-supplemented sea cucumbers in the laboratory and versus individuals freshly collected from the field reflected a consistent feeding strategy for *C. frondosa*. Although sea cucumbers were fed twice daily during the 84-d trial, it was not enough to completely alter the fatty acid profile of their tissues, which might be attributed to the low metabolism of *C. frondosa*. However, sea cucumbers supplemented with fish eggs had an increase in body length and higher specific growth rates than un-supplemented sea cucumbers, which showed a reduction in body length and negative specific growth rate

over the feeding experiment. Fatty acid ratios of DHA:EPA and ARA:EPA also suggest that supplemented sea cucumbers may maintain better reproductive performance. The development of diets with high lipid contents might be an option to enhance growth in captivity, although further investigation is needed to determine optimal feeding rates and levels of amino and fatty acids and minerals. The findings presented here provide new information about the feeding ecology of a cold-water sea cucumber, which will hopefully help develop broodstock-conditioning protocols as aquaculture programs for sea cucumbers expand in temperate and subpolar environments.

4.7 Acknowledgments

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4.9 Tables

Table 4.1: Comparison of final contracted body length among treatments at the end of a 84-d feeding experiment. Sea cucumbers were supplemented with either live diatoms, *Chaetoceros muelleri* (Fed diatoms), or a commercial preparation of fish eggs (Fed fish eggs), while the control group did not receive any prepared food (Un-supplemented). Results of one-way ANOVA followed by Holm-Sidak test are shown.

Treatments	**F**	**df**	**p**
Un-supplemented x Fed diatoms x Fed fish eggs	5.929	2	0.004
Fed fish eggs > Un-supplemented			0.001
Fed diatoms > Un-supplemented			0.012
Fed diatoms = Fed fish eggs			0.205

Table 4.2: Comparison of specific growth rates (SGR) among sea cucumbers supplemented with live diatoms (Fed diatoms) or fish eggs (Fed fish eggs) and un-supplemented individuals after the 84-d feeding trial. Results of one-way ANOVA on ranks followed by Tukey test are shown.

Treatments	**H**	**df**	**p**
Un-supplemented x Fed diatoms x Fed fish eggs	7.200	2	0.004
Fed fish eggs > Un-supplemented			<0.001
Fed diatoms > Un-supplemented			0.002
Fed fish eggs > Fed diatoms			0.547

Table 4.3: Comparison of total lipids in diets and tissues of *C. frondosa*. Sea cucumbers were supplemented with diatoms (Fed diatoms) and fish eggs (Fed fish eggs) for a total of 84 d. The control group did not receive any additional food (Un-supplemented). Those were also compared with sea cucumbers collected in the field (Field). Results of t-test and ANOVA on ranks followed by Dunn's test are showed.

Samples	**Treatments**	**H**	**t**	**df**	**p**
Diets	Diatom x Fish egg				
	Fish egg > Diatom		22.312	4	<0.001
Gonad	Un-supplemented x Fed diatoms x Fed fish eggs x Field	13.862		3	0.024
	Un-supplemented > Fed diatoms				<0.001
	Fed fish eggs > Fed diatoms				<0.001
	Field > Fed diatoms				<0.001
	Un-supplemented = Fed fish eggs				0.977
	Un-supplemented = Field				0.796
	Fed fish eggs = Field				0.667
Muscle	Un-supplemented x Fed diatoms x Fed fish eggs x Field	13.777		3	0.003
	Fed diatoms > Fed fish eggs				0.003
	Fed diatoms > Un-supplemented				<0.001
	Field > Un-supplemented				0.041
	Field > Fed fish eggs				0.049
	Un-supplemented = Fed fish eggs				0.086
	Field = Fed diatoms				0.095

Table 4.4: Lipids in muscle and gonadal tissue of *Cucumaria frondosa* exposed to 3 treatments (un-supplemented, fed diatoms, fed fish eggs; n=12 for each). Lipids in the two diets (diatom, fish egg; n=3 for each) and in sea cucumbers collected from the field (n=5). Data shown as mean ± se in mg g^{-1} wet weight for tissues and fish egg diet and as mg ml^{-1} wet weight in diatom diet.

Samples	**Muscle**				**Gonad**				**Diets**	
Lipid Classes	**Un-supplemented**	**Fed diatoms**	**Fed fish eggs**	**Field**	**Un-supplemented**	**Fed diatoms**	**Fed fish eggs**	**Field**	**Diatom**	**Fish egg**
Hydrocarbons	0.04 ± 0.01	0.03 ± 0.00	0.06 ± 0.01	0.07 ± 0.02	0.24 ± 0.06	0.68 ± 0.25	0.45 ± 0.15	0.02 ± 0.01	-	0.032 ± 0.01
Steryl Esters/ Wax Esters	0.03 ± 0.01	0.05 ± 0.02	0.07 ± 0.04	-	0.24 ± 0.11	0.36 ± 0.28	0.214 ± 0.12	0.014 ± 0.01	-	0.01 ± 0.00
Ethyl Ketones	0.03 ± 0.00	0.39 ± 0.24	0.11 ± 0.06	0.01 ± 0.01	15.11 ± 9.21	2.60 ± 1.53	0.284 ± 0.18	-	-	0.60 ± 0.25
Triacylglycerols	0.58 ± 0.15	0.93 ± 0.19	0.23 ± 0.09	0.24 ± 0.16	27.65 ± 8.48	14.00 ± 3.55	34.45 ± 8.24	20.41 ± 1.27	0.03 ± 0.02	0.19 ± 0.04
Free Fatty Acids	0.46 ± 0.06	0.68 ± 0.16	0.46 ± 0.15	2.61 ± 0.47	13.83 ± 3.53	15.21 ± 5.31	18.17 ± 3.45	41.01 ± 4.88	0.02 ± 0.01	0.88 ± 0.12
Alcohols	0.22 ± 0.04	0.2 ± 0.03	0.12 ± 0.03	-	0.36 ± 0.15	0.77 ± 0.04	0.74 ± 0.43	-	-	0.14 ± 0.05
Sterols	0.64 ± 0.07	0.6 ± 0.06	0.59 ± 0.05	1.37 ± 0.12	9.07 ± 3.14	3.17 ± 1.03	8.52 ± 2.30	5.95 ± 4.06	0.01± 0.01	0.58 ± 0.24
Acetone Mobile Polar Lipids	2.49 ± 0.14	3.11 ± 0.31	2.07 ± 0.15	2.52 ± 0.07	3.42 ± 0.91	1.15 ± 0.18	2.05 ± 0.53	1.288 ± 0.42	0.06 ± 0.003	0.28 ± 0.11
Phospholipids	5.42 ± 2.77	6.94 ± 0.24	5.59 ± 0.51	5.90 ± 0.21	10.67 ± 1.55	9.67 ± 1.41	12.08 ± 1.57	19.51 ± 2.09	0.03 ± 0.00	0.59 ± 0.07

Table 4.5: Results of PERMANOVA analysis (% similarity) conducted on fatty acid profiles of diets and tissues of sea cucumbers. Diets consisted of the diatom *Chaetoceros muelleri* and fish eggs. Sea cucumbers were fed with *C. muelleri* (Fed diatoms) and fish eggs (Fed fish eggs) twice a day for a total of 84 d. Those were compared with un-supplemented sea cucumbers and individuals collected in the field. Percentage similarity in bold indicates that the fatty acid profile between groups is statistically different ($p<0.05$).

Samples	**Treatments/diets**	**Un-supplemented**	**Fed diatoms**	**Fed fish eggs**	**Field**	**Diatom**	**Fish egg**
Muscle	Un-supplemented	78.142					
	Fed diatoms	74.246	72.061				
	Fed fish eggs	75.853	74.076	75.413			
	Field	**71.904**	**67.094**	**68.082**	82.338		
Diets	Diatom	**35.002**	**32.183**	**31.931**	**45.471**	85.335	
	Fish egg	**32.609**	**32.256**	**32.414**	**36.777**	**41.200**	89.941
Gonad	Un-supplemented	59.094					
	Fed diatoms	62.061	68.056				
	Fed fish eggs	59.755	64.023	60.853			
	Field	**55.368**	**58.810**	**56.889**	69.757		
Diets	Diatom	**45.848**	**50.259**	**48.209**	**54.751**	85.335	
	Fish egg diet	**34.688**	**35.334**	**34.300**	**35.652**	**41.200**	89.941

Table 4.6: Percentage of the fatty acid composition (mean ± se %) of muscle and gonadal tissue of *Cucumaria frondosa* exposed to 3 treatments (un-supplemented, fed diatoms, fed fish eggs; n=12 for each), sea cucumbers collected from the field (n=5) and of diets (diatom and fish egg; n=3 for each).

Samples	**Diets**		**Muscle**				**Gonad**			
Fatty Acid	**Diatom**	**Fish egg**	**Un-supplemented**	**Fed diatoms**	**Fed fish eggs**	**Field**	**Un-supplemented**	**Fed diatoms**	**Fed fish eggs**	**Field**
14:0	8.03 ± 1.00	2.49 ± 0.59	1.00 ± 0.11	0.88 ± 0.12	1.00 ± 0.31	3.55 ± 0.53	3.90 ± 0.55	3.61 ± 0.41	2.82 ± 0.20	4.53 ± 0.42
***i*15:0**	0.01 ± 0.00	0.13 ± 0.06	0.17 ± 0.12	0.15 ± 0.11	0.09 ± 0.05	0.01 ± 0.00	1.13 ± 1.00	0.06 ± 0.01	1.11 ± 0.65	4.05 ± 2.23
***ai*15:0**	0.22 ± 0.10	-	1.67 ± 0.32	1.45 ± 0.30	1.16 ± 0.27	1.50 ± 0.32	12.2 ± 3.14	15.9 ± 2.18	13.8 ± 2.65	9.29 ± 4.16
15:0	0.66 ± 0.06	0.5 ± 0.11	0.08 ± 0.03	0.31 ± 0.23	0.07 ± 0.01	0.01 ± 0.04	2.24 ± 0.60	1.18 ± 0.16	1.43 ± 0.20	1.18 ± 0.20
***i*16:0**	0.58 ± 0.26	-	0.77 ± 0.30	0.64 ± 0.32	0.44 ± 0.21	0.24 ± 0.06	2.73 ± 0.80	1.78 ± 0.40	2.10 ± 0.48	1.53 ± 0.35
***ai*16:0**	-	0.03 ± 0.01	1.83 ± 0.64	1.95 ±0.45	2.72 ± 0.52	-	1.09 ± 0.54	0.96 ± 0.48	1.04 ± 0.43	-
16:0	12.73 ± 5.21	18.34 ± 0.08	1.31 ± 0.45	0.38 ± 0.27	0.48 ± 0.29	4.32 ± 0.34	2.15 ± 0.65	2.64 ± 0.70	2.36 ± 0.46	3.61 ± 0.36
16:1ω9	0.07 ± 0.00	1.78 ± 0.50	0.75 ± 0.37	1.23 ± 0.50	1.63 ± 0.65	0.01 ± 0.00	2.30 ± 1.46	1.26 ± 1.03	1.00 ± 0.88	0.03 ± 0.01
16:1ω7	32.2 ± 1.37	2.40 ± 0.75	2.91 ± 0.45	2.51 ± 0.19	1.61 ± 0.12	8.87 ± 0.97	14.0 ± 2.86	18.7 ± 1.85	18.0 ± 2.58	21.2 ± 0.88
***ai*17:0**	4.02 ± 0.12	0.18 ± 0.06	0.31 ± 0.05	0.30 ± 0.07	0.32 ± 0.05	0.41 ± 0.10	1.43 ± 0.24	1.35 ± 0.16	1.73 ± 0.30	1.01 ± 0.07
16:3ω4	10.4 ± 0.62	0.13 ± 0.05	2.58 ± 0.91	0.99 ± 0.32	2.53 ± 0.72	1.00 ± 0.82	0.87 ± 0.38	0.50 ± 0.13	0.56 ± 0.20	0.24 ± 0.21
17:1	0.76 ± 0.05	0.14 ± 0.07	3.23 ± 0.99	3.52 ± 1.11	3.07 ± 1.17	3.5 ± 1.02	1.56 ± 0.28	1.80 ± 0.30	1.80 ± 0.34	1.14 ± 0.26
16:4ω1	0.11 ± 0.04	-	0.51 ± 0.33	0.50 ± 0.25	0.29 ± 0.18	1.26 ± 0.47	1.10 ± 0.21	1.31 ± 0.26	0.79 ± 0.11	2.23 ± 0.13
18:0	3.29 ± 0.30	3.73 ± 1.32	2.82 ± 0.32	1.97 ± 0.35	2.57 ± 0.24	2.85 ± 0.70	2.35 ± 0.46	0.96 ± 0.26	2.00 ± 0.38	2.68 ± 0.30
18:1ω9	0.95 ± 0.49	9.98 ± 1.28	1.40 ± 0.36	0.85 ± 0.08	1.80 ± 0.44	1.81 ± 0.41	4.77 ± 0.85	4.00 ± 0.70	2.78 ± 0.60	2.89 ± 0.42
18:1ω7	1.22 ± 0.11	4.08 ± 0.03	2.62 ± 0.51	2.80 ± 0.22	2.20 ± 0.43	2.73 ± 0.90	4.74 ± 1.13	3.70 ± 0.70	5.74 ± 0.95	4.00 ± 0.22
18:5ω3	-	-	3.46 ± 1.18	2.69 ± 0.90	2.97 ± 1.02	-	0.69 ± 0.34	1.57 ± 0.88	0.54 ± 0.25	0.41 ± 0.36
20:1ω11	0.07 ± 0.06	0.25 ± 0.10	4.48 ± 1.06	4.74 ± 1.06	4.20 ± 0.94	5.72 ± 0.4	1.98 ± 0.30	1.26 ± 0.14	2.16 ± 0.40	-
20:4ω6	2.57 ± 0.23	2.10 ± 1.71	6.60 ± 0.42	8.67 ± 1.57	9.44 ± 1.80	3.25 ± 0.52	0.94 ± 0.19	1.94 ± 0.68	0.65 ± 0.09	0.55 ± 0.10
20:4ω3	-	1.74 ± 0.00	0.06 ± 0.03	1.10 ± 0.57	0.12 ± 0.05	0.16 ± 0.08	3.02 ± 2.40	0.21 ± 0.05	1.74 ± 1.50	5.29 ± 4.73
20:5ω3	17.9 ± 0.77	15.4 ± 0.09	35.9 ± 3.38	32.1 ± 2.00	32.3 ± 2.75	33.9 ± 1.11	12.2 ± 2.00	14.5 ± 1.43	16.9 ± 2.36	20.1 ± 4.57
22:1ω7	-	0.03 ± 0.03	1.06 ± 0.25	1.04 ± 0.17	2.48 ± 1.55	1.27 ± 0.06	0.46 ± 0.10	0.48 ± 0.08	0.38 ± 0.08	0.53 ± 0.10
21:5ω3	-	0.42 ± 0.31	3.17 ± 2.95	1.91 ± 1.06	0.08 ± 0.03	-	0.5 ± 0.10	0.98 ± 0.33	0.21 ± 0.04	-
22:4ω6	-	-	8.93 ± 2.19	14.12 ± 1.08	15.37 ± 1.92	10.03 ± 0.9	3.47 ± 0.88	3.74 ± 0.82	2.96 ± 0.54	1.55 ± 0.38
22:6ω3	-	29.2 ± 0.04	0.70 ± 0.22	0.91 ± 0.16	1.75 ± 0.28	0.9 ± 0.1	1.18 ± 0.19	0.87 ± 0.12	1.10 ± 0.30	1.0 ± 0.10
24:1	-	0.70 ± 0.28	1.24 ± 0.22	1.22 ± 0.11	1.65 ± 0.21	2.74 ± 0.13	2.07 ± 0.37	1.97 ± 0.28	1.58 ± 0.25	1.82 ± 0.44

Table 4.7: Multivariate dispersion index for fatty acid profiles among treatments and diets. Sea cucumbers were fed with diatoms (Fed diatoms) and fish eggs (Fed fish eggs) twice a day for a total of 84 d. The control group did not receive any additional food (Un-supplemented) and individuals collected from the field (Field) were also analysed.

Samples	**Diets/Treatments**	**Index**
Diets	Diatom	0.390
	Fish egg	0.202
Muscle	Un-supplemented	0.648
	Fed diatoms	0.899
	Fed fish eggs	0.773
	Field	0.447
Gonad	Un-supplemented	1.388
	Fed diatoms	1.077
	Fed fish eggs	1.333
	Field	0.978

Table 4.8: Percentage of DHA and EPA in diets and fatty acid ratio of DHA:EPA in tissue of *C. frondosa* among treatments. Sea cucumbers were fed with diatoms (Fed diatoms) and fish eggs (Fed fish eggs) for a total of 84 d. The control group did not receive any additional food (Un-supplemented). Those were also compared with sea cucumbers collected in the field (Field). Results of t-test and ANOVA on ranks followed by Dunn's test are shown.

Samples		**Treatments**	**H**	**t**	**df**	**p**
Diets	**DHA**	Diatom x Fish egg				
		Fish egg > Diatom		117.422	4	<0.001
	EPA	Diatom x Fish egg				
		Diatom > Fish egg		97.265	4	0.032
Gonad		Un-supplemented x Fed diatoms x Fed fish eggs x Field	12.365		3	0.001
		Fed fish eggs > Un-supplemented				0.001
		Fed fish eggs > Fed diatoms				0.039
		Fed fish eggs > Field				0.040
		Un-supplemented = Fed diatoms				0.068
		Un-supplemented = Field				0.094
		Field = Fed diatoms				0.758
Muscle		Un-supplemented x Fed diatoms x Fed fish eggs x Field	15.689		3	0.001
		Fed diatoms > Un-supplemented				0.009
		Fed diatoms > Field				0.047
		Fed fish eggs > Un-supplemented				0.005
		Fed fish eggs > Field				0.002
		Fed fish eggs = Fed diatoms				0.568
		Field = Un-supplemented				0.475

Table 4.9: Comparison of ARA:EPA in diets and tissues of *C. frondosa*. Sea cucumbers were fed with diatoms (Fed diatoms) and fish eggs (Fed fish eggs) for a total of 84 d. The control group did not receive any additional food (Un-supplemented). Those were also compared with sea cucumbers collected in the field (Field). Results of *t*-test and ANOVA on ranks followed by Dunn's test are shown.

Samples	**Treatments**	**H**	**t**	**df**	**p**
Diets	Diatom x Fish egg				
	Fish egg = diatom		0.0363	4	0.973
Gonad	Un-supplemented x Fed diatoms x Fed fish eggs x Field	12.469		3	0.017
	Fed fish eggs > Un-supplemented				0.037
	Fed fish eggs > Fed diatoms				0.030
	Field > Un-supplemented				0.027
	Field > Fed diatoms				0.043
	Field = Fed fish eggs				0.127
	Fed diatoms = Un-supplemented				0.258
Muscle	Un-supplemented x Fed diatoms x Fed fish eggs x Field	11.080		3	0.011
	Fed diatoms > Field				0.013
	Fed diatoms > Un-supplemented				0.039
	Fed fish eggs > Field				0.009
	Fed fish eggs > Un-supplemented				0.024
	Fed diatoms = Fed fish eggs				0.568
	Field = Un-supplemented				0.059

4.10 Figures

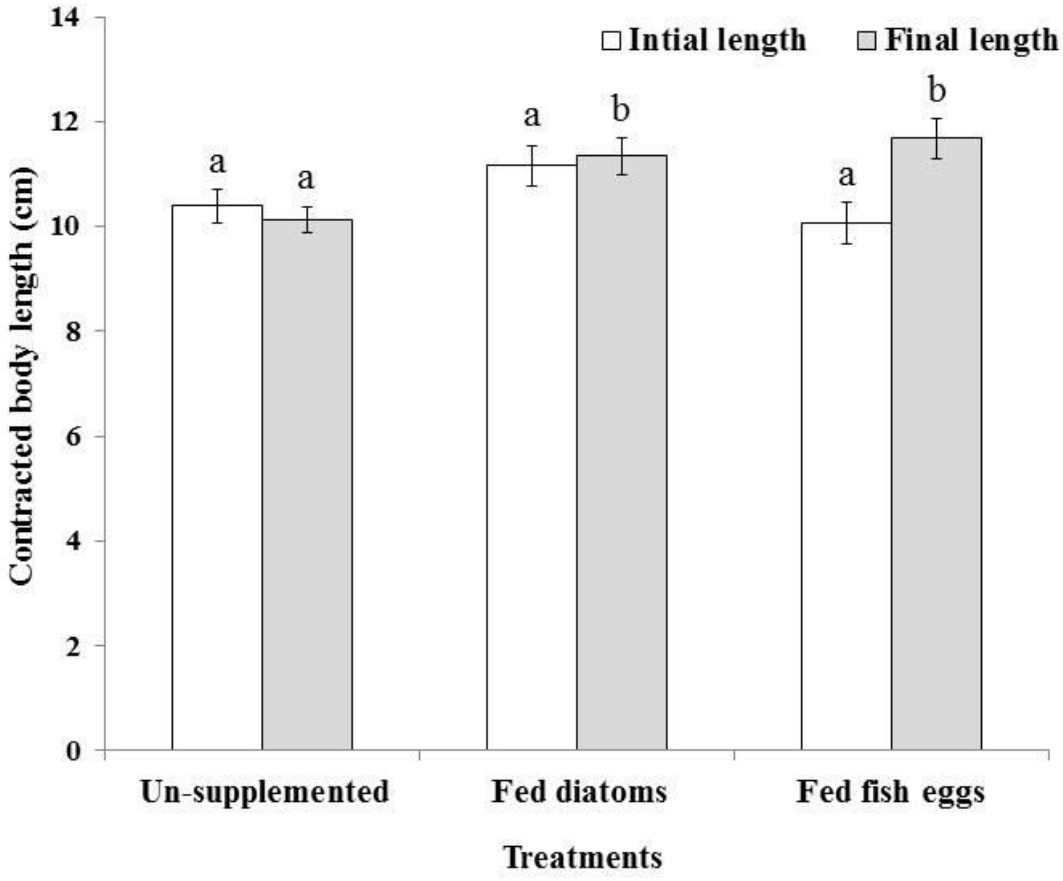


Figure 4.1: Measurements of initial and final contracted body length of un-supplemented sea cucumbers (having access to micro/nanoplankton in ambient running seawater), and sea cucumbers fed with a supplement of either live diatoms (*Chaetoceros muelleri*) or commercial fish eggs over 84 days. Data are mean ± se (n=3). Means with different letters are significantly different See Table 4.1 for statistical results.

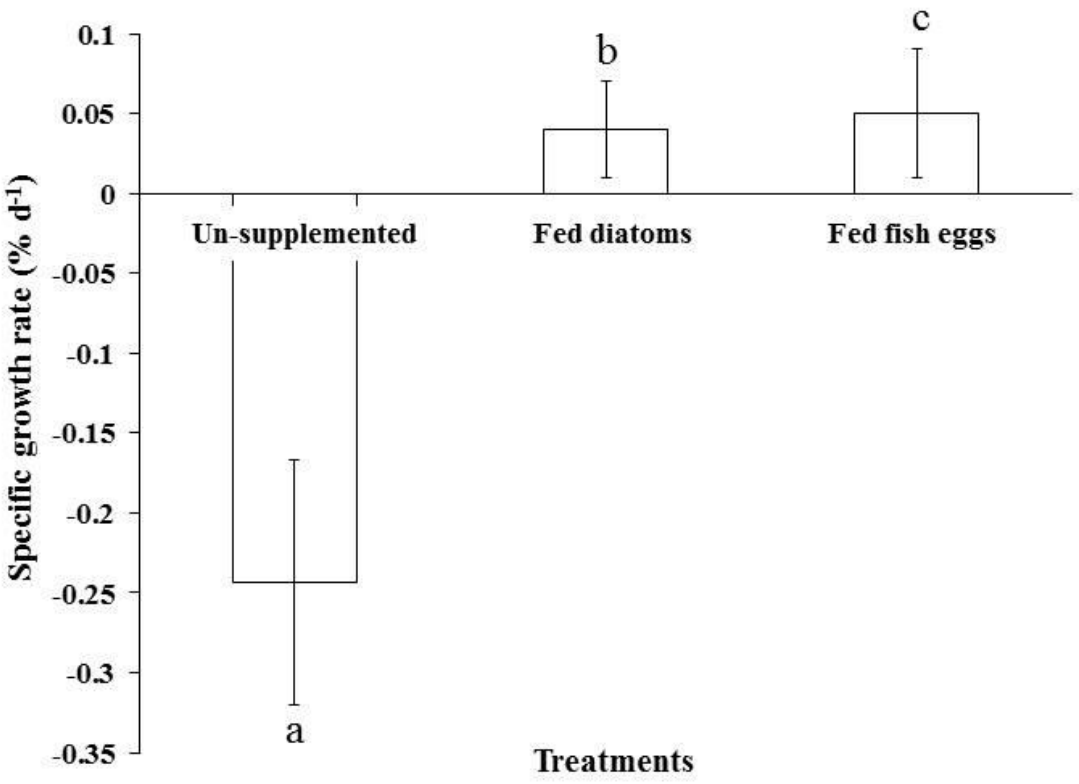


Figure 4.2: Specific growth rate (SGR) of *Cucumaria frondosa* fed with either live diatoms or commercial fish eggs compared to un-supplemented individuals over 84 days. Data are mean ± se (n=3). Means with different letters are significantly different. See Table 4.2 for statistical results.

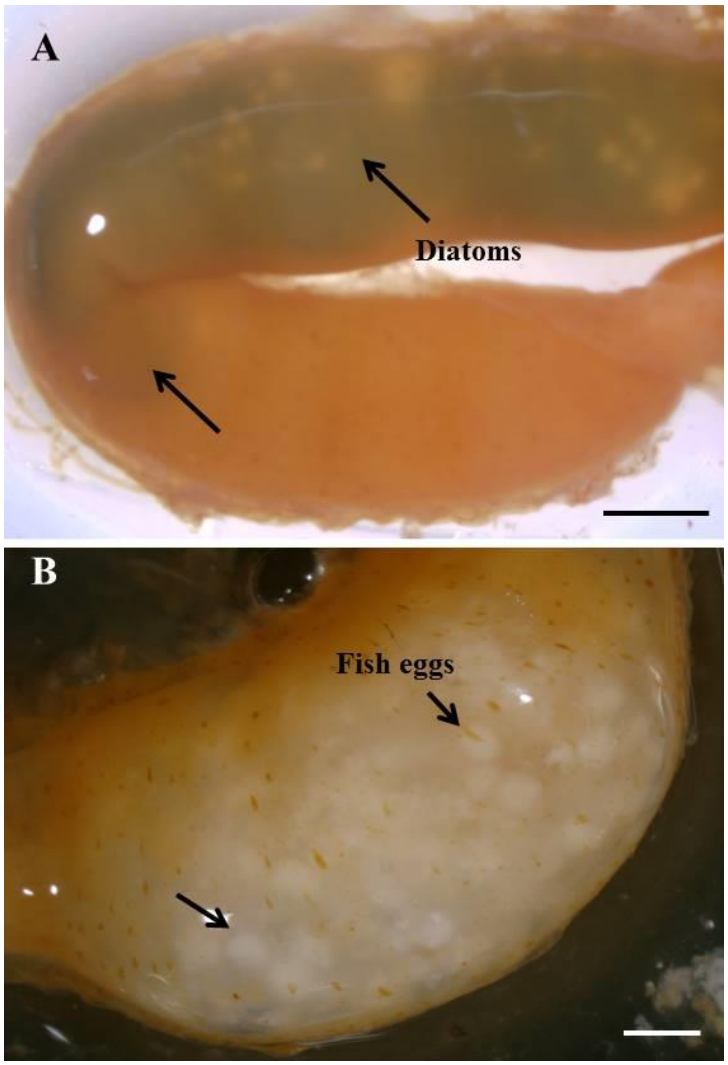
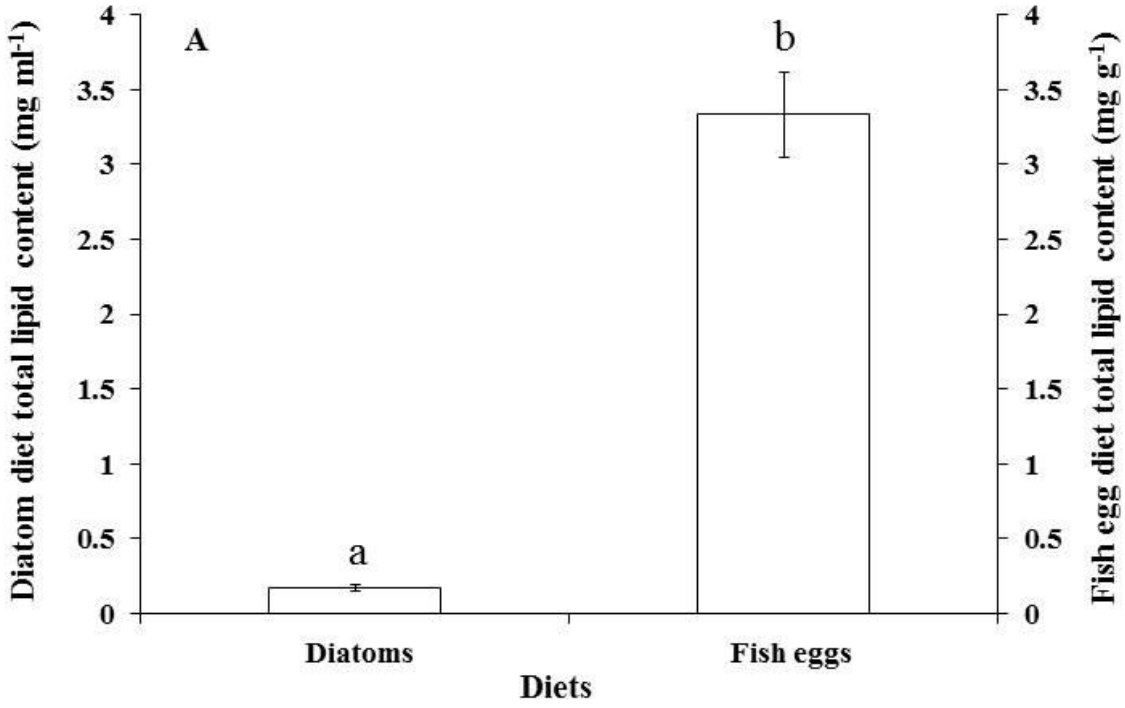


Figure 4.3: Intestinal content of *Cucumaria frondosa*. (A) Sea cucumbers fed with live diatoms *Chaetoceros muelleri* exhibited a greenish mass of phytoplankton in the intestinal tract. (B) Individuals fed with commercial fish eggs exhibited white/yellow spots in the intestinal tract, corresponding to ingested eggs. Pictures were taken 3 h after the food had been added to the experimental tanks during the last feeding period. Scale bars represent 3 mm.


A
Diatom diet total lipid content (mg ml-1)
Fish egg diet total lipid content (mg g-1)
0
0.5
1
1.5
2
2.5
3
3.5
4
a
b
Diatoms
Fish eggs
Diets

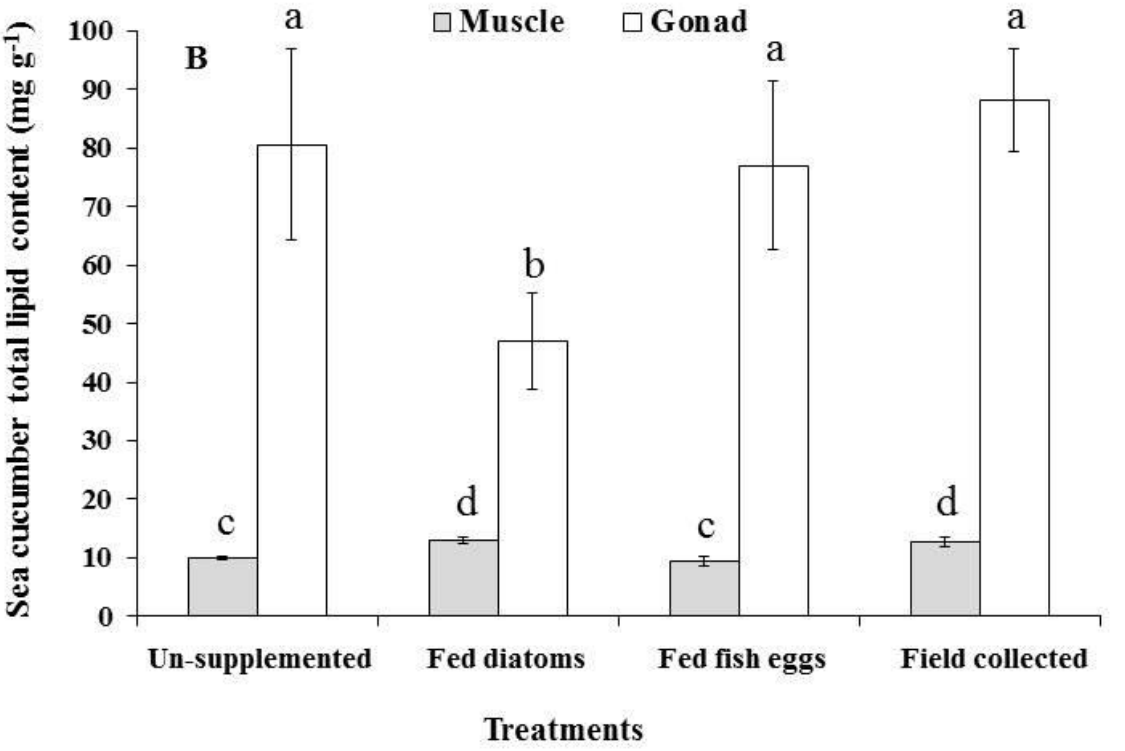

B
Muscle
Gonad
Sea cucumber total lipid content (mg g-1)
0
10
20
30
40
50
60
70
80
90
100
a
b
c
d
a
c
a
d
Un-supplemented
Fed diatoms
Fed fish eggs
Field collected
Treatments

Figure 4.4 (previous page): Total lipid content in diets and tissues of *Cucumaria frondosa*. (A) Total lipid content in the diatom *Chaetoceros muelleri* (n=3) and a commercial preparation of fish eggs (n=3). (B) Total lipid in muscle and gonadal tissue of *C. frondosa* fed with diatom (n=12) or fish eggs (n=12). Comparisons were made with un-supplemented sea cucumbers (n=12) and individuals freshly collected from the field (n=5). Data are mean ± se. Means with different letters are significantly different. See Table 4.3 for statistical results.

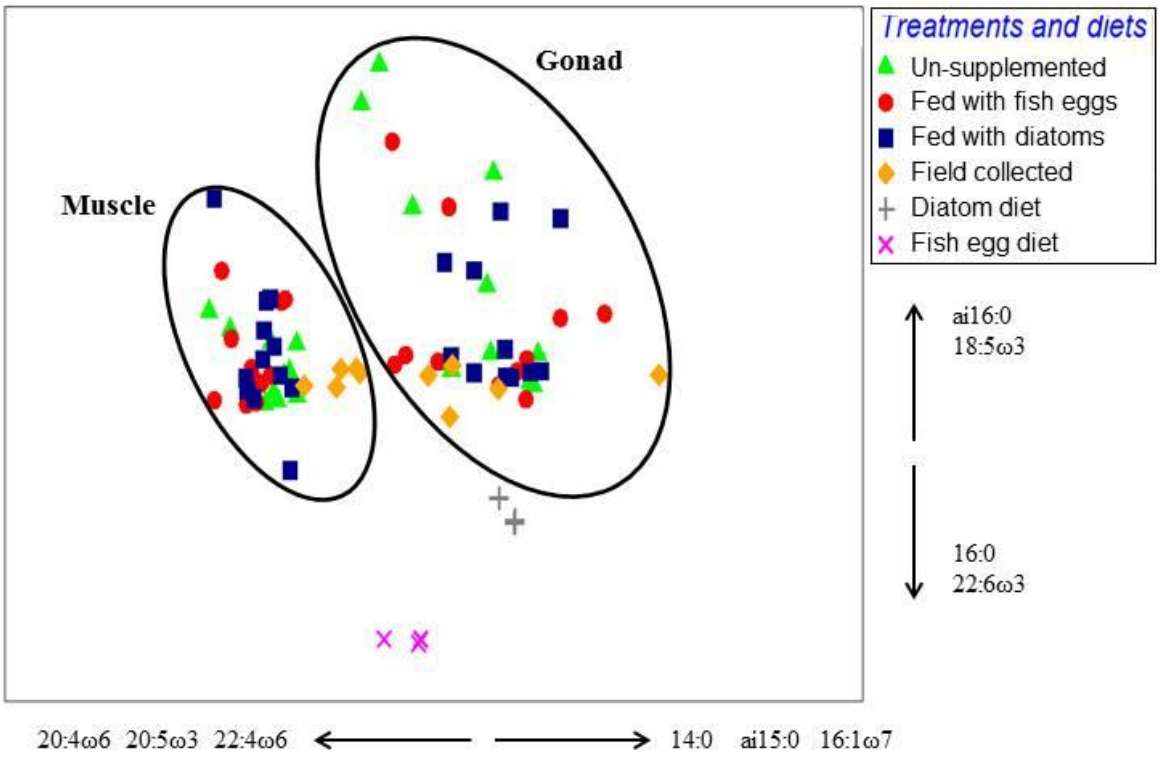


Figure 4.5: nMDS plot of fatty acid contents in diets and tissues of *C. frondosa*. Diets consisted of live diatoms *Chaetoceros muelleri* (Diatom diet) or a commercial fish eggs (Fish egg diet). Sea cucumbers were supplemented with diatoms (Fed diatoms) or fish eggs (Fed fish eggs) whereas the control group did not receive an additional food (Un-supplemented) during the 84-d feeding experiment. Sea cucumbers were also collected in the field (Field), at the end of the trial. Arrows and fatty acids indicate the main components responsible for group dissimilarities based on Pearson correlations > 0.5.

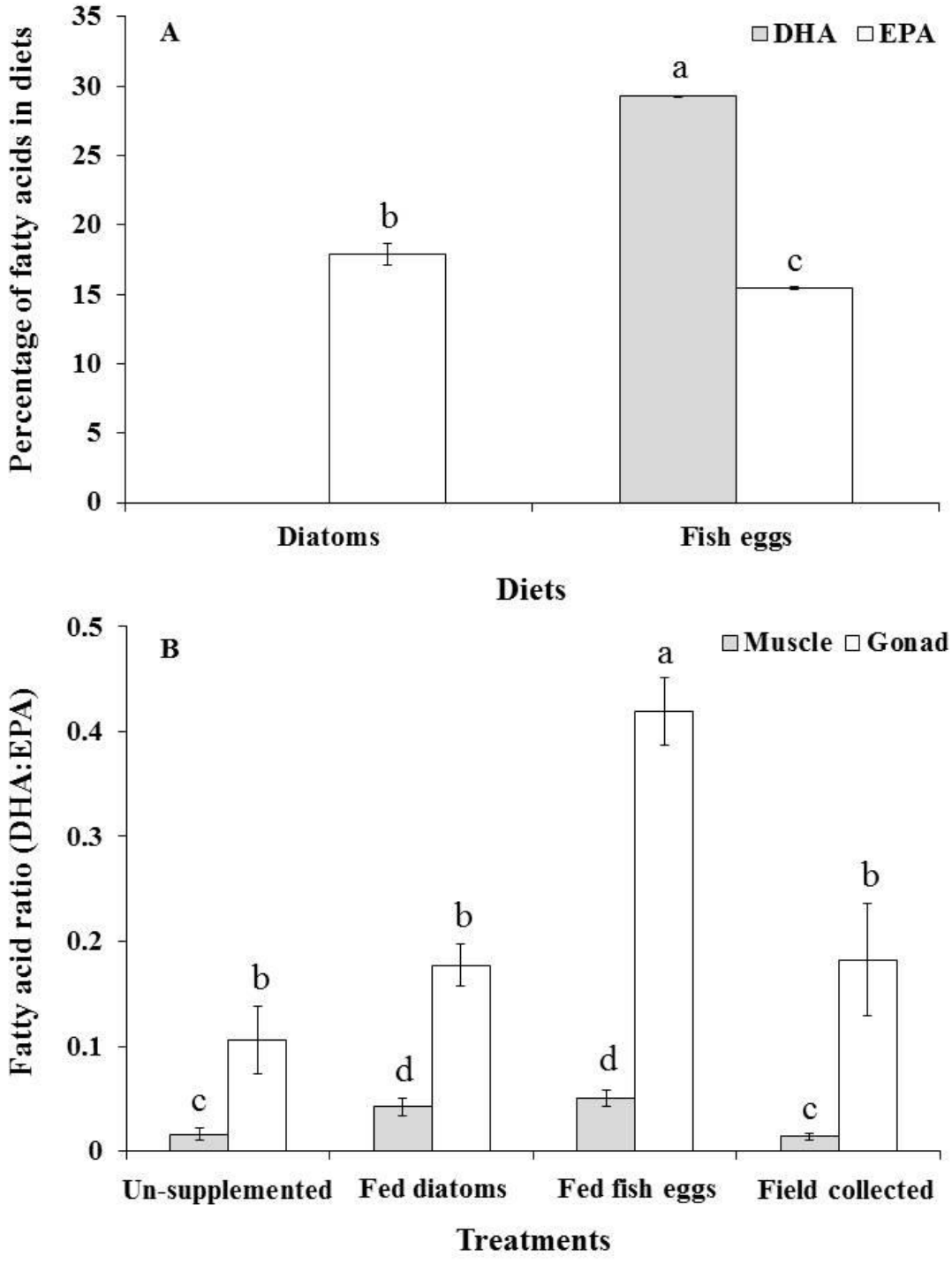

A
DHA
EPA
Percentage of fatty acids in diets
35
30
25
20
15
10
5
0
a
b
c
Diatoms
Fish eggs
Diets
B
Muscle
Gonad
Fatty acid ratio (DHA:EPA)
0.5
0.4
0.3
0.2
0.1
0
a
b
b
b
c
d
d
c
Un-supplemented
Fed diatoms
Fed fish eggs
Field collected
Treatments

Figure 4.6 (previous page): Fatty acids DHA (docosahexaenoic) and EPA (eicosapentaenoic) in diets and their ratio: DHA:EPA in tissues of *Cucumaria frondosa*. (A) Percentage (± se) of DHA and EPA in the diatom *Chaetoceros muelleri* (Diatom diet, n=3) and the commercial preparation of fish eggs (Fish egg diet, n=3). (B) DHA:EPA in muscle and gonadal tissues of *C. frondosa* fed with diatoms (Fed diatoms, n=12) and fish eggs (Fed fish eggs, n=12), un-supplemented sea cucumbers (Un-supplemented, n=12) during the 84-d feeding experiment and individuals collected in the field (Field, n=5). Means with different letters are significantly different. Data are mean ± se. See Table 4.8 for statistical results.

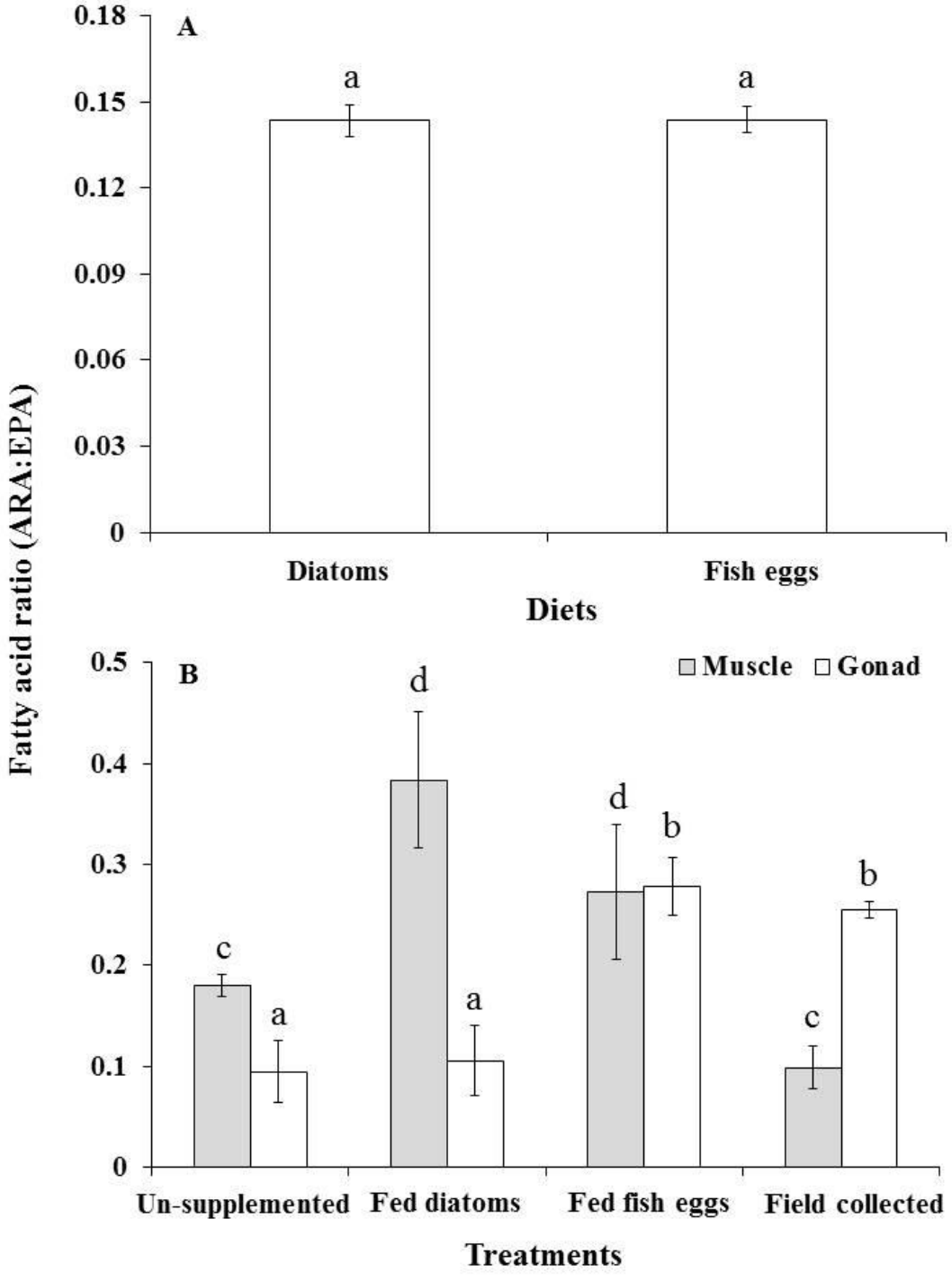

A
0.18
0.15
0.12
0.09
0.06
0.03
0
a
a
Diatoms
Fish eggs
Diets
Fatty acid ratio (ARA:EPA)
B
Muscle
Gonad
0.5
0.4
0.3
0.2
0.1
0
c
a
d
a
d
b
c
b
Un-supplemented
Fed diatoms
Fed fish eggs
Field collected
Treatments

Figure 4.7 (previous page)**:** Ratio of eicosapentaenoic:arachidonic acid (ARA:EPA) in the diets and tissues of *Cucumaria frondosa*. (A) ARA:EPA in the diatom *Chaetoceros muelleri* (Diatom diet, n=3) and the commercial preparation of fish eggs (Fish egg diet, n=3). (B) ARA:EPA in muscle and gonadal tissue of *C. frondosa* fed with diatoms (Fed diatoms, n=12) and fish eggs (Fed fish eggs, n=12), un-supplemented sea cucumbers (Un-supplemented, n=12) during the 84-d feeding experiment and individuals collected in the field (Field, n=5 Means with different letters are significantly different. Data are mean ± se. See Table 4.9 for statistical results.

Chapter 5: General Conclusions

Sea cucumbers have been consumed and used in the traditional medicine in Asia for centuries. Recent studies have demonstrated that sea cucumbers contain high levels of protein and low levels of fat (Zhong *et al.*, 2007) and are important sources of high-valued compound with multiple biological benefits to human health, including anticoagulant, anticancer, antioxidant, anti-inflammatory and antibacterial properties (Bordbar *et al.*, 2011; Kiew and Don, 2012). Over the last decades, sea cucumbers have become one of the most valuable seafoods in the world (Purcell, 2014), being commonly served at weddings, banquets and corporate events (Fabinyi, 2012) in many East Asian countries. The increasing demand and high market prices have led to the growth of sea cucumber fisheries and, consequently, to the depletion of wild stocks of high-value species from Asia and the Indo-Pacific (Anderson *et al.*, 2011; Conand, 2004; Purcell *et al.*, 2013). In recent years, the overexploitation of wild stocks has spurred the development of new sea cucumber fisheries targeting underutilized species around the world, including in eastern Canada (Grant, 2006; Grant *et al.*, 2006; Rowe *et al.*, 2009).

The sea cucumber *Cucumaria frondosa* is widely distributed in cold waters and has been the focus of an emerging fishery in the North Atlantic (Hamel and Mercier, 2008a). It is considered to have potential for aquaculture due to its high marketability for food, nutraceutical and pharmaceutical products and much of its life cycle is well documented (Hamel and Mercier, 1996; Hamel and Mercier, 2008b; So *et al.*, 2010). *C. frondosa* is also currently being explored as an extractive species for integrated multi-trophic aquaculture (Nelson *et al.*, 2012).

Chapter 2 explored the efficacy of passive integrated transponder (PIT) tags for individually marking sea cucumbers. The implantation of PIT tags at the base of an oral

tentacle to reach the vesicles of the aquapharyngeal bulb was identified as a valuable technique, yielding promisingly high retention rates over the long term. The advantage of this technique over previously tested ones is that it allows individual marking and repeated identification without any major side effect to the sea cucumbers. In addition, it does not involve any complicated manipulations/analyses and the tags can be re-used multiple times. This technique could be very useful for fishery-oriented, ecological and aquaculture studies using mark-recapture to assess seasonal migration patterns, temporal changes in individual growth, survival and mortality rates, habitat preference and identification of broodstock and juveniles for sea ranching.

Chapter 3 investigated the use of different media for refrigeration during live storage of *C. frondosa*. Among all media tested, iced seawater (a mix of freshwater ice with cold seawater) was the most successful. Individuals exposed to iced seawater exhibited the best overall condition, with no mortality or skin damage, yielding high quality sea cucumbers. Instead of following industry standards largely developed for shellfish with hard exoskeletons, the use of a storage medium adapted to sea cucumbers will optimize live storage. This will greatly benefit the fishery and aquaculture industries involving temperate sea cucumbers, helping improve the quality of end products and consequently maximizing their selling price.

Finally, Chapter 4 investigated the influence of different diets on growth metrics, and lipid class and fatty acid composition of muscle and gonadal tissue of *C. frondosa*. This experiment confirmed that *C. frondosa* is able to capture and ingest suspended organic particles of various sizes, ranging from a few µm (diatoms) to ~1 mm (fish eggs). Also, supplemented individuals showed better growth and their lipid profiles suggested

that they could maintain better reproductive performance than un-supplemented individuals. This study improved the understanding of the feeding ecology of *C. frondosa* under controlled/captive condition and will hopefully help the development of broodstock-conditioning protocols as aquaculture programs for sea cucumbers expand in temperate and subpolar environments.

5.1 Future directions

Although much of the life cycle of *C. frondosa* is well documented, various aspects of its biology and physiology still need to be investigated with a perspective on resource management and aquaculture development. Additional research on this species could provide important information favouring long-term sustainability of wild stocks and aquaculture programs. Some areas that deserve further attention are outlined below.

- Retention rates and side effects of PIT tags implanted in different tissues/organs still need to be investigated in temperate and tropical sea cucumbers in the order Aspirochirotida.

- *C. frondosa* has been considered a promising aquaculture species and the development of artificial spawning techniques could guarantee reliable and stable production of juveniles yearlong.

- Few studies have investigated the development and survival rate of *C. frondosa* embryos and larvae with an aquaculture perspective. Therefore, identification of optimal conditions for the embryonic development, hatching success and

larval development/quality of *C. frondosa* would provide a useful tool for the development of a scalable protocol in the aquaculture industry.

- As phytoplankton is the main source of food for juveniles of *C. frondosa*, the evaluation of growth and survival of juveniles fed live phytoplankton and commercial prepared pastes need to be conducted and production cost estimated.

- Feeding and nutritional requirements of juveniles and adults of *C. frondosa* need to be investigated in order to develop formulated diets and captivity-rearing protocols.

- Sea cucumbers and lobsters play a fundamental role in the recycling of nutrients in marine ecosystems and have both been identified by DFO as potential candidates for integrated multi-trophic aquaculture (IMTA). Therefore, the comparison of sea cucumber and lobster growth, health and survival in integrated multi-trophic aquaculture needs to be explored.

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Appendix

Table A1: Complete fatty acid profile (mean ± se) of muscle and gonadal tissues of *Cucumaria frondosa* supplemented with diatoms (Fed diatoms, n=12), fish eggs (Fed fish eggs, n=12), un-supplemented sea cucumbers (n=12), individuals freshly collected in the field (Field, n=5) and diets (diatom and fish egg, n=3 for each).

Samples	**Diets**		**Muscle**				**Gonad**			
Fatty acid	**Diatoms**	**Fish eggs**	**Un-supplemented**	**Fed Diatoms**	**Fed Fish eggs**	**Field**	**Un-supplemented**	**Fed Diatoms**	**Fed Fish eggs**	**Field**
14:0	8.03 ± 1.00	2.49 ± 0.59	1.00 ± 0.11	0.88 ± 0.12	1.00 ± 0.31	3.55 ± 0.53	3.90 ± 0.55	3.61 ± 0.41	2.82 ± 0.20	4.53 ± 0.42
14:1	0.11 ± 0.03	0.00	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.12 ± 0.05	1.63 ± 0.76	0.48 ± 0.09	0.51 ± 0.09	0.89 ± 0.16
***i*15:0**	0.01 ± 0.00	0.13 ± 0.06	0.17 ± 0.12	0.15 ± 0.11	0.09 ± 0.05	0.01 ± 0.00	1.13 ± 1.00	0.06 ± 0.01	1.11 ± 0.65	4.05 ± 2.23
a*i*15:0	0.22 ± 0.10	0.00	1.67 ± 0.32	1.45 ± 0.30	1.16 ± 0.27	1.50 ± 0.32	12.23 ± 3.14	15.90 ± 2.18	13.83 ± 2.65	9.29 ± 4.16
15:0	0.66 ± 0.06	0.50 ± 0.11	0.08 ± 0.03	0.31 ± 0.23	0.07 ± 0.01	0.01 ± 0.04	2.24 ± 0.60	1.18 ± 0.16	1.43 ± 0.20	1.18 ± 0.20
15:1	0.13 ± 0.05	0.36 ± 0.30	0.08 ± 0.04	0.12 ± 0.05	0.12 ± 0.05	0.05 ± 0.04	1.11 ± 0.71	0.92 ± 0.40	1.27 ± 0.63	0.16 ± 0.04
***i*16:0**	0.58 ± 0.26	0.00	0.77 ± 0.3	0.64 ± 0.32)	0.44 ± 0.21	0.24 ± 0.06	2.73 ± 0.80	1.78 ± 0.40	2.10 ± 0.48	1.53 ± 0.35
***ai*16:0**	0.00	0.03 ± 0.01	1.83 ± 0.64	1.95 ± 0.45	2.72 ± 0.52	0.00	1.09 ± 0.54	0.96 ± 0.48	1.04 ± 0.43	0.00
16:0	12.73 ± 5.21	18.34 ± 0.08	1.31 ± 0.45	0.38 ± 0.27	0.48 ± 0.29	4.32 ± 0.34	2.15 ± 0.65	2.64 ± 0.70	2.36 ± 0.46	3.61 ± 0.36
16:1ω11	0.20 ± 0.02	0.07 ± 0.03	0.01 ± 0.00	0.00	0.01 ± 0.00	0.00	0.04 ± 0.02	0.07 ± 0.06	0.06 ± 0.03	0.00
16:1ω9	0.07 ± 0.007	1.78 ± 0.50	0.75 ± 0.37	1.23 ± 0.50	1.63 ± 0.65	0.01 ± 0.00	2.3 ± 1.46	1.26 ± 1.03	1.00 ± 0.88	0.03 ± 0.01
16:1ω7	32.24 ± 1.37	2.40 ± 0.75	2.91 ± 0.45	2.51 ± 0.19	1.61 ± 0.12	8.87 ± 0.97	14.00 ± 2.86	18.78 ± 1.85	18.00 ± 2.58	21.23 ± 0.88
16:1ω5	0.17 ± 0.01	0.22 ± 0.09	0.67 ± 0.19	1.07 ± 0.18	0.80 ± 0.28	0.98 ± 0.38	1.69 ± 0.51	0.74 ± 0.4	0.81 ± 0.20	1.28 ± 0.24
***i*17:0**	0.69 ± 0.01	0.22 ± 0.09	0.03 ± 0.01	0.06 ± 0.02	0.02 ± 0.01	0.12 ± 0.08	0.01 ± 0.00	0.01 ± 0.00	0.00	0.02 ± 0.00
***ai*17:0**	4.02 ± 0.12	0.18 ± 0.06	0.31 ± 0.05	0.30 ± 0.07	0.32 ± 0.05	0.41 ± 0.08	1.43 ± 0.24	1.35 ± 0.16	1.73 ± 0.30	1.01 ± 0.07
16:2ω4	1.31 ± 0.62	0.40 ± 0.09	0.16 ± 0.05	0.33 ± 0.08	0.22 ± 0.05	0.24 ± 0.07	0.82 ± 0.24	0.73 ± 0.11	0.51 ± 0.06	0.58 ± 0.11
phytanic	0.14 ± 0.03	0.17 ± 0.09	0.15 ± 0.04	0.13 ± 0.03	0.14 ± 0.03	0.17 ± 0.06	0.26 ± 0.07	0.16 ± 0.06	0.14 ± 0.05	0.08 ± 0.03
17:0	0.15 ± 0.00	0.22 ± 0.07	1.73 ± 1.09	0.08 ± 0.01	0.30 ± 0.24	0.12 ± 0.06	0.25 ± 0.05	0.15 ± 0.03	0.20 ± 0.04	0.28 ± 0.06
16:3ω4	10.47 ± 0.62	0.13 ± 0.05	2.58 ± 0.91	0.99 ± 0.32	2.53 ± 0.72	1.00 ± 0.82	0.87 ± 0.38	0.50 ± 0.13	0.56 ± 0.20	0.24 ± 0.21
17:1	0.76 ± 0.05	0.14 ± 0.07	3.23 ± 0.99	3.52 ± 1.11	3.07 ± 1.17	3.50 ± 1.02	1.56 ± 0.28	1.80 ± 0.30	1.80 ± 0.34	1.14 ± 0.26

16:3ω3	0.05 ± 0.02	0.08 ± 0.03	0.40 ± 0.33	1.55 ± 0.44	0.02 ± 0.01	0.82 ± 0.40	0.07 ± 0.01	0.13 ± 0.10	0.10 ± 0.02	0.07 ± 0.01
16:4ω3	0.00	0.00	0.99 ± 0.55	1.00 ± 0.60	2.03 ± 0.73	1.05 ± 0.57	0.57 ± 0.30	0.70 ± 0.23	0.88 ± 0.37	0.07 ± 0.02
16:4ω1	0.11 ± 0.04	0.00	0.51 ± 0.33	0.50 ± 0.25	0.29 ± 0.18	1.26 ± 0.47	1.10 ± 0.21	1.31 ± 0.26	0.79 ± 0.11	2.23 ± 0.13
18:0	3.29 ± 0.30	3.73 ± 1.32	2.82 ± 0.32	1.97 ± 0.35	2.57 ± 0.24	2.85 ± 0.70	2.35 ± 0.46	0.96 ± 0.26	2.00 ± 0.38	2.68 ± 0.30
18:1ω11	0.04 ± 0.02	0.00	0.59 ± 0.38	0.20 ± 0.11	0.55 ± 0.23	1.17 ± 0.63	1.02 ± 0.52	1.00 ± 0.52	1.32 ± 0.69	0.01 ± 0.00
18:1ω9	0.95 ± 0.49	9.98 ± 1.28	1.40 ± 0.36	0.85 ± 0.08	1.80 ± 0.44	1.81 ± 0.41	4.77 ± 0.85	4.00 ± 0.70	2.78 ± 0.60	2.89 ± 0.42
18:1ω7	1.22 ± 0.11	4.08 ± 0.03	2.62 ± 0.51	2.80 ± 0.22	2.20 ± 0.43	2.73 ± 0.90	4.74 ± 1.13	3.70 ± 0.70	5.74 ± 0.95	4.00 ± 0.22
18:1ω6	0.00	0.47 ± 0.01	0.04 ± 0.03	0.02 ± 0.00	0.002 ± 0.00	0.00	0.07 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.14 ± 0.10
18:1ω5	0.02 ± 0.01	0.61 ± 0.24	0.04 ± 0.03	0.06 ± 0.03	0.06 ± 0.05	0.02 ± 0.01	0.23 ± 0.15	0.12 ± 0.05	0.05 ± 0.01	0.08 ± 0.03
18:2ω6	0.16 ± 0.11	0.00	0.41 ± 0.04	0.44 ± 0.14	0.19 ± 0.38	0.51 ± 0.02	0.72 ± 0.11	0.65 ± 0.12	0.61 ± 0.11	0.50 ± 0.02
18:2ω4	0.00	0.02 ± 0.01	0.04 ± 0.01	0.16 ± 0.07	0.06 ± 0.03	0.15 ± 0.04	0.60 ± 0.11	0.43 ± 0.06	0.35 ± 0.03	0.34 ± 0.01
18:3ω6	0.41 ± 0.33	0.00	0.18 ± 0.06	0.12 ± 0.03	0.21 ± 0.03	0.19 ± 0.04	0.21 ± 0.05	0.20 ± 0.08	0.18 ± 0.03	0.20 ± 0.04
19:0	0.00	0.00	0.00	0.00	0.004 ± 0.00	0.00	0.02 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.00
18:3ω4	0.10 ± 0.06	0.00	0.01 ± 0.00	0.07 ± 0.02	0.17 ± 0.07	0.07 ± 0.02	0.21 ± 0.07	0.23 ± 0.07	0.36 ± 0.13	0.09 ± 0.01
18:3ω3	0.02 ± 0.01	0.16 ± 0.06	0.03 ± 0.02	0.019 ± 0.01	0.07 ± 0.04	0.14 ± 0.04	0.27 ± 0.09	0.50 ± 0.15	0.24 ± 0.08	0.09 ± 0.02
18:4ω3	0.00	0.41 ± 0.17	0.15 ± 0.05	0.36 ± 0.09	0.36 ± 0.12	0.00	1.20 ± 0.20	0.85 ± 0.26	0.71 ± 0.09	0.00
18:4ω1	0.00	0.00	0.03 ± 0.02	0.01 ± 0.01	0.00	0.00	0.18 ± 0.04	0.44 ± 0.20	0.15 ± 0.06	0.00
20:0	0.11 ± 0.01	0.00	0.53 ± 0.10	0.67 ± 0.09	0.36 ± 0.10	0.76 ± 0.07	0.14 ± 0.07	0.51 ± 0.16	0.27 ± 0.07	0.50 ± 0.11
18:5ω3	0.00	0.00	3.46 ± 1.18	2.69 ± 0.90	2.97 ± 1.02	0.00	0.69 ± 0.34	1.57 ± 0.88	0.54 ± 0.25	0.41 ± 0.36
20:1ω11	0.07 ± 0.06	0.25 ± 0.10	4.48 ± 1.06	4.74 ± 1.06	4.20 ± 0.94	5.72 ± 0.40)	1.98 ± 0.30	1.26 ± 0.14	2.16 ± 0.40	0.00
20:1ω9	0.02 ± 0.01	1.98 ± 0.80	0.40 ± 0.09	0.28 ± 0.12	0.37 ± 0.12	0.00	0.91 ± 0.16	1.06 ± 0.34	0.52 ± 0.13	1.89 ± 0.52
20:1ω7	0.00	0.06 ± 0.02	0.03 ± 0.02	0.22 ± 0.10	0.05 ± 0.03	0.07 ± 0.06	0.14 ± 0.05	0.15 ± 0.07	0.15 ± 0.05	0.00
20:2ω6	0.00	0.09 ± 0.03	0.89 ± 0.10	1.00 ± 0.13	1.03 ± 0.08	0.76 ± 0.05	0.50 ± 0.08	0.50 ± 0.08	0.53 ± 0.13	0.31 ± 0.02
20:3ω6	0.12 ± 0.05	0.00	0.13 ± 0.08	0.12 ± 0.06	0.11 ± 0.07	0.01 ± 0.00	0.07 ± 0.04	0.13 ± 0.06	0.02 ± 0.01	0.02 ± 0.00
0.875	0.00	0.00	0.22 ± 0.07	1.13 ± 0.50	1.08 ± 0.50	0.15 ± 0.04	0.11 ± 0.03	0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.01
20:4ω6	2.57 ± 0.23	2.10 ± 1.71	6.60 ± 0.42	8.67 ± 1.57	9.44 ± 1.80	3.25 ± 0.52	0.94 ± 0.19	1.94 ± 0.68	0.65 ± 0.09	0.55 ± 0.10
20:3ω3	0.08 ± 0.06	0.01 ± 0.01	0.01 ± 0.00	0.06 ± 0.02	2.72 ± 2.50	0.05 ± 0.02	0.04 ± 0.02	0.13 ± 0.10	0.03 ± 0.01	0.02 ± 0.00
20:4ω3	0.00	1.74 ± 0.009	0.06 ± 0.03	1.10 ± 0.57	0.12 ± 0.05	0.16 ± 0.08	3.02 ± 2.40	0.21 ± 0.05	1.74 ± 1.50	5.29 ± 4.73

20:5ω3	17.91 ± 0.77	15.41 ± 0.09	35.96 ± 3.38	32.19 ± 2.00	32.31 ± 2.78	33.94 ± 1.11	12.27 ± 2.00	14.48 ± 1.43	16.92 ± 2.36	20.10 ± 4.57
22:0	0.07 ± 0.06	0.00	0.19 ± 0.08	0.36 ± 0.06	0.25 ± 0.08	0.65 ± 0.06	0.33 ± 0.10	0.35 ± 0.05	0.19 ± 0.05	0.22 ± 0.10
22:1ω11	0.05 ± 0.04	0.17 ± 0.14	0.16 ± 0.09	0.07 ± 0.02	0.44 ± 0.16	0.10 ± 0.02	0.31 ± 0.11	0.24 ± 0.10	0.41 ± 0.16	0.11 ± 0.05
22:1ω9	0.01 ± 0.00	0.20 ± 0.16	0.49 ± 0.17	0.49 ± 0.09	0.68 ± 0.16	0.93 ± 0.05	0.67 ± 0.10	0.51 ± 0.12	0.50 ± 0.08	0.64 ± 0.16
22:1ω7	0.00	0.03 ± 0.03	1.06 ± 0.25	1.04 ± 0.17	2.48 ± 1.55	1.27 ± 0.06	0.46 ± 0.10	0.48 ± 0.08	0.38 ± 0.08	0.53 ± 0.10
21:5ω3	0.00	0.42 ± 0.31	3.17 ± 2.92	1.91 ± 1.06	0.08 ± 0.03	0.00	0.50 ± 0.10	0.98 ± 0.33	0.21 ± 0.04	0.00
23:0	0.00	0.00	0.03 ± 0.15	0.13 ± 0.09	0.03 ± 0.02	0.13 ± 0.02	0.52 ± 0.44	0.07 ± 0.02	0.80 ± 0.46	0.04 ± 0.01
22:4ω6	0.00	0.00	8.93 ± 2.19	14.12 ± 1.08	15.37 ± 1.92	10.03 ± 0.90	3.47 ± 0.88	3.74 ± 0.82	2.96 ± 0.54	1.55 ± 0.38
22:5ω6	0.00	0.04 ± 0.03	0.05 ± 0.04	0.00	0.06 ± 0.05	0.00	0.10 ± 0.04	0.03 ± 0.02	0.03 ± 0.01	0.00
22:4ω3	0.00	0.00	0.06 ± 0.04	0.08 ± 0.04	0.21 ± 0.08	0.00	0.03 ± 0.02	0.14 ± 0.10	0.16 ± 0.13	0.00
22:5ω3	0.00	0.40 ± 0.32	1.32 ± 1.12	0.34 ± 0.21	0.25 ± 0.03	0.00	0.12 ± 0.05	0.23 ± 0.05	0.16 ± 0.04	0.00
24:0	0.00	0.00	0.001 ± 0.00	0.34 ± 0.17	0.00	1.13 ± 0.11	0.11 ± 0.09	0.00	0.00	1.36 ± 0.17
22:6ω3	0.00	29.26 ± 0.04	0.70 ±0.22	0.91 ± 0.16	1.75 ± 0.28	0.90 ± 0.10	1.18 ± 0.19	0.87 ± 0.12	1.10 ± 0.30	1.00 ± 0.12
24:1	0.00	0.70 ± 0.28	1.24 ± 0.22	1.22 ± 0.11	1.65 ± 0.21	2.74 ± 0.13	2.07 ± 0.37	1.97 ± 0.28	1.58 ± 0.25	1.82 ± 0.44