The reproductive biology of warty sea cucumber, *Neostichopus grammatus* Clark (Holothuroidea: Echinodermata) under natural and integrated multi-trophic aquaculture (IMTA) conditions in the Eastern Cape Province, South Africa.

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“Leave no stone unturned.” - Ancient Greek proverb

Abstract

This study investigated the reproductive cycle of the warty sea cucumber, *Neostichopus grammatus,* in the wild and in a farming environment, to test its suitability for co-culture with abalone *Haliotis midae* in an IMTA system. The research formed part of a broader EU H2020 program, Aquavitae, and was conducted at Wild Coast Abalone in South Africa. The study investigated several environmental parameters, such as temperature, day length, and substrate, to determine if these have any effects on the gonadal development and reproductive cycle. The natural gametogenic cycle of wild warty sea cucumbers was determined by monitoring the Gonadosomatic Index (GI) values monthly for a period of 16 months. The GI index was validated by means of other maturity assessment techniques including measurement of the size and colour of the gonads, oocyte diameter measurements and gonad histology sections. Wild collected sea cucumbers were maintained in abalone farm rearing tanks on a diet of abalone feed and faecal waste for a period of 10 months, with GI values being determined monthly. The wild and farmed sea cucumber GI data was correlated with environmental parameters.

Seasonal water temperature was strongly correlated with the GI values of wild warty sea cucumbers, suggesting that water temperature likely influences gonadal development. Sea cucumbers matured sexually during colder months (May to September) and spawned from September to February, during summer. The results of oocyte measurements, macroscopic and microscopic analyses, and histological analyses in combination with the GI values, provides a practical indicator of sexual maturity for captive breeding purposes.

Farmed warty sea cucumbers followed the same annual reproductive cycle as wild sea cucumbers, however they came into spawning condition approximately one month later than wild conspecifics. As the farm water temperature was significantly higher than the ambient ocean water temperature, it was hypothesised that this may have affected the gonadal development of the farmed sea cucumbers. Furthermore, the farm-reared sea cucumbers lost weight and condition, indicating a nutritional deficiency. Previous studies indicated that the presence of a sand sediment facilitated the assimilation of organic detrital matter by sea cucumbers. An additional trial was thus set up to determine the effect of a sand sediment and cooler ambient temperature on the growth and gonadal development of the warty sea cucumbers.

The growth and GI values of sea cucumbers in the cool water treatment did not differ significantly from the ambient temperature control groups. However, the addition of a sand substrate to the sea cucumber tanks had a significant positive growth effect on sea cucumber body mass and GI values. Sea cucumbers fed a diet of abalone waste material on the sand substrate exhibited final average GI values of 2.99 % (SE ±0.56). In contrast, sea cucumbers kept in bare tanks and only received abalone waste as food had significantly lower average GI values of 1.36 % (SE±0.2). This suggests that the provision of a sand sustrate in sea cucumber rearing containers is essential for the adequate nutrition and gonadal development.

The results of this study indicate that the warty sea cucumber is a promising candidate for inlcusion in an IMTA system with South African perlemoen abalone.

Keywords: sea cucumber, gonads, gonadosomatic index, IMTA, abalone, sediment, temperature.

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Chapter 1: General introduction

1.1 Introduction

Integrated multitrophic aquaculture, or IMTA, was developed as an environmentally sustainable approach to mitigate the damaging effects of intensive aquaculture practices (Correia *et al.,* 2020). Abalone farms in South Africa have established a commercial-scale IMTA system culturing seaweed as abalone feed in the farm effluent. The potential exists to culture other complementary detritivorous and filter-feeding species. The present research formed part of a broader European Union Horizon2020 program known as 'Aquavitae' to develop culture technology for low-trophic species in existing IMTA systems. The research was undertaken at Wild Coast Abalone (WCA), an abalone farm on the East Coast of South Africa.

Preliminary investigations revealed that the local sea cucumber *Neostichopus grammatus* was a suitable candidate species to evaluate for inclusion in the integrated multitrophic aquaculture (IMTA) system at WCA. While the seaweed currently part of the IMTA system removes a proportion of the dissolved waste nutrients from the effluent, waste solids were discharged directly back into the ocean with no treatment. The farm management hypothesized that sea cucumbers could offer a practical and sustainable solution for abalone tank solids removal while producing an additional high-value crop. A series of trials were thus designed to evaluate the performance of the species under aquaculture conditions, including investigations into its environmental and nutritional requirements and reproductive biology. The present research investigated the effect of selected environmental parameters on its reproductive cycle, as well as establishing the natural gametogenic cycle of the species, to lay a knowledge foundation for its captive breeding.

1.2 Low trophic aquaculture species

Several low trophic-level aquatic species have been identified as species of interest in the aquaculture sector. Primary producers such as micro and macroalgae are widely produced, creating a global output of 35 million tonnes of algae per annum (FAO, 2022). Low-level consumers, such as bivalves, sponges and sea cucumbers, are of particular interest because their diets are of an extractive or unfed nature (Slater and James, 2023). Other high-value species, such as abalone, have been farmed extensively in several countries due to its popularity in Asia. The popularity of abalone products is ever-increasing, and the vast majority of abalone is produced through aquaculture (Cook, 2023).

The perlemoen abalone (*Haliotis midae*) is one of the most valuable farmed species in South Africa (Adeleke *et al.,* 2020). By 2016, South Africa was the world's third largest producer of abalone *Haliotis midae*, with an estimated 1685 metric tons produced per year on its 16 commercial abalone farms (Cook, 2019; DAFF, 2018). In 2016, abalone sales in South Africa made up approximately 76 percent of the revenue generated by the aquaculture sector (Britz and Venter, 2016). There are several reasons for its success, including an established market niche, production technology supported by ongoing research and financial and regulatory support from the public sector. The leading importer of South African abalone is China, and the strong demand is prompting investment in and expansion of farms. Asian countries such as Korea and China have been increasing abalone (*Haliotis discus hannai*) production in recent years and are in competition with the South African market, influencing market prices negatively (Britz and Venter, 2016). The South African abalone industry, therefore, needs to explore ways to increase sales and/or lower production costs in order to create a more profitable product. Sea cucumber and macroalgae production in an IMTA system in co-culture with abalone offers a plausible and practical solution to lower production costs and increase the profit margin of South African abalone sales.

Sea cucumber products are sought after in many Asian countries, and sea cucumber numbers are declining drastically worldwide as a consequence. Many countries are overharvesting sea cucumbers from the wild and are performing unsustainable fishing and farming practices. For many in third-world countries, harvesting and selling sea cucumbers is their only source of income, and their livelihood depends on these organisms (Anderson *et al.,* 2011[b]; Hasan, 2019). As stocks decline, these individuals are becoming more desperate, catching and selling smaller sea cucumbers, effectively eliminating wild stocks. This is not only a problem for the people depending on these wild stocks for an income, but also for the species itself. Previous studies have shown that heavily exploited sea cucumber stocks can take more than 50 years to recover (Preston 1993; Battaglene *et al.,* 1999; Friedman *et al.,* 2011). These organisms are not only a source of income, but also play important, if not crucial, ecological roles.

The aquaculture production of several valuable sea cucumber species has increased in recent decades following the overexploitation of wild stocks of sought-after sea cucumber species (Zamora *et al.,* 2016). The sandfish, *Holothuria scabra* Jaeger and Japanese spiky sea cucumber *Apostichopus japonicus* Selenka are two of the most sought-after sea cucumber species and are being farmed extensively in various parts of the world (Battaglene *et al.,* 1999; Conand and Byrne, 1993; Preston, 1993). As of 2016, there are more than 70 species of sea cucumber being exploited commercially worldwide (Purcell *et al.,* 2016). China and Japan were among the first countries to culture sea cucumbers in the 1980s (Chen, 2004; Ito and Kitamura, 1997). Spawning and larval rearing protocols were developed for *Apostichopus japonicus* as early as the 1950's. By 2013, *A. japonicus*, *Holothuria scabra,* and *Isostichopus* *fuscus* were the only three sea cucumber species commercially bred in hatcheries (Mercier and Hamel, 2013). Sea cucumber products were used to supply the bêche-de-mer industry as well as enhance wild populations through sea ranching, pond farming, and restocking wild stocks (Agudo, 2006; Ivy and Giraspy, 2006). As of 2012, sea cucumbers were being produced in several (at least 70) countries worldwide, including Canada, the United States, Fiji, India, Mexico, New Zealand, and several other smaller contender countries (Purcell *et al.,* 2012, 2013, Toral-Granda *et al.,* 2008).

1.3 Sea cucumbers in polyculture

Sea cucumbers are particularly well-suited for polyculture with other species as they are detritivores, feeding on the waste of other species. Sea cucumbers, being able to utilise various types of organic materials as a food source, are an appealing option for polyculture systems (Kim *et al.,* 2014; Zhou *et al*., 2006). These deposit-feeding species are exceptionally well suited for co-culture as no additional feed is required, only wastes of a higher trophic level species (Lutz, 2003; Zamora *et al.,* 2016). The warty sea cucumber, *Neostichopus grammatus* Clark, was identified to have potential as a candidate to be integrated into the IMTA system at WCA. *N. grammatus* is an indigenous deposit-feeding sea cucumber that has the same environmental requirements as *H. midae*, as both naturally co-occur in the same habitat on the Eastern Cape province coastline. The two species are ecologically compatible, meaning they have similar environmental requirements but do not compete for resources (Milstein, 1992; Kang *et al.,* 2003; Neori et al., 2004; Kim *et al.,* 2014). In a recent six-week pilot study, Onomu *et al.* (2023) successfully demonstrated that abalone (*H. midae*) waste products could be used as a food source for *N. grammatus* sea cucumbers in a land-based flow-through system.

It was hypothesised that the co-culture of the warty sea cucumber and farmed abalone could reduce waste organics in abalone tanks, improve water quality and result in an additional high value crop.

1.4 Sea cucumber culture in captivity

The most common practice is to retrieve mature broodstock from the ocean and induce spawning on land. This can only be done once the gonads are sufficiently mature. The gonadal maturity can be determined by employing one or several maturity assessment techniques. The most common methods include the gonadosomatic index value, histological analyses of the gonads and macroscopic assessment of the gonads (Conand, 1981). Tubule length and thickness measurements can also be an appropriate method for some species (Ramofafia *et al,* 2003). Ovary and oocyte measurements can also assist in determining the fecundity of the females (Dissanayake and Stefansson, 2010). These methods can be used to establish baseline reference conditions for a species and can be used as a guide to determine the maturity of new specimens. It is important to note that these reference conditions will differ among species and cannot be used as a universal guide. The maturity assessment techniques can be used to determine a species' gonadal development pattern and, subsequently, to attempt to determine which environmental conditions are favourable to gonadal maturation.

There is a need for accurate knowledge of the reproductive cycle and influencing environmental factors in order to condition and breed a species in captivity (Morgan, 2000; Agudo, 2006; Purcell *et al,* 2012). Significant challenges are faced when trying to condition broodstock in captivity. The maturation of gonads can be influenced by several factors, including, but not limited to, temperature, food quality and availability, light intensity, lunar cycle, photoperiod, and tidal flux (Conand, 1981; Ramofafia *et al.,* 2000; Hamel and Mercier, 2004; Mercier and Hamel, 2009; Pasquini *et al.,* 2022). Controlling these factors in an artificial environment can prove to be very challenging. Furthermore, the culture of any species in higher-than-normal densities can lead to the spread of disease and a general decline in health. The handling of animals and air exposure for tank cleaning can cause stress to the animals (Wang *et al.,* 2023).

1.5 Aquaculture at Wild Coast Abalone

Wild Coast Abalone (WCA) (Haga Haga, Eastern Cape of South Africa) is a commercial abalone farm producing 180 tons of abalone per annum for exports of fresh and dried products, mainly to Hong Kong. WCA utilizes water pumped ashore from the ocean and returns effluent waters back to the ocean. Abalone are fed a diet of pelleted feed (Abfeed, produced by Marifeed) and cultured IMTA seaweed that is produced on site. WCA implemented an IMTA system that utilizes abalone waste as a fertilizer for the growth of macroalgal species, particularly *Ulva* and *Gracilaria,* in 2003 (Amosu *et al.,* 2013; Bolton *et al.,* 2013). This yields algae with high protein content (30% of dry weight), promoting increased abalone growth rates (Bolton *et al.,* 2013; Robertson-Anderson *et al.,* 2009). Culturing the algae in farm effluent reduces dissolved waste nitrogen and phosphorus levels, producing algal biomass for abalone food. Nonetheless, abalone tank waste solids from tank cleaning are released in the effluent water with no treatment. The weekly cleaning of abalone raceways to remove the solid organic wastes cost the Wild Coast Abalone farm approximately ZAR100,000 per month in 2020 (D. Taylor, WCA farm manager, personal communication).

Figure 1.1: Wild Coast Abalone, situated northeast of Marshstrand. (GPS coordinates 32°45'03S/ 28°16'25E)

1.6 Importance of integrated multitrophic aquaculture (IMTA)

The production of valuable aquaculture species often has negative but manageable impacts on the surrounding marine environment. The most common practice in South Africa, including at WCA, is to pump water from the ocean and run it through the tanks and raceways after which the water is discharged back into the ocean. These systems are referred to as flow-through systems. While the seaweed cultured in abalone effluent on some farms removes a proportion of the dissolved waste nutrients, the solid wastes generated, comprising of abalone faeces and uneaten food debris, are not treated (Kemp *et al.,* 2015). Organic wastes released into adjacent waters can lead to several environmental problems, including but not limited to eutrophication, oxygen depletion, and toxicity of ammonia and nitrate (Xia *et al.,* 2017). The abovementioned problems arose as conventional aquaculture is focused on one species only, with no remedial actions other than releasing effluents into adjacent waters. Ecosystem services are effectively used as a bioremediation tool and are often unable to keep up with the high volumes of waste entering the water bodies (Kemp *et al.,* 2015; Xia *et al.,* 2017).

Sustainable aquaculture needs to be "environmentally sustainable, protecting the quality of the environment for other users, must maximize benefits and minimize detriments and other negative impacts on the natural and social environment", as defined by Frankic and Hershner (2003). Simply put, it means receiving goods and services from the earth for the benefit of humans while "not harming the continued provision of these goods and services" (McNevin, 2020). As aquaculture production in South Africa and the rest of the world increases, IMTA systems offer practical solutions for sustainable, low-discharge production. IMTA aims to address the abovementioned issues by producing two or more species of different trophic levels in the same environment, using the waste of one species to feed another using an ecosystem-based approach (Zamora *et al.,* 2016). The inclusion of a species that feeds on detritus, such as sea cucumbers, has potential ecological and financial benefits for commercial abalone farms. Sea cucumbers can act as "cleaners", removing solid waste from the system, decreasing labour costs. They also take on the role of nutrient recyclers, as organic wastes are consumed, and nutrients are returned to adjacent waters (Kim *et al.,* 2014). Another benefit of adding sea cucumbers to an IMTA system is the production of another high-value exportable crop, i.e., sea cucumber biomass.

1.7 General introduction of *Neostichopus grammatus* (H.L. Clark, 1923)

Neostichopus grammatus Clark, commonly known as the warty sea cucumber, is a dioecious species found in the shallow intertidal zone of the rocky shores of South Africa (Forster, 1994). It is one of 163 currently recognized nominal species in southern Africa, south of the Tropic of Capricorn. Southern Africa is exceptionally biodiverse and is home to representatives of all five extant orders of Holothurians (Thandar, 2015). The class Holothuroidea is classified under Phylum Echinodermata, which proves its close relation to the sea stars, brittle stars, and sea urchins. Unlike other Echinoderms, which have pentaradial symmetry along the vertical axis, sea cucumbers, having elongate worm-like bodies, have pentaradial symmetry along the horizontal axis (Smirnov, 2012). Holothurians are divided into six orders, of which one is extinct, based on the shape of the calcareous ring at the anterior end, the presence or absence of a respiratory tree, specific muscle groups, and the form of the tentacles. Order Aspidochirotida (shield-tentacle) are characterized by the presence of calcareous rings without projections to the rear, flattened tentacle extremities, the presence of respiratory trees, longitudinal muscles arranged into five double bands, and are mostly soft-bodied species found in shallow waters (Slater, 2006). The family Stichopodidae is characterized by the gonads being divided into two tufts (as described by Haeckel, 1896 in Thandar, 1987).

Figure 1.2: *N. grammatus* sea cucumbers clinging to the underside of a dome shelter at Wild Coast Abalone.

Previous research on *N. grammatus* is limited. The focus of previous research was on the changes in spicule composition with age (Thandar, 1987), gut length and content, as well as comparisons of the structure of intestines and tentacles (Foster and Hodgson, 1995), intertidal densities, distribution and biomass, as well as the seasonality of reproduction (Foster, 1994).

A recent study conducted at Wild Coast Abalone by Onomu *et al.* (2023) provided insight into the survivorship of captive-fed warty sea cucumbers. However, not enough research has been performed on the suitability of the species in the context of its potential as a farmed aquaculture species.

N. grammatus is distributed along the coast of South Africa from Cape Agulhas (Western Cape Province) to Cape Vidal (KwaZulu-Natal Province) (Foster, 1994). The organisms are gelatinous when alive and usually red in colour, but can vary to brown, cream, white, pink, grey, and mottled green. Five rows of tube feet are arranged along the ventral side, and few papillae are present. Body wall spicules in juveniles are reduced or absent in mature individuals. The spicules are replaced by minute plates in older specimens (as described by Deichmann, 1948 in Thandar, 1987). The regression of spicules, changing drastically with growth from juvenile to adult, has led to the belief that they are two different species (Thandar, 1987). *N. grammatus* is a deposit feeder, feeding on benthic sediments in the intertidal zone (Foster, 1994). According to Foster (1994) and Foster and Hodgson (1995), *N. grammatus* reaches sexual maturity at a body size of 3.0-3.9cm^3, but it is unknown at what age this is reached. It reaches a maximum length of approximately 12cm (Thandar, 1987). Foster, 1994 observed that *N. grammatus* undergoes gonadal maturation from July to September, with gonad maturity being maintained until spawning takes place from December to February.

1.7.1 Classification

Kingdom Animalia

Phylum Echinodermata (Bruguiere, 1791)

Class Holothuroidea (Blainville, 1834)

Order Aspidochirotida (Grube, 1840)

Family Stichopodidae (Haeckel, 1896)

Genus Neostichopus (Deichmann, 1948)

Species *Neostichopus grammatus* (Clark, 1923) (Thandar, 2015; Reich, 2019)

1.8 Motivation of research approach

In order to implement the warty sea cucumber as a bioremediation tool at Wild Coast Abalone as part of an IMTA system, its mass propagation under captive conditions is required. This thesis aims to describe the reproductive cycle of N. grammatus and the environmental parameters affecting reproductive maturation. In this section, relevant literature is reviewed in order to motivate the thesis research approach.

The increased popularity of ocean products, along with the growing human population, has placed enormous pressure on fishing industries. More than 50 percent of the world's finfish fisheries have reached their maximum sustainable limit as of 2010 (FAO, 2010). This has led to increased pressure on non-finfish resources (Anderson *et al.*, 2011[a]). Low-trophic level resources, already vulnerable due to anthropogenic stressors, are now being exploited faster than what is sustainable (Purcell *et al.,* 2013). This includes sea cucumber exploitation, which has increased drastically in recent years (Anderson *et al.,* 2011[a]), leading to overexploitation of natural stocks. The decimation of these species is to such a degree that improved fishing governance and regulatory measures alone may not be enough to reverse the damage already done (Friedman *et al*., 2011; Purcell *et al.,* 2012). Fortunately, mariculture may provide some relief through the production of valuable overexploited sea cucumber species, lessening the impact on wild stocks. Restocking of exploited species is also made possible through sea ranching and deliberate culture-based stocking (Bell *et al.,* 2005).

Several studies on the co-culture of abalone and sea cucumbers have been conducted and have yielded promising results. A study done by Kang *et al.* (2003) found that where abalone (*Haliotis discus hannai)* and sea cucumbers (*Stichopus japonicus*) (Reclassified as *Apostichopus japonicus*) were co-cultured, consistently lower levels of inorganic nitrogen were found (in comparison to the culture of abalone alone). The same study also found that when abalone and sea cucumber were produced in co-culture, the abalone and sea cucumber both had a larger biomass than when cultured independently. Although the Dissolved Oxygen (DO) was lower when the two species were co-cultured, this was easily remedied. This study shows that the co-culture of these two species is not only beneficial for abalone growth, but for sea cucumber growth as well, which is a promising prospect for the aquaculture industry.

Stocking densities have also been examined in a study done by Qi *et al.* in 2013. The difference in growth and survival of the abalone *Haliotis discus hannai* and the sea cucumber *Apostichopus japonicus* in different stocking densities, namely abalone: sea cucumber in ratios of 3:1 and 6:1 was examined. Both abalone and sea cucumbers grew well, and no mortalities were recorded. No additional feed was provided for the sea cucumbers, indicating that a diet of abalone waste material provided sufficient nutrition for *A. japonicus.*

A large variety of anaerobic bacteria have been found in the gut of *A. japonicus,* all of which occur naturally in the sediment where the specimens were collected (Bogatyrenko and Buzoleva, 2016). The microbiota in the gut of the sea cucumbers was also more abundant than in the sediment, indicating that the microbiota are able to colonize the digestive tract. This may function as a digestive aid, as certain strains found inside the gut were capable of amylolytic and lipolytic activity (Bogatyrenko and Buzoleva, 2016). Watanabe *et al.* (2012) theorised that sand may aid in digestion of food in sea cucumbers, while Schagerström (2003) observed that juvenile sandfish (*Holothuria scabra*) reared in the presence of sand had thicker body walls compared to conspecifics raised on biofilm alone. Robinson *et al.* (2015) found that *H. scabra* sea cucumbers were attracted to and grew well over anoxic sand sediments (Robinson *et al.,* 2015)*,* while aeration of the sediments led to decreased growth rates. This is likely due to the growth of anaerobic bacteria aiding the digestion of organic material in the sediments. N. grammatus sea cucumbers likely require similar nutritional gut microorganisms and sediments for healthy growth and reproduction.

Closing the reproductive cycle of a new aquaculture species is an essential step in commercialising its production, and therefore, an understanding of its reproductive style and factors affecting the reproductive cycle is required. *N. grammatus* sea cucumbers are annual breeders, coming into spawning condition in summer and maturing during winter months. Sexually mature warty sea cucumbers found in the wild have been recorded to spawn between December and February each year (Foster, 1994). This coincides with the summer season in southern Africa. Gonadal maturation starts in July and usually reaches ripeness by September at the onset of the spawning season (Foster and Hodgson, 1995; Foster, 1994). Sea cucumber reproduction and sexual maturation are controlled by exogenous stimuli, including light intensity, photoperiod, temperature, salinity, and food availability (Giese and Pearse, 1974; Grahame and Branch, 1985). Not all species respond to the same stimuli. For example, Foster (1994) found evidence indicating that sexual maturation in the South African warty sea cucumber was correlated to photoperiod while spawning was thought to be initiated by a rise in sea water temperature. This contrasts with two sympatric species, *Pseudocnella sykion* and *Roweia stephansoni*, whose gonadal maturation and spawning events were primarily influenced by sea water temperature (Foster 1994). *Australostichopus mollis*, a temperate species found in Australasian waters, is naturally conditioned and triggered to spawn by lunar phases and phytoplankton blooms (Morgan, 2009[a]). In contrast, in a study on *Holothuria atra* in Sri Lankan waters, a strong correlation was observed between temperature and the gonadosomatic index (GI) (Dissanayake and Stefansson, 2010). Photoperiod has been shown to be an important cue for gonadal development in many marine invertebrates in temperate areas, where changes in day length are more pronounced (Pearse, 1981).

Various characteristics of the warty sea cucumber make it an exceptionally well-suited candidate for co-culture with abalone. The main reason for choosing this species is because of its ecological compatibility with the abalone being farmed at WCA. Another reason is, as a deposit feeder, it is hypothesized that this species will reduce the organic wastes produced by abalone by consuming faeces and food debris, effectively acting as a bioremediation tool. This could result in cleaner effluent being returned to adjacent waters, lessening the impact that abalone farming has on the oceans and other ocean inhabitants. This could also lead to the production of another high-value crop, i.e., sea cucumber biomass, with a very low production cost.

It is hoped that South African sea cucumber products will be attractive to buyers in the international markets for several reasons. Traders generally believe that products from South Africa, Australia, and South America are of good quality (FAO, 2008). Secondly, as the demand for sea cucumbers is on the rise and will likely continue to rise over the coming years (Han *et al.,* 2016), another competitor species will likely do well in the international market. Thirdly, as sea cucumbers with a warty or spiky appearance are preferred in many countries (FAO, 2008), the warty appearance of *N. grammatus* could enhance its market acceptability. Members of the families Stichopodidae and Holothuriidae are the most traded sea cucumbers and are also considered the most valuable (FAO, 2008). *N. grammatus* belongs to the family Stichopodidae, which could also increase its appeal on the international market. However, as medium to large sized sea cucumbers are preferred in most international markets (Ferdouse, 2004), *N. grammatus* might not be a popular option as its maximum length is only 12 cm.

Apostichopus japonicus and *Holothuria scabra* are the two best-selling sea cucumber species particularly because of their large size (FAO, 2008).

Figure 1.3: The spiky/warty appearance of the warty sea cucumber.

1.9 Research aim and objectives

The study is focused on *N. grammatus* reproductive biology with a view to breeding in captivity. The research in this study forms part of a broader research effort by the Aquavitae Project to implement the species into the IMTA program currently run at Wild Coast Abalone.

Chapter 2 of the thesis presents an investigation into the natural gonadal cycle of the warty sea cucumber and the environmental parameters that correlate with it. Gonadosomatic Index (GI) values were used to determine the gonad maturity, and subsequently, the stage of development of the gonads. Oocytes were also used to determine the stage of development in females. The objectives were to:

- Determine the natural gametogenic cycle of the warty sea cucumber, focusing on gonad maturity monthly.

- Determine the physical appearance of the gonads throughout the gametogenic cycle and its various stages of development by assessing macroscopic, microscopic and histological data.
- Determine which exogenous factors, if any, have an influence on gonadal maturation, focusing on diurnal rhythm and sea water temperature.

Chapter 3 presents an investigation into the warty cucumber gonadal cycle in the farming environment, focusing on water temperature and daylength as possible conditioning factors. The objectives were to:

- Compare the gonadal maturation cycle of farmed and wild sea cucumbers, focusing on GI values.
- Determine if the warmer on-farm water temperatures would have an effect on gonadal maturation.
- Examine the gametogenic cycle of farmed sea cucumbers to assess their suitability for breeding in a farming environment.

Chapter 4 presents a controlled trial undertaken to investigate the effect of water temperature and the presence of sand sediment on the growth and reproductive development of warty sea cucumbers kept in captivity. The objectives were to:

- Compare the effect of colder (16°C) on-farm water temperature with ambient water temperature (approximately 19 °C - 21 °C) on the growth and gonadal development of warty sea cucumbers.
- Compare the effect of the presence and absence of sand in sea cucumber tanks on the growth and gonadal development of warty sea cucumbers.

Chapter 2: Investigation of the natural gametogenic cycle, histological analysis and staging of *Neostichopus grammatus* gonads

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2.1. Introduction

The gonads of the warty sea cucumber undergo marked seasonal changes (Foster, 1994). Immature gonads are small, with the female gonads appearing light pink and the male gonads appearing white. The gonads are large at maturity, and sex can easily be determined with the naked eye. The mature male gonad is translucent yellow in appearance, while the mature female gonad appears translucent white (Foster, 1994). Warty sea cucumber gonad tubules are formed as two branches that lead to two clusters of tubules, each tubule branching out further, creating a maze of intertwined tubules as the individual matures sexually. This can make tubule quantification very challenging in this species (Foster, 1994). Furthermore, tubule diameter is difficult to determine in *N. grammatus* because of varying tubule sizes, thicknesses, and lengths. Once the animals are spent, the gonads start to recede, and it becomes increasingly difficult to determine the sex. Microscopic examination may help to determine the sex, although this is not always possible. When the animals are completely spawned (spent), the gonad is resorbed and becomes difficult to locate. A similar finding was reported by Sewell (1992) for *Australostichopus* (*Stichopus) mollis,* where the gonads were completely resorbed to such an extent that no gonadal tissue could be identified for several months of the year. Using a combination of maturity assessment techniques has been described as a successful indicative tool for accurate determination of sexual maturity on the population level (Yanigasawa, 1999; Reichenbach, 1999; Ramofafia *et al.,* 2000; Ramofafia *et al.,* 2001).

Several environmental parameters can concurrently act as cues for sea cucumber gonadal recrudescence. Sea cucumbers such as *Holothuria* (*Microthele) fuscogilva* (Conand, 1981) and

Actinopyga mauritiana (Hopper *et al.,* 1998) were found to mature sexually during the colder months and spawn during summer months, although not all were correlated with temperature. Sea cucumbers found in temperate areas, such as *H. fuscogilva* in New Caledonia (Conand, 1981), were found to undergo sexual maturation and spawning in correlation with changes in water temperature. Foster (1994) found that day length had an influence on gonadal maturation in *N. grammatus.* Spawning during warmer months is the most common reproductive pattern found in holothurians (Conand, 1981). Gonadal maturation has to be initiated at a certain time of the year in order for the animal to be sufficiently ripe once the conditions conducive to spawning have come about. The predictability of seasonal variations allows for the onset of spawning and/or maturation, synchronizing conspecifics and leading to spawning events within a population (McClanahan, 1988, Muthiga & Kawaka, 2008).

Only one study has been conducted on the reproductive cycle of *Neostichopus grammatus.* Foster (1994) found that the Gonadosomatic Index (GI) value decreases rapidly after spawning, as remaining gametes are reabsorbed by the body and the gonad regresses. It was found that tubule diameters and GI values increased drastically during periods of gonad maturation. Furthermore, it was concluded that the mean mature oocyte diameter was 300-350µm, declining rapidly in January, supposedly because the mature oocytes had been shed. It was also noted that there were no sperm present in male tubules from January to June, suggesting that all spermatozoa had been shed.

Echinodermata have a range of reproductive strategies, but all are broadcast spawners, i.e., oocytes and sperm are released into the water where fertilization takes place (Boolootian, 1966; Smiley *et al.,* 1991). The Holothurian reproductive system is different from that of all other Echinoderms in that it comprises of only one gonad, whereas all other Echinoderms possess more than one gonad (Boolootian, 1966; Tyler and Gage, 1983; Costelloe, 1985; Smiley and

Cloney, 1985; Cameron and Fankboner, 1986; Barnes, 1987; Hamel *et al.,* 1993). Typically, temperate Holothurians only reproduce once a year, but spawning may take place rhythmically or sporadically throughout the year or only at certain times of the year, depending on the species (Giese, 1959). It has been established that the warty sea cucumber only spawns once a year in summer, employing several spawning events during this period (Foster, 1994).

2.1.1 Gonadosomatic Index

The gonadosomatic index (GI) expresses the gonad weight as a percentage of body weight and is used widely for describing the stages of development of the reproductive cycle of marine species. The higher the GI, the more mature the gonads (Cayre and Laloe, 1986). GI values differ widely among holothuroid species. In some species the GI can be low, even when the organisms are sexually mature and ready to spawn. For example, in the case of *Australostichopus mollis,* the maximum GI recorded was 5% (Morgan, 2009 [b]), whereas for *Holothuria atra,* the highest GI recorded was approximately 16% (Dissanayake and Stefansson, 2010). The GI values are used as an indication of when sexual maturation ensues, how long maturity is maintained before spawning starts, what type of spawning takes place, and how long before the recrudescent cycle restarts. This information is crucial in establishing a baseline for the reproductive cycle of the warty sea cucumber with a view to breeding the species for aquaculture. The gonadosomatic index was used as a measure of the maturity of the gonads in the current and previous studies. The current study was conducted over 16 months, providing valuable insights into gonadal development over two summer recrudescence cycles.

The aim of the study was to examine the natural annual reproductive cycle of *N. grammatus* in a long-term study over 16 months, and to determine what effects the natural environment has on the gonadal maturation of these animals. The objectives were to: 1) determine the natural gametogenic cycle of the warty sea cucumber; focusing on gonad maturity on a monthly basis;

2) determine the physical appearance of the gonads throughout the various stages of development of the gametogenic cycle by assessing macroscopic, microscopic and histological data; and 3) to determine which exogenous factors, if any, have an influence on gonadal maturation, focusing on diurnal rhythm and sea water temperature.

2.2 Materials and methods

2.2.1 Collection and processing of specimens

Wild sea cucumbers were collected monthly between August 2020 and November 2021 from Marshstrand (32.7602º S, 28.2512º E), East London's Nahoon Reef (32.9932º S, 27.9485º E), Gonubie Black Rock (32.9439º S, 28.0272º E), and Port Elizabeth (Gqeberha) at Cape Recife (34.0209º S, 25.6944º E). The animals were transported live in buckets to the Wild Coast Abalone farm where they were euthanized by hypothermia, drained of excess water, and dissected. Dissection took place approximately three hours after collection of specimens. The gonads were removed and weighed (±0.01g) after which they were examined microscopically to determine the stage of gonadal development and placed in a 10% buffered formalin solution. Approximately 10 to 25 specimens were collected monthly, depending on how many could be retrieved from the ocean. A total of 297 specimens were collected over the sampling period. Specimen weights ranged from 4.42g to 40.44g.

2.2.2 Gonadosomatic Index (GI)

Gonad maturity was measured using the gonadosomatic index. Specimens were longitudinally incised on the dorsal side and the coelomic fluid, viscera and gonads were removed. The wet weight of the body (d_{wt}) and gonads (g_{wt}) were used in the calculations. All data was collected as fresh wet weights because drying the gonads resulted in inaccurate measurements at certain times of the year because of the size of the gonads (Foster, 1994). The GI was calculated using the following equation and expressed as a percentage (Ramofafia *et al.,* 2000; Santos *et al.,* 2016):

$$GI = \frac{g_{wt}}{d_{wt}} * 100$$

Female and male GI data was pooled independently each month, whereafter monthly means were compared to examine synchrony between the sexes.

2.2.3 Macroscopic gonad analysis

Gonads were examined macroscopically to determine the sex and the stage of development. Preliminary observations of gonad colour and size during the stages of gonadal development were used to develop macroscopic staging descriptions, as the findings in this study were significantly different to previous studies done on this species. The colour of gonad tubules and gonadosomatic index were used to determine the stage of maturity. Preliminary findings were used to create a basic descriptive guide for tubule appearance during each stage of development. Gonad weights were pooled monthly to calculate the average weight per month. Unfortunately, as the gonad matures, the tubules branch out and ramify, creating a network of intertwined tubules. Tubule length measurements were excluded from this study.

2.2.4 Oocyte measurements

Oocytes were measured monthly to determine the mean oocyte diameter over the course of the experiment (16 months). 10 oocytes were teased from the tubules of five females per month. A total of 800 oocytes were measured over the course of the experiment. The monthly oocyte measurements (n=50) were pooled to calculate the mean monthly oocyte diameter. Oocytes were observed using an Optika B-190 series microscope with an Optika C-P8 camera attached. Optika Proview software was used to measure oocyte diameters.

2.2.5 Histology

Gonad tubules were removed from both sexes during different stages of gonadal development and processed for histological analysis. During the months when gonad weight was low (<1g), the whole gonad was collected and processed. During the months when the gonads were larger (>1g), only a portion of the gonad was collected for analysis. A total of 165 samples were taken, of which 79 were male and 86 female. Samples were fixed in 10% formalin for 24h. Thereafter, the tubules were placed in graded alcohol solutions for dehydration and cleared in xylene. Tissue dehydration and clearing was done following these steps: Samples were placed in a 70% ethanol solution for 20 m, thereafter in a 95% ethanol solution for 20 m, repeated in a fresh solution. Hereafter samples were placed in absolute ethanol for 20 m and repeated in a fresh solution. Samples were cleared in xylene for 20 m, repeated twice. Finally, tissue samples were removed from the cassettes and placed in molten paraffin (58-60ºC) for 30 m. The tissue was then placed in a mould with liquid paraffin and allowed to harden (Adopted from Canene-Adams, 2013). Transverse sections of ±6.0 µm were cut using a microtome and stained with Haematoxylin and Eosin by Amanzi Biosecurity. Maturity stages, oogenesis and spermatogenesis were examined. The physical appearance of the gonads was also recorded. Micrographs were taken using an Optika B-190 series microscope with an Optika C-P8 camera attached.

2.2.6 Stages of gonad maturity

Once the gonads were dissected, weighed, and examined, they were categorized into one of five categories depending on the level of maturity (Stage I – Indeterminate; Stage II – growing; Stage III – Mature; Stage IV – partly spawned and stage V – spent). Macroscopic and microscopic characteristics, as well as histology, were used as maturity assessment techniques. Gonads classified as stage I (Indeterminate) were either small, almost invisible gonads that could not be sexed through microscopy, or no gonad was present. This can be due to evisceration, gonads being spent, or gonads being too minute to trace. If gonads were present, they were generally translucent- white in colour. Gonads classified as stage II were visible to the naked eye, visibly undergoing oogenesis or spermatogenesis under the microscope. These gonad tubules typically contained many small oocytes or spermatozoa. Female gonads were light pink and male gonads were white. Gonads classified as stage III were large, tubules visibly swollen, with oocytes being visible through the tubule wall in females. Under the microscope, the female tubules were filled with ripe oocytes, and the male tubules were filled with spermatozoa. Female gonads were a bright salmon colour, while male gonads were white or milky yellow in appearance. Gonads classified as stage IV were large, tubules were slightly deflated, and some mature oocytes and spermatozoa were present in the tubules. The lumens generally contained few gametes, and no gametogenesis was visible. Female gonads were dark salmon coloured and male gonads were milky yellow or dark yellow. Gonads classified as stage V were shrivelled and smaller in appearance, or appeared deflated, with few relict oocytes and spermatozoa present in the tubules. Gonads were darker in colour, with female gonads appearing a dark salmon colour, and male gonads were dark yellow.

2.2.7 Environmental data

The annual natural gonadosomatic cycle was compared to two environmental parameter cycles, namely water temperature and day length. Water temperature (ºC) data was compiled by the South African Weather Service (SAWS). Water temperature recordings were taken at the coastal station at East London Orient Beach (Climate Number: 0059572B8; -33.0350ºS, 27.8160ºE). Historic daylength data (2020-2021) was compiled by Time and Date AS (Norway) and daylength data was collected from their website (https://www.timeanddate.com/) for comparison. The measurements were pooled monthly to create an average temperature and daylength per month, which was compared with the gonadosomatic cycle of the sea cucumbers to elucidate if it has any effect on the maturation of the gonads.

2.2.8 Statistical analyses

All male and female farmed sea cucumber GI value data were compared and tested for statistically significant differences and similarities. Male and female sea cucumber monthly average GI values were compared using a repeated measures ANOVA to examine significant differences in GI values ($p<0.05$). A multiple regression analysis was performed on sea cucumber GI and seawater temperature and photoperiod to determine if any of the two parameters influenced gonadal development. Oocyte measurements were pooled monthly, and a repeated measures ANOVA was performed to examine size differences over the 16-month sampling period ($p<0.05$).

2.3 Results

2.3.1 The Gonadosomatic Index (GI)

The sampled sea cucumber gonads matured from early winter (April / May) through to early spring (September), with the peak GI values recorded in September of both years (Figure 2.1, Table 2.1). From October, the GI values declined significantly through the summer months until February of the following year, indicating when spawning season ensued.

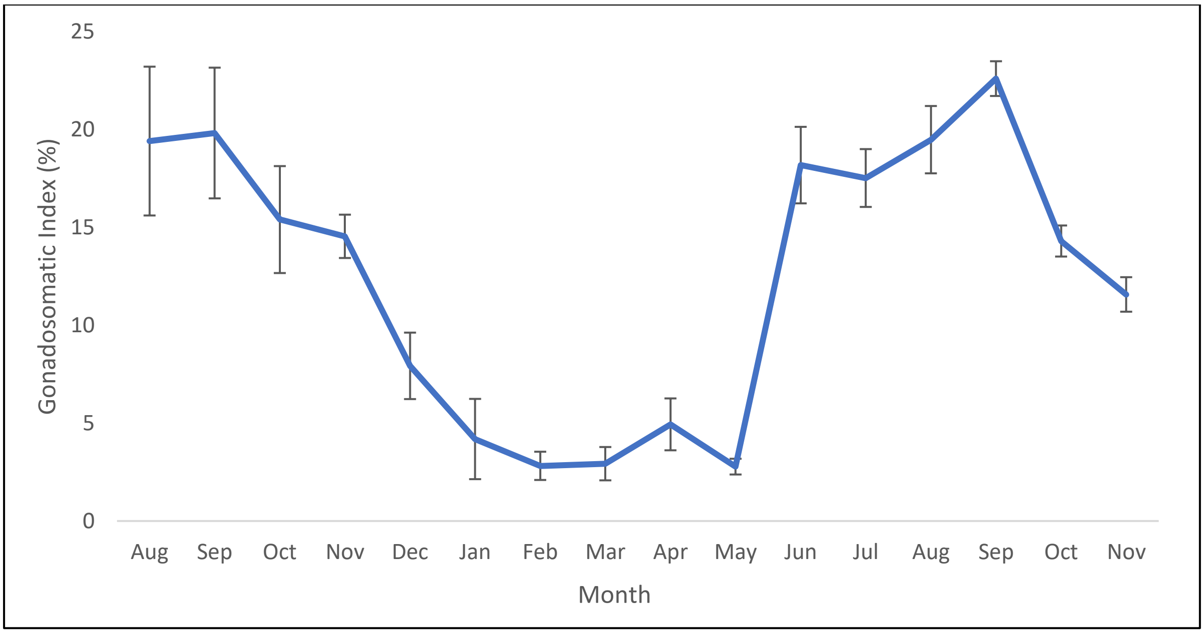


Figure 2.1: The monthly average gonadosomatic index values of wild warty sea cucumbers collected over the course of a 16-month sampling period. (SE±, n= 297).

Table 2.1: Monthly gonadosomatic index values of wild collected warty sea cucumbers over a 16-month sampling period.

Year	Month	Body weight (d_{wt}) Average, g	Gonad weight (g_{wt}) Average, g	GI average, %	SE	Count
2020	August	9.22	2.0	19.48	3.8	13
	September	6.49	1.51	19.83	3.34	12
	October	9.27	1.59	15.44	2.73	10
	November	10.84	1.58	14.55	1.11	10
	December	11.81	1.0	7.93	1.7	10
2021	January	7.96	0.34	4.19	2.05	10
	February	10.32	0.32	2.82	0.72	23
	March	14.05	0.56	2.93	0.85	17
	April	9.96	0.65	4.94	1.33	24
	May	11.31	0.32	2.78	0.4	22
	June	23.82	8.21	18.19	1.96	19
	July	16.08	3.04	17.53	1.48	23
	August	13.71	2.86	19.49	1.72	26
	September	7.23	1.67	22.61	0.89	27
	October	14.51	2.15	14.31	0.79	26
	November	8.13	0.89	11.58	0.88	25
Total						297

Female sea cucumbers reached peak GI values in August of both 2020 and 2021, with averages of 23.6% (SE±6.36) and 26.3% (SE±1.9) respectively (Figure 2.2; Table 2.2). Male sea cucumber GI values reached peaks in September of both years, with the highest average values of 22.5% (SE±2.7) and 22.2% (SE±1.2), respectively (Figure 2.2, Table 2.2). Female sea cucumbers reached higher GI values than males in most months while gonadal maturation took place (February – October), whilst males had higher GI values during the months after spawning started (November – January). Minimum GI values in females were reached in May (3.49, SE± 0.51), whereas the minimum GI value for males was seen in February (0.85, SE± 0.18).

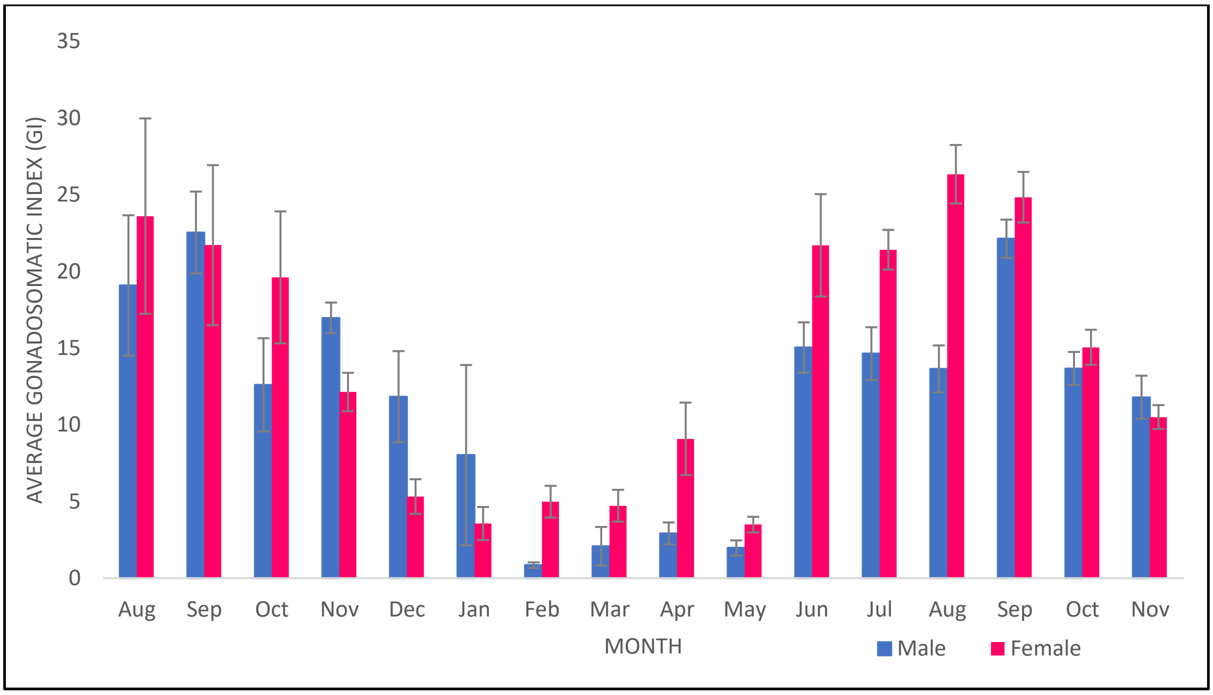


Figure 2.2: Male and female monthly average gonadosomatic index values over a 16-month sampling period (±SE, female n=145, male n=138, total n= 283).

Table 2.2: Male and female gonadosomatic index (GI) values of wild-collected warty sea cucumber specimens, pooled monthly.

		Male			Female			Total count
Year	Month	GI	SE	Count	GI	SE	Count	
2020	Aug	19,08	4,58	7	23,6	6,37	5	12
	Sep	22,53	2,67	5	21,71	5,22	6	11
	Oct	12,60	3,04	6	19,61	4,30	4	10
	Nov	16,97	1,00	5	12,14	1,25	5	10
	Dec	11,83	2,97	4	5,32	1,13	6	10
2021	Jan	8,02	5,88	3	3,56	1,08	5	8
	Feb	0,85	0,18	6	4,98	1,04	12	18
	Mar	2,08	1,26	8	4,72	1,04	8	16
	Apr	2,92	0,72	11	9,08	2,36	11	22
	May	1,97	0,49	8	3,49	0,51	13	21
	Jun	15,04	1,64	10	21,7	3,33	9	19
	Jul	14,64	1,72	10	21,41	1,29	12	22
	Aug	13,64	1,53	14	26,33	1,91	12	26
	Sep	22,13	1,25	13	24,83	1,65	14	27
	Oct	13,67	1,07	14	15,05	1,14	12	26
	Nov	11,80	1,40	14	10,5	0,78	11	25
Total				138			145	283

2.3.2 Macroscopic gonad analysis

Macroscopic gonadal characteristics, combined with gonadosomatic index values, produced valuable information when determining the stage of development of the individual sea cucumbers. GI values, compared to the gonad colour and appearance, were used as preliminary classification characteristics (Table 2.3).

Table 2.3: Summary of the Gonadosomatic Index (GI) and tubule colour descriptions during the five stages of gonadal development.

Maturity stage	Sex	Gonadosomatic index (GI), average	Colour
I (Indeterminate)	N/A	0.17 (SE±0.12)	Translucent white
II (Growing)	F	12.49 (SE±0.95)	Light pink
	M	11.16 (SE±0.85)	White
III (Mature)	F	32.02 (SE±1.51)	Salmon
	M	29.44 (SE±1.79)	White, milky yellow
IV (Partly spent)	F	12.3 (SE±0.76)	Dark salmon
	M	13.22 (SE±0.93)	Milky yellow to dark yellow
V (Spent)	F	4.79 (SE±1.35)	Dark salmon
	M	3.24 (SE±1.92)	Dark yellow

Growing (stage II) male gonads have short, thin tubules that are in the beginning stages of spermatogenesis. More mature, thicker tubules may or may not be present in the same gonad sample. Growing male gonads appear white to light yellow. Once maturity (stage III) has been reached, all tubules are visibly swollen and turgid. Mature male gonads range from light yellow to yellow in colour (Figure 2.3, right). Partly spent (stage IV) gonads have some tubules that have been emptied during spawning and other tubules that still contain viable spermatozoa. Partly spent gonads appear yellow. Spent (stage V) male gonads in appear thin and stretched out. Little to no spermatozoa were present in the tubules. In other spent samples, the tubules had not started to regress, and the tubules were visibly larger and thicker. Spent tubules appear dark yellow in colour.

Female gonads in the process of growing (stage II) contained small oocytes that were sparsely packed in the lumen. These tubules were thinner and smaller in size and correlated with the lower GI values of stage II females. Growing tubules were light pink in colour. Mature (stage III) gonad tubules were densely packed with large oocytes. Tubules were visibly turgid. Mature female gonads appeared salmon coloured (Figure 2.3, left). Partly spent (stage IV) gonad tubules contained large oocytes but were not as densely packed as some of the oocytes had been shed. The tubules were still large but had become flaccid due to the loss in volume. Partly spent gonads appeared dark salmon in colour. Spent female gonads (stage V) contained little to no oocytes in the lumen of the tubules. The tubules appeared flaccid as most oocytes had been shed. Spent tubules also appeared dark salmon in colour.

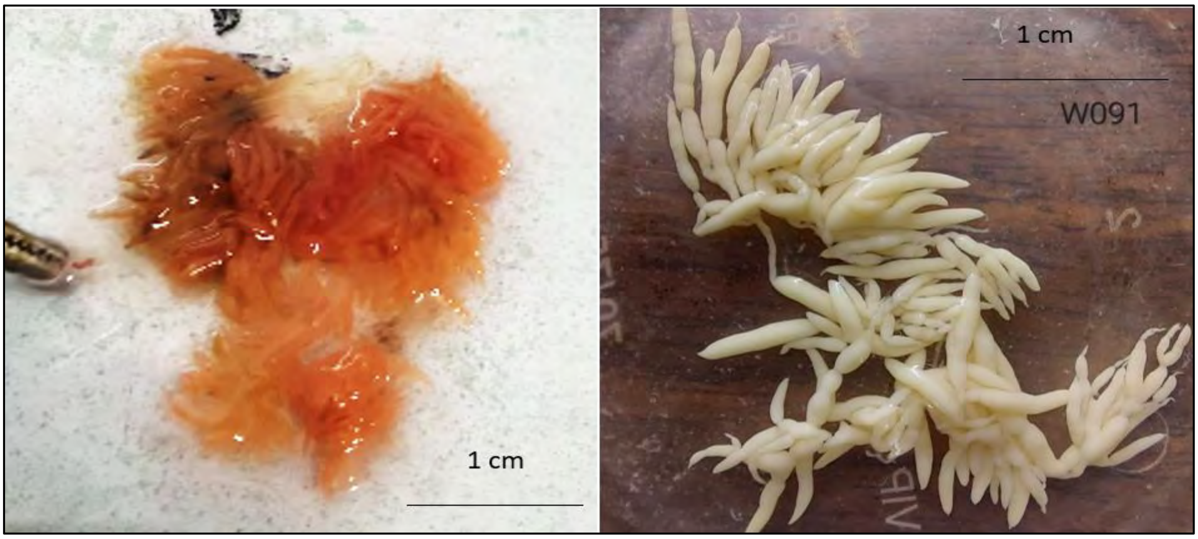


Figure 2.3: Mature female (left) and male (right) gonads. Female gonads are a bright salmon pink, while male gonads appear a pale, milky yellow.

2.3.4 Histological analyses and gonadal staging

Histological analyses revealed that male and female sea cucumber gonad tubules comprised three layers of tissue: the tubule wall, connective tissue, and germinal epithelium. Gametogenesis was clearly in progress in stage II (growing) in both sexes. Tubule walls are thin and stretched out in the mature stage (stage III), as tubules are filled with gametes. Some gametes are present during the partly spent stage (stage IV). The Spent (V) stage was characterized by relict gametes and thickened connective tissue in both sexes (Figures 2.4, a-d; 2.5 a-d).

Gonadosomatic stages of development:

The gonad stages of development (histological analysis) are classified based on visual observations.

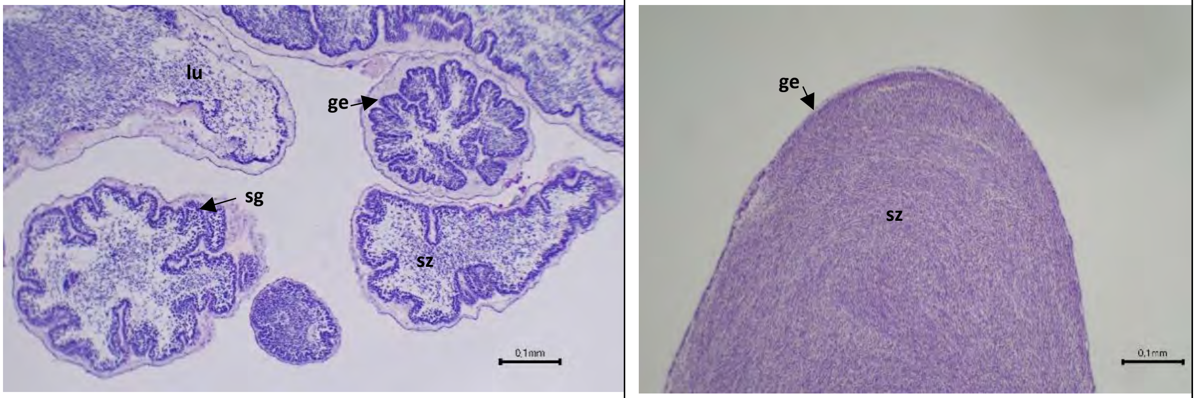
Males

Stage I: Indeterminate. Gonads are not visible in many cases. Small gonads are not able to be sexed.

a) *Stage II: Growing.* Spermatogenesis is in progress. Spermatogonia and spermatocytes line the inside of the germinal epithelium. The germinal epithelium contains many infolds. Spermatozoa are present in the lumen of the tubules. Spermatogenesis is stratified, with less mature spermatozoa in the periphery of the tubule.
b) *Stage III: Mature.* The tubule wall, connective tissue and germinal epithelium are thin and stretched. The lumen is filled with spermatozoa. Spermatogonia and spermatocytes are present along the germinal epithelium but are not as abundantly visible as in stage II.
c) *Stage IV: Partly spent.* Most of the spermatozoa have been expelled from the lumen. Some spermatozoa can be seen in the lumen. The connective tissue between the tubule wall and germinal epithelium is thickening. Some tubules contain many spermatozoa, while others are flaccid and mostly empty.
d) *Stage V: Spent.* The lumen of the tubule is mostly empty, apart from some relict spermatozoa. The connective tissue is visibly thickened, and the germinal epithelium contains some infolds.

a) Stage II: Growing

b) Stage III: Mature



c) Stage IV: Partly spent

d) Stage V: Spent

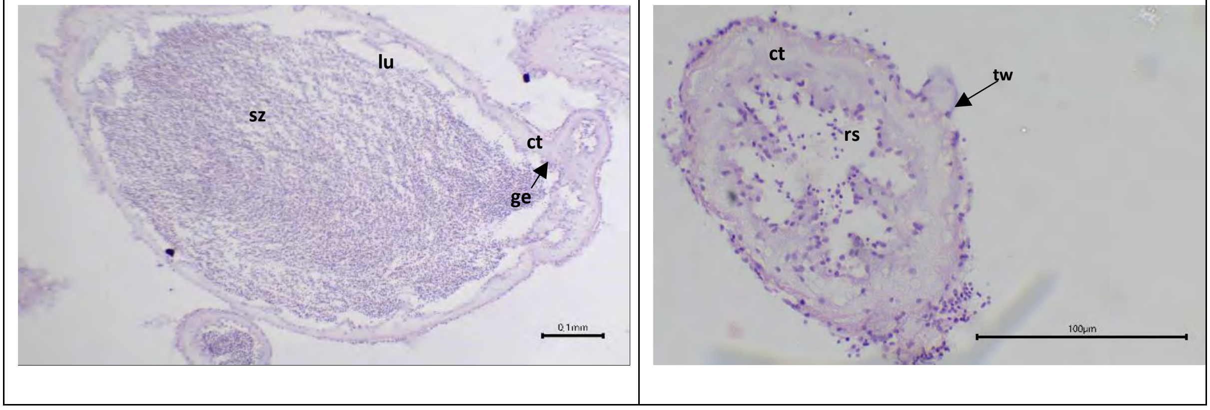


Figure 2.4: *Spermatogenesis.* Micrographs of the typical developmental stages found during the male gonadal maturation process. **Sg**: spermatogonia; **ge**: germinal epithelium; **tw**: tubule wall; **sz**: spermatozoa; **lu**: lumen; **ct**: connective tissue; **rs**: relict spermatozoa. Scale bars: a, b &c = 0.1mm; d =100µm.

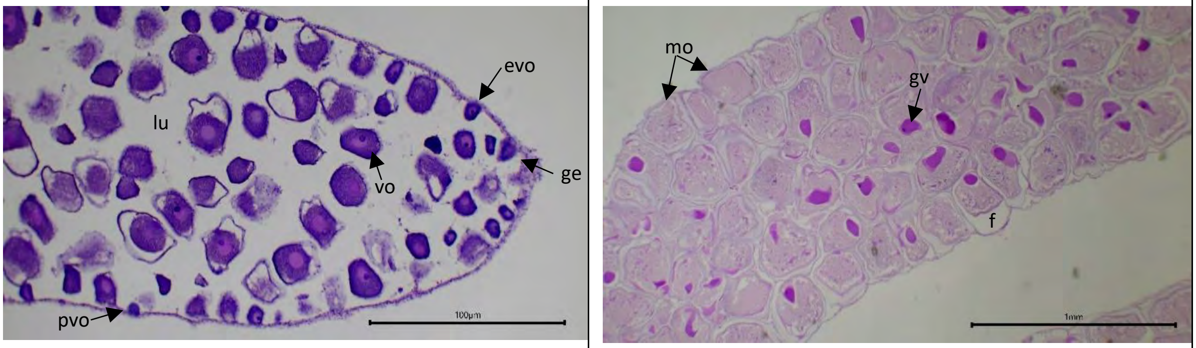
Females

Stage I: Indeterminate. Gonads are not present or visible in most cases. Gonads are not able to be sexed.

a) *Stage II: Growing*. Unripe oocytes are sparsely present in the lumen of the tubule. Pre-vitellogenic oocytes are present in the periphery of the tubule, close to the germinal epithelium. Larger, early vitellogenic oocytes can also be seen close to the germinal epithelium. Small and large vitellogenic oocytes are found closer to the centre of the tubule.
b) *Stage III: Mature*. Mature oocytes are densely packed in the lumen of the tubule. The germinal vesicle is visible in some oocytes and a distinct nucleolus can be seen. The germinal vesicles are located to the periphery inside the oocytes. All mature oocytes are contained within a follicle.
c) *Stage IV: Partly spent.* Tubule walls are thickening, and the connective tissue is clearly visible. Some mature oocytes are still present in the lumen, along with nutritive phagocytes. Some tubules are empty, while other tubules from the same individual still contain many ripe oocytes.
d) *Stage V: Spent.* A few relic oocytes are present in the lumen of the tubule. The connective tissue of the tubule wall is thick and nutritive phagocytes are present.

a) Stage II: Growing

b) Stage III: Mature



c) Stage IV: Partly spent

d) Stage V: Spent

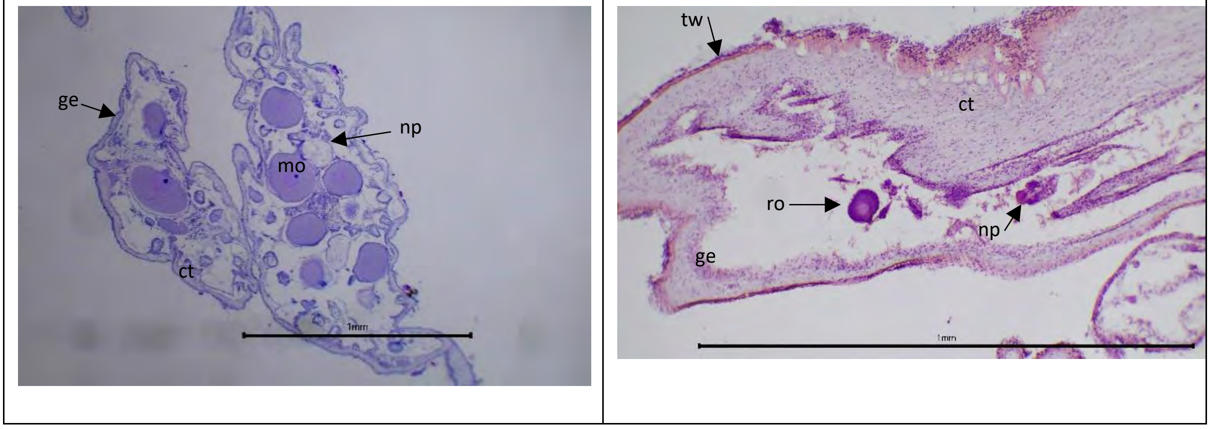


Figure 2.5: *Oogenesis.* Micrographs of the typical developmental stages found during the female gonadal maturation process. **pvo**: pre-vitellogenic oocytes; **evo**: early vitellogenic oocytes; **vo**: vitellogenic oocytes; **lu**: lumen; **mo**: mature oocytes; **ge**: germinal epithelium; **gv**: germinal vesicles; **f**: follicle; **ct**: connective tissue; **mo**: mature oocytes; **np**: nutritive phagocytes; **ro**: relict oocytes; **tw**: tubule wall. Scale bars: a =100µm; b, c &d = 1mm.

Gonad recrudescence occurred predominantly from winter into spring, after which gonads reached maturity and gametes were released (Figure 2.6). The macroscopic visual and histological observation indicated that not all gametes were expelled from the gonads at once, as some gametes were still present in tubules of partially spent animals. From late summer, a high proportion of spent animals were present.

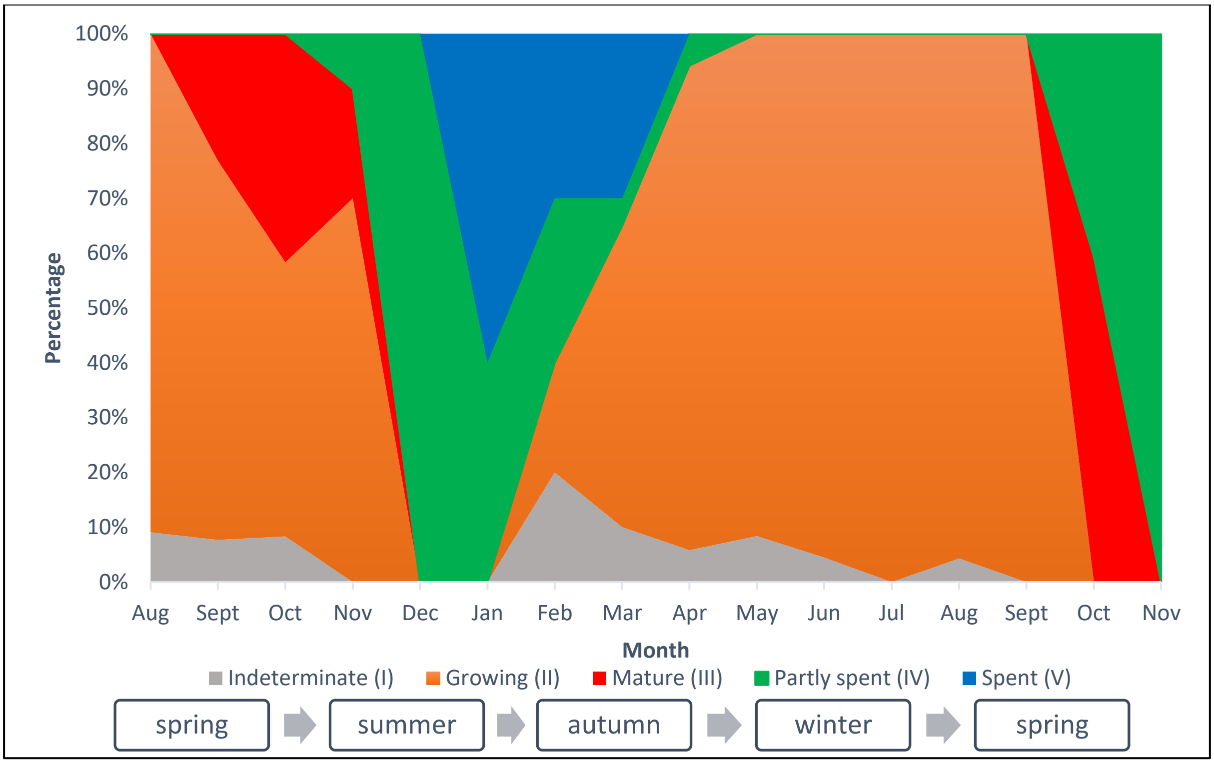


Figure 2.6: Gonadal development stages based on macroscopic evaluation, histological analysis, and GI values over a 16-month sampling period.

2.3.3 Oocyte measurements

The monthly oocyte diameter measurements (Figure 2.7) followed a similar trend to the Gonadosomatic Index values. The largest oocytes were found in December 2020, with average values of 360µm (SE±1.16). Oocyte diameter in January and February 2021 was very small, averaging 16 µm (SE±2.95) and then gradually increase in size, reaching 350 µm from September – November 2021.

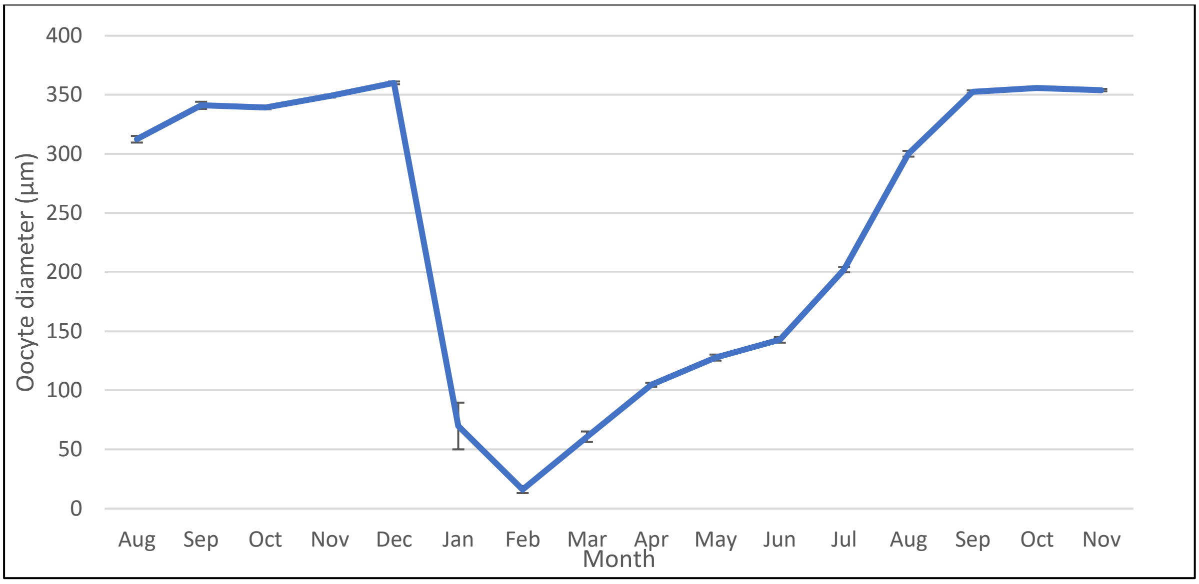


Figure 2.7: Average monthly oocyte diameter over 16 months (SE±, n=800), $p<0.05$.

2.3.5 Environmental correlates with N. grammatus gonadal cycle

Monthly mean sea temperatures reached a maximum of 19.6°C in January, February, and March (SE± 0.23, 0.29 and 0.27, respectively) of 2021. A minimum average temperature of 16.4°C (±0.09) was recorded in August of 2021. Maximum average daylength was recorded in December, having 876 minutes (11.4 h) of daylight on average. The shortest days on average were recorded during May and June, having an average of 600 minutes (10 h) of daylight (Figure 2.8).

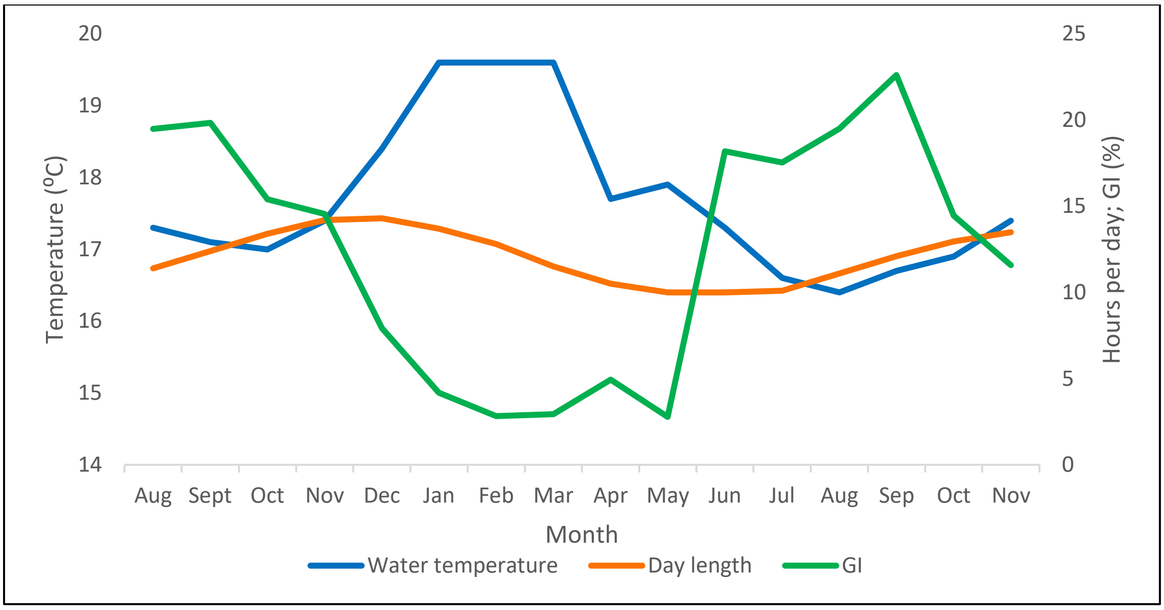


Figure 2.8: Seasonal variation of water temperature and hours of daylight (diurnal rhythm), as well as fluctuations in GI values – monthly averages, during the 16-month study period. Photoperiod data collected from Time and Date (AS) Norway. Water temperature data collected from the South African Weather Service (SAWS).

Gonadosomatic Index values declined annually from September onwards, as sea water temperatures rose. A multiple regressions analysis (ANOVA) showed that the only significant relationship between GI values was with sea water temperature ($y= -5.548x+110.53$, $F=11.942$, $p<0.001$, $R^2=0.749$; Figure 2.9). An increase in GI values was evident as sea water temperature decreased. No significant relationship was observed when GI values were correlated with daylength ($p> 0.19$).

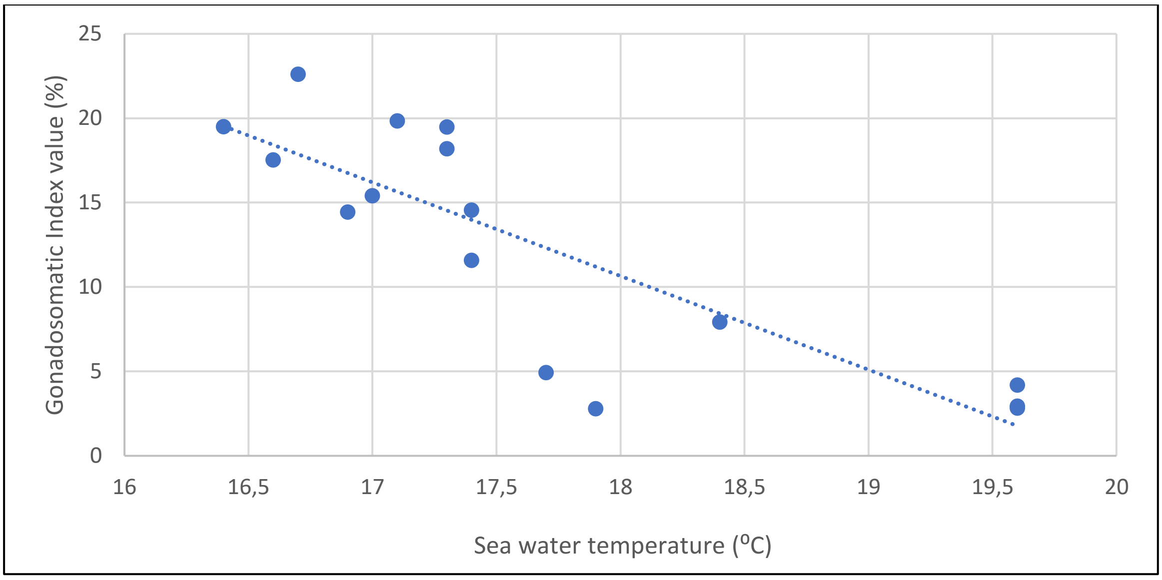


Figure 2.9: Linear regression model showing a negative relationship between seawater temperature and GI values of wild sea cucumbers.

2.4 Discussion

The natural gonadal maturation cycle of *Neostichopus grammatus* followed an annual pattern. This was evidenced by the peak GI values being reached in September of both years of this study, followed by declines in GI values through summer, indicating spawning activity. Recrudescence took place from late summer or early autumn, through to late spring. Gametogenesis restarted as early as January for some specimens, with individuals entering the growing stage increasing steadily as the months progress. This pattern of development, i.e., reaching maturity in spring and spawning during the summer months, is typical for many Aspidochirotid holothurians (Conand, 1981; Smiley *et.al.*, 1991; Hopper *et al.,* 1998; Ramofafia *et al.,* 2000).

During the spawning period, GI values decreased steadily for several months, having reached the lowest GI values in late summer. Macroscopic and histological observations of the gonads indicated that they were not emptied fully during the spawning period, with oocytes and spermatozoa still present in some gonad tubules. This indicates that a series of spawning events likely took place through the summer, as is evident by the “partly spent” phase. During this phase, gametes were released into the environment on several separate occasions from late September to February of the following year. Partial spawning behaviour is typical for several holothurian species, such as *Holothuria whitmaei* (Shiell and Uthicke, 2006), *H. forskali* (Santos *et al.,* 2016), *H. fuscogilva* (Ramofafia *et al.,* 2000) and *Actinopyga mauritiana* (Ramofafia *et al.,* 2001). As warty sea cucumbers are broadcast spawners with pelagic larvae, partial spawning may be a beneficial ‘bet-hedging’ strategy in that more opportunities are created for fertilization and successful settlement in suitable habitats. Reproductive timing may be influenced by factors such as food availability as well as optimal settlement conditions (Ramofafia *et al.,* 2003).

The highest number of mature individuals was recorded in October of both years. Several stages of gonadal development were identified within the monthly samples, indicating that some individuals mature earlier than others, and other individuals will spawn later than some. Nonetheless, a clear overall seasonal cycle of gonad recrudescence, spawning and resting was evident.

The observed GI values corresponded with the stages of gonadal development indicated by the histological results and oocyte diameters. For example, individuals characterised by lower GI values, when analysed microscopically, were found to be in the process of gametogenesis. This is further evidenced by the rise in GI values from late winter and spawning during summer, with a corresponding pattern observed in histology samples and for oocyte dimensions. This indicates that GI values and/or oocyte dimensions and/or macroscopic observations and/or microscopic analyses can be used as a maturity assessment technique for breeding purposes.

The warty sea cucumber was observed to be diecious with no sexual dimorphism. Macroscopic examinations of the gonads can reveal important information on the sex and the sexual maturity of an animal. Macroscopic analyses of the gonads revealed that male gonads appear milky white when mature, and female gonads appear a bright salmon pink. This contrasts with what was found by Foster (1994).

The sex ratio was approximately 1:1, which is a common feature in sexually reproducing holothurians (Hamel *et al.,* 1993). During the growth phase, females had considerably higher GI values than males, whilst post-spawning, the GI values of the males were higher. Males maintained relatively high GI values during this time and a decrease in male GI values was first observed in October of both years. Male sea cucumbers have been reported to spawn for longer periods of time than females (Battaglene *et al.,* 2002), which might explain the higher GI values of males in the post-spawning phase. Females generally had higher body mass and larger gonads than males. This phenomenon has been observed in several sea cucumber species such as *Holothuria arenacava* (Muthiga, 2006), *H. leucospilota* (Gaudron *et al.,* 2008) and *H. atra* (Dissanayake and Stefansson, 2010).

The reproductive tubule structure followed a typical holothurian pattern, comprising three layers of tissue (germinal epithelium, connective tissue, and tubule wall) in males and females (Hamel *et al.,* 1993). Spermatogenesis takes place along the germinal epithelium of the tubules in males. The germinal epithelium contained many infolds during the growth phase. Infolds are suggested to increase the surface area for spermatozoa to proliferate (Cameron and Fankboner, 1986) and may also play a role in providing a nutrient reservoir for spermatogenesis (Smiley *et al.,* 1991). Spermatogonia were located at the periphery of the tubules, and more mature spermatozoa were present in the lumen of the tubules. Once mature, the lumen was packed with millions of spermatozoa. As with the females, not all gametes were released in one spawning event. During the partly spent phase, many spermatozoa were still present in the lumen of some of the tubules. Relict spermatozoa were still present during the spent phase. During the spent phase, the thickness of the connective tissue increased.

Oocytes were produced from the germinal epithelium, starting out as pre-vitellogenic oocytes. Vitellogenic oocytes were observed closer to the centre of the lumen, with vitellogenesis starting in oocytes as small as 30 µm. This is not consistent with what was found by Foster (1994), who stated that vitellogenesis was initiated in oocytes measuring 180 µm. In the present study, larger, more mature vitellogenic oocytes were observed closer to the centre of the tubule. Once mature, the oocytes were densely packed inside the tubule wall. The wall was thin and stretched, and oocytes were visible to the naked eye. Germinal vesicles were visible in some oocytes, depending on the plane of the cross-section. Each mature oocyte was covered by a follicle. Mature oocytes reached maximum diameters of 300-360 µm. Nutritive phagocytes were visible in the partly spent and spent phases, indicating that phagocytosis of oocytes had started during mid-summer while spawning was still a frequent occurrence in other individuals. During the partly spent phase, many mature oocytes were still present inside tubules. These results indicated that the stages of development described in this chapter correspond to Foster's findings (1994), confirming that spawning occurs during the warmer months.

The oocytes were found to range from average values of 16 µm at the onset of the growth phase. The largest oocytes (~360 µm) were found in females during the height of the summer. The oocyte measurements confirm the gonadosomatic index data, indicating that the GI values and oocyte measurements accurately indicate reproductive maturity. The type of larval development in *N. grammatus* is unknown, but oocyte size can be used to determine the type of development that follows fertilization (Hamel *et al,* 1993). Generally, echinoderm oocytes smaller than 100 µm will follow planktotrophic development, and oocytes larger than 1000 µm will follow direct development. Intermediate oocytes generally follow lecithotrophic development. It has been reported by Smiley *et al* (1991) that most holothurian larvae follow direct development, except for members of the order Aspidochirotida and Apodida. It remains to be seen which type of larval development occurs in *N. grammatus* juveniles.

Both female and male gonads regressed after becoming spent, becoming nearly impossible to locate in many cases. In many indeterminate cases, no gonads were located, suggesting that the animal had eviscerated its gonad, or that the gonad had regressed or had been reabsorbed to an extreme extent. The seasonal reabsorption of gonads is observed in other sea cucumber species, such as *Holothuria leucospilota* (Purwati and Luong-van, 2003). The reabsorption of tubules is likely to be useful as an additional source of energy and may be stored in the gut as suggested by Fish (1967). It has been suggested by Herrero-Pérezrul *et al.* (1999) that the evisceration behaviour common in sea cucumbers when placed under environmental stress (Ren *et al.,* 2020; Zhang *et al.,* 2021; Huo *et al.,* 2022) and could be the cause of gonads being absent. However, it was noted during dissection that all other organs were present, indicating that evisceration was not a likely cause of undetectable gonads in resting-phase individuals.

As with many Holothurians, exogenous factors play a significant role in the development of gonads and spawning. Seasonal reproductive cycles in Echinoderms are influenced by some type of environmental cue/s (Pearse & Cameron, 1991). Water temperature was significantly correlated the gonadal cycle in *Neostichopus grammatus*, while no correlation between GI value and photoperiod was evident in the sampled animals. Foster (1994), however, found that day length had a significant effect on the gonadal development of *Neostichopus grammatus.* The limited data sets of both Foster (1994) and the present study preclude any definitive statement on the influence of temperature and photoperiod on gonad maturation. However, it is worth noting that spawning during warmer months is the most common reproductive pattern found in holothurians (Conand, 1981).

2.5 Conclusion

In conclusion, the natural reproductive cycle of *Neostichopus grammatus* followed an annual pattern of recrudescence and depletion. Maturation is initiated in early autumn (May) and continues until late spring (September). Mature gonads were observed in September, followed by spawning taking place between September and February. This was followed by a resting or indeterminate phase where the gonads regressed and were difficult to detect, occurring from March to May. The results of this chapter provide an insight into the natural reproductive cycle and influence of environmental factors on the reproduction of the warty sea cucumber. If these environmental conditions can be replicated and applied in a farming environment, captive breeding might be possible. This is examined in chapters 3 and 4.

Chapter 3: Comparison of the reproductive cycles of wild and farm conditioned *Neostichopus grammatus* sea cucumbers

3.1 Introduction

Understanding the reproductive cycle of a candidate aquaculture species is an essential step in the development of technology for commercial mass production. Before any species can be considered for captive breeding, the gametogenic cycle and the environmental parameters responsible for sexual maturation and spawning should be well understood. Captive breeding of a species usually requires control over environmental cues for sexual maturation and spawning (Morgan, 2000). Several commercially valuable sea cucumbers from the Family Stichopodidae have been successfully bred in captivity (Yang *et al.,* 2015). In Chapter 2, a description of the reproductive cycle of *N. grammatus* was provided, and a significant correlation was found with temperature. In this chapter, the reproductive cycle of *N. grammatus* was monitored under aquaculture conditions on the Wild Coast Abalone farm in a controlled environment.

Several environmental factors are important to consider when attempting to breed a species in an artificial environment. Temperature can affect growth, physiological performance and metabolism of sea cucumbers, and can lead to aestivation when temperatures are too high, or hibernation when temperatures are too low (Yang *et al.,* 2005; Dong *et al,* 2006; Zhao *et al,* 2022). High stocking densities can lead to intraspecific competition and ultimately lead to suboptimal growth rates (Tolon *et al,* 2017). Food quality and availability are also important factors that can influence the fitness and growth of sea cucumbers (Ciriminna *et al*, 2023).

The present study represents the first attempt to condition wild collected *N. grammatus* sea cucumbers in a pump ashore, flow-through aquaculture facility. The sea cucumbers were held in abalone rearing tanks and provided with a diet of abalone waste and faecal matter. Initial tests indicated that wild-collected N. grammatus adapted well to the farm conditions, however it was recognised maintaining them under altered environmental conditions and diet might lead to changes in gonad development and, consequently, spawning periodicity. For example, the on-farm ambient water temperature was 1-3 degrees warmer than the ambient sea temperature, as the reticulation pipes and shallow tanks absorb solar heat (Warf, 2010). It was thus hypothesised that warmer conditions on-farm might advance gonadal maturation during the winter months and the sea cucumbers would come into spawning condition sooner than their wild conspecifics.

The aim of this chapter was to examine gonad maturation of warty sea cucumbers conditioned in an artificial environment and compare their GI cycle to wild warty sea cucumbers. The objectives were to: 1) compare the gonadal maturation cycle of farmed and wild sea cucumbers, focusing on GI values; 2) determine if the warmer on-farm water temperatures would have an effect on gonadal maturation; and 3) examine the gametogenic cycle of farmed sea cucumbers to assess suitability of breeding in a farming environment.

3.2 Materials and methods

This chapter provides a comparison of wild and farmed sea cucumbers gonadal development and thus the wild sea cucumber GI analysis and its associated methodology described in chapter 2 are reiterated.

3.2.1 Farmed sea cucumber enclosures

The sea cucumber specimens were collected from Marshstrand (32.7602º S, 28.2512º E) adjacent to the Wild Coast Abalone farm in February 2020 and held in uninhabited abalone rearing raceways (L 5.0 m x W 2.5 m x H 1.3 m), for six months prior to the start of the gonad monitoring experiment. Outflow pipes were positioned at 450 mm from the bottom of the tank, maintaining water depth at 450 mm. The raceways were covered by shade cloth and the sea cucumbers were provided with refuge in the form of 20 dome-shaped shelters (L 30 cm x W 18 cm x H 9.0 cm). Aeration was supplied via a bottom-suspended polyvinyl chloride (PVC) pipe aeration system.

Figure 3.1: Abalone raceways repurposed to house sea cucumbers during the conditioning phase.

A diet of abalone food and faecal waste was provided to the animals once a week in slight excess of what they appeared to consume – approximately one litre of abalone waste. The raceways were supplied with a continual flow of fresh seawater (60L/min) at ambient temperature. The experimental animals were transferred to an adjacent clean and filled tank every week while the soiled tank was drained and cleaned. Sea water pumped onto the farm was gravity fed to the sea cucumber tanks from a header tank. The seawater supplied to the tanks was an average of 2.1 °C (SE 0.39) warmer than the ambient sea temperature (Table 3.1). No significant changes in air temperature or photoperiod were caused by the farming conditions.

Table 3.1: Differences in ocean and farm water temperature over the 10-month study period.

Month	Ocean water temperature (°C)	Farm water temperature (°C)	Difference (°C)
August	17,3	18,5	1,2
September	17,1	18,0	0,9
October	17,0	19,5	2,5
November	17,4	20,5	3,1
December	18,4	22,0	3,6
January	19,6	20,5	0,9
February	19,6	23,5	3,9
March	19,6	19,5	-0,1
April	17,7	20,0	2,3
May	17,9	20,5	2,6

3.2.2 Processing of wild and farmed specimens

Approximately 10 to 15 farmed specimens were sampled from the experimental tanks monthly from August 2020 to May 2021 which spanned the peak gonad maturation and spawning period. The wild sea cucumbers were collected and processed at the same time as the farmed sea cucumbers were collected and processed (see chapter 2, section 2.2.1). The data collected from farmed specimens was compared with the data from the wild specimens presented in Chapter 2. A total of 128 farmed specimens and 151 wild specimens were collected over the 10-month sampling period (total n = 279).

3.2.3 Gonadosomatic Index (GI)

The Gonadosomatic Index of the sea cucumbers was determined as described in Chapter 2.2 (Section 2.2.2).

3.2.4 Environmental effects

Sea water temperature was measured daily at 9 am and 2 pm on the farm at Wild Coast Abalone. The water temperature was compiled into monthly averages and were used to determine the effect of farm water temperature on the gonadosomatic cycle of farmed sea cucumbers. Photoperiod data was compiled by Time and Date AS (Norway) and daylength data was collected from their website (https://www.timeanddate.com/). Monthly averages were used to determine the effect of diurnal rhythm on the gonadal development.

3.2.5 Statistical analyses

All male and female, wild and farmed sea cucumber GI value data were compared and tested for statistically significant differences and similarities. A Mann Whitney U test was conducted on all possible cohort combinations, e.g., male farmed vs wild, female vs male wild, etc. to determine which sample groups are significantly different from one another ($p<0.05$). Male and female farmed sea cucumber monthly average GI values were compared using a repeated measures ANOVA to examine significant differences in GI values ($p<0.05$). A correlation analysis was performed on farmed and wild sea cucumber GI values to determine the similarities at a 95% confidence interval and Pearson $r > 0.07$. A multiple regression analysis was performed on farmed sea cucumber GI and farm water temperature and photoperiod to determine if any of the two parameters influenced gonadal development.

3.3 Results

3.3.1 Gonadosomatic Index comparison

A similar seasonal reproductive development pattern was observed for wild and farmed sea cucumbers (Figure 3.2). Maximum GI values were recorded at the end of spring, indicating that gonadal maturation took place during the winter. Spawning started in late spring and continued through the summer. The maximum average GI value for farmed sea cucumbers was reached in October, with a monthly average of 21.72% (SE±3.54), whereas wild sea cucumbers reached a maximum average GI value of 19.83% (SE±3.34) in September (Figure 3.2, Table

3.2). Differences in farmed and wild sea cucumber GI values were not statistically significant (p>0.05). A large variation in individual sea cucumber GI values was seen within monthly samples from August to October, with October GI values ranging from 8.42 to 42.8% in farmed sea cucumbers. A similar range of values was seen in wild sea cucumbers during the mature stage, with individual GI values ranging from 8.4% to 41.6% in September. The farm-treated sea cucumbers lost weight and condition throughout the trial, leading to the suspension of the trial after 10 months.

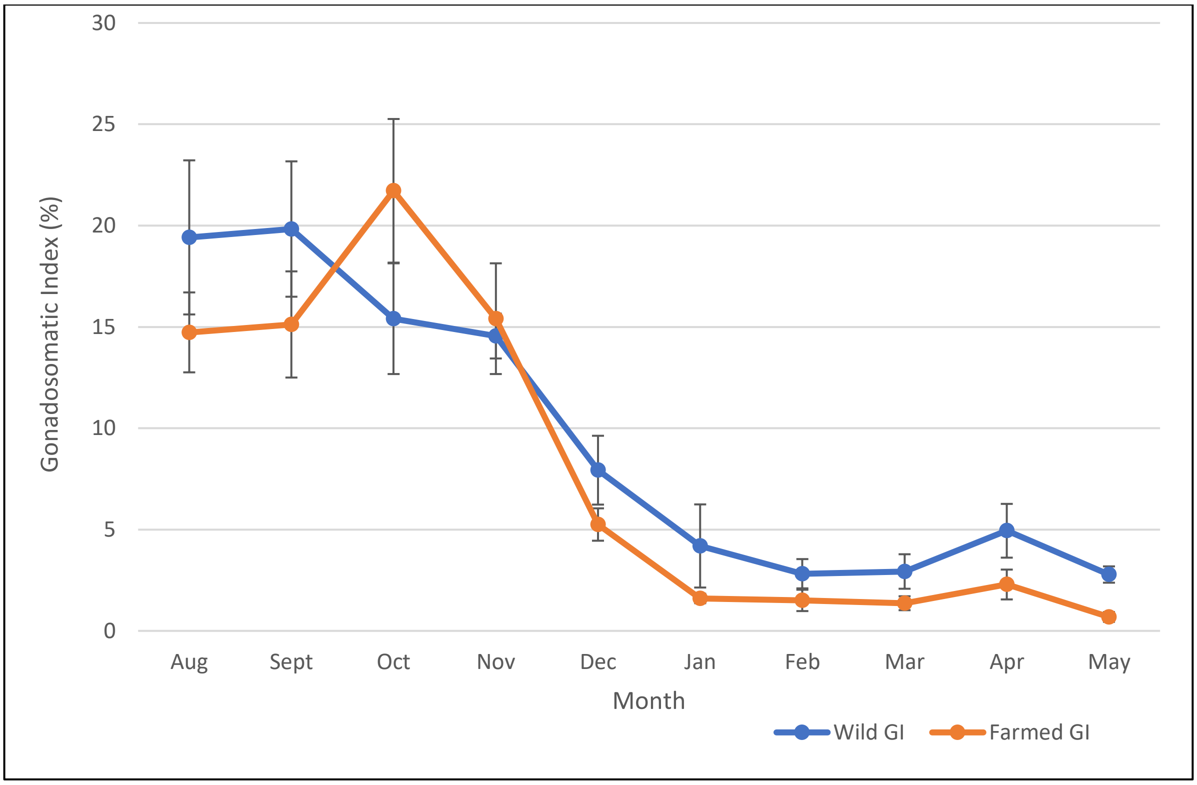


Figure 3.2: The average monthly Gonadosomatic Index values for wild and farmed warty sea cucumbers. Error bars =±SE, n=279.

Table 3.2: Gonadosomatic index values of wild and farmed warty sea cucumbers over a 10-month sampling period.

Month	Wild sea cucumbers			Farmed sea cucumbers		
	Gonadosomatic Index	SE	Count	Gonadosomatic Index	SE	Count
Aug	19,42	3,80	13	14,73	1,97	14
Sept	19,83	3,34	12	15,12	2,62	10
Oct	15,41	2,73	10	21,72	3,54	11
Nov	14,55	1,11	10	15,41	2,73	10
Dec	7,93	1,70	10	5,25	0,80	11
Jan	4,19	2,05	10	1,60	0,23	11
Feb	2,82	0,72	23	1,50	0,53	12
Mar	2,93	0,85	17	1,36	0,34	12
Apr	4,94	1,33	24	2,29	0,74	10
May	2,78	0,40	22	0,69	0,24	27
Total:			151			128

A good correlation was observed between wild and farmed warty sea cucumber GI values, indicating that a similar pattern of sexual maturation occurred in farmed and wild sea cucumbers (P<0.05, r>0.7; Figure 3.3).

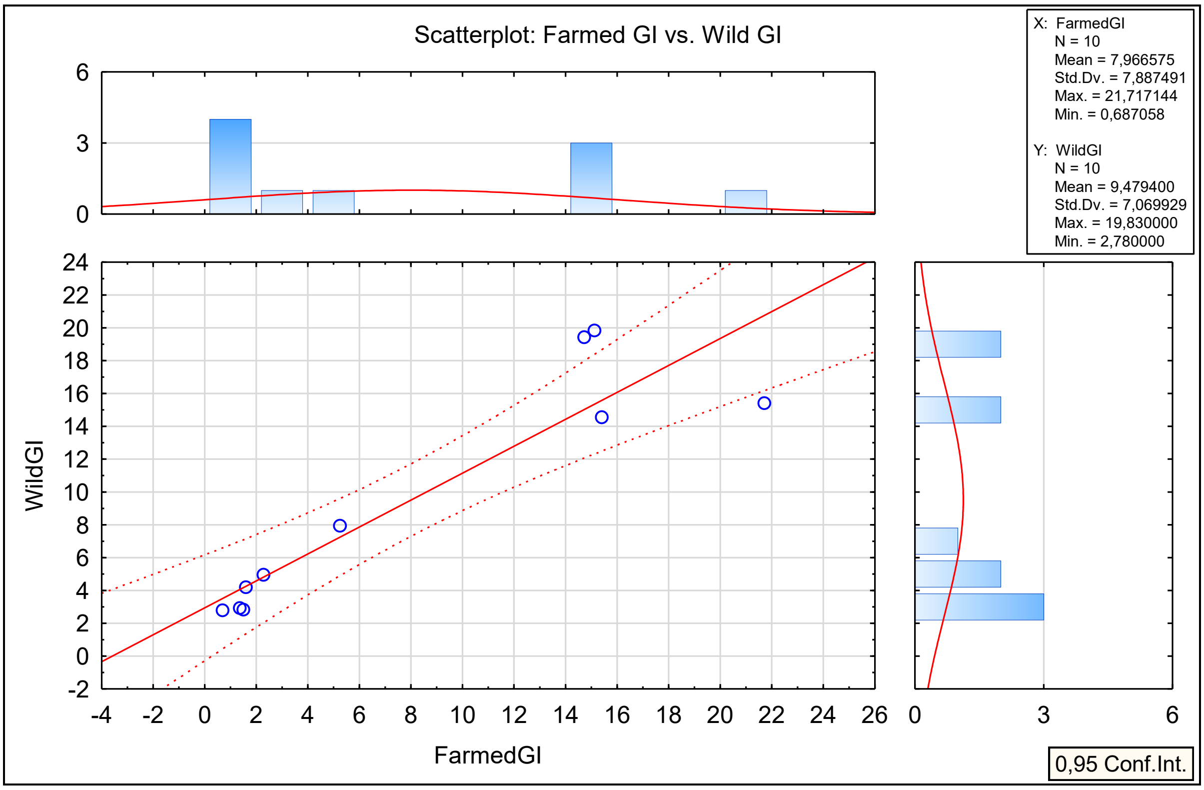


Figure 3.3: A correlation scatterplot: Wild and farmed sea cucumber Gonadosomatic Index values over a 10-month period, with a 0.95 confidence interval, Pearson r =0.92 (n=279).

Male and female captive warty sea cucumbers followed the same annual pattern of development, with both sexes reaching maximum GI values in October (Figures 3.2; 3.4). Maximum recorded GI value for farmed males was 16.03% (SE± 4.95) and 24.96% (SE± 4.06) for farmed females. A decrease in the GI values was observed in December for both male and female specimens signalling the end of the spawning season approaching. Farmed female sea cucumbers reached a minimum average GI value of 1.33% (SE± 0.41) in March, while minimum average GI values for males were observed in February, with a value of 0.72% (SE± 0.25). Wild female sea cucumbers reached peak GI values in August reaching averages of 23.6% (SE±6.36). Wild male sea cucumber GI vales reached peaks in September, with the highest average values 22.5% (SE±2.7). Female sea cucumbers were found to have higher average GI values than males ($p<0.04$).

Comparing all wild and farmed sea cucumber average GI values, all four groups were found to develop high GI values prior to spawning season and had relatively low GI values after the summer spawning season. Variation between development in males and females, as well as wild and farmed sea cucumbers was evidenced by high standard error values (Figure 3.4, Table 3.3).

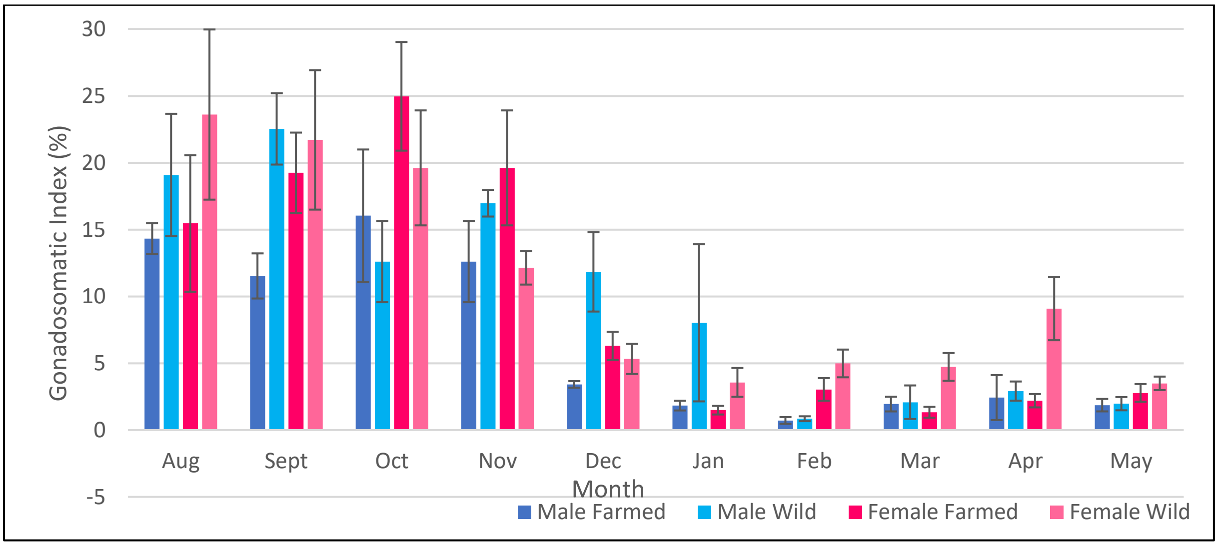


Figure 3.4: A comparison of all male and female sea cucumber Gonadosomatic Index values, sampled over the 10-month sampling period, from wild and farm. Error bars= ±SE.

Table 3.3: Male and female gonadosomatic index values of farm and wild collected sea cucumber specimens, collected over a 10-month period.

		Wild collected specimens							Farm collected specimens						
		Male			Female			Total count	Male			Female			Total count
Year	Month	GI	SE	Count	GI	SE	Count		GI	SE	Count	GI	SE	Count	
2020	Aug	19,08	4,58	7	23,6	6,37	5	13	14,33	1,15	9	15,46	5,11	5	14
	Sep	22,53	2,67	5	21,71	5,22	6	12	11,53	1,69	3	19,24	3,01	6	9
	Oct	12,60	3,04	6	19,61	4,30	4	10	16,04	4,95	4	24,96	4,06	7	11
	Nov	16,97	1,00	5	12,14	1,25	5	10	12,60	3,04	6	19,61	4,30	4	10
	Dec	11,83	2,97	4	5,32	1,13	6	10	3,41	0,24	4	6,30	1,06	7	11
2021	Jan	8,02	5,88	3	3,56	1,08	5	10	1,83	0,36	5	1,49	0,32	5	10
	Feb	0,85	0,18	6	4,98	1,04	12	23	0,72	0,25	4	3,04	0,85	5	9
	Mar	2,08	1,26	8	4,72	1,04	8	17	1,94	0,55	5	1,33	0,41	5	10
	Apr	2,92	0,72	11	9,08	2,36	11	24	2,43	1,68	4	2,20	0,50	6	10
	May	1,97	0,49	8	3,49	0,51	13	22	1,86	0,47	4	2,78	0,66	4	8
Total				63			75	138			48			54	102

3.3.2 Environmental effects

The farm water temperature (daily average 20.3 ± 0.48 °C) was significantly warmer than ocean water temperature (daily average 18.16 ± 0.32 °C) over the experimental period (Student's T-test, $p<0.001$; Figure 3.5, Table 3.1).

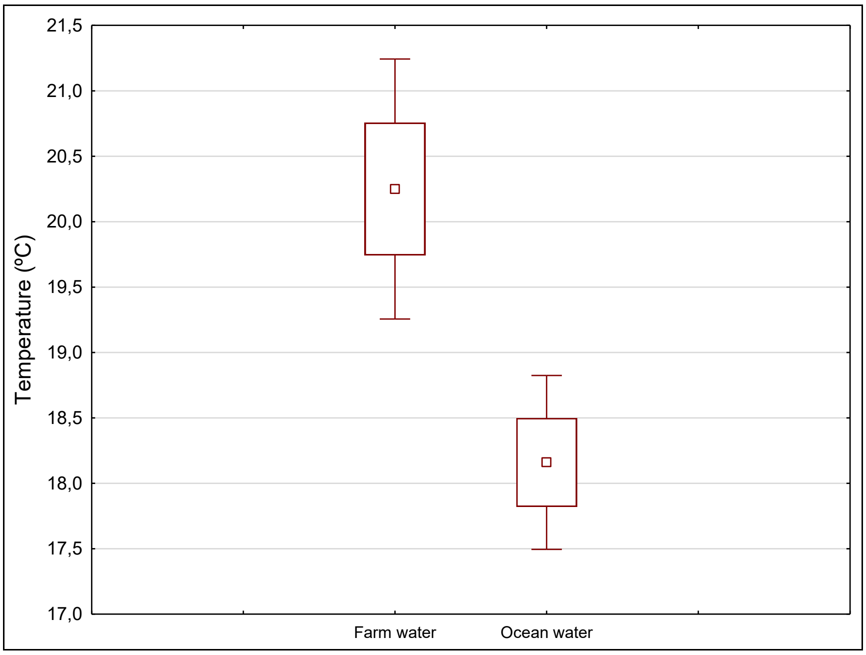


Figure 3.5: Comparison of mean monthly sea water temperature on-farm and in the ocean at Marshstrand (original source), $p= 0.0007$, ▫= Mean, ☐= Mean ± SE, ⌶ = Mean ± 1.96*SE.

Maximum daylength was recorded during the summer in December (14.3 h/d). The maximum average water temperature was recorded in February (23.5°C) and the minimum average water temperature was recorded in September (18.0°C) (Figure 3.6).

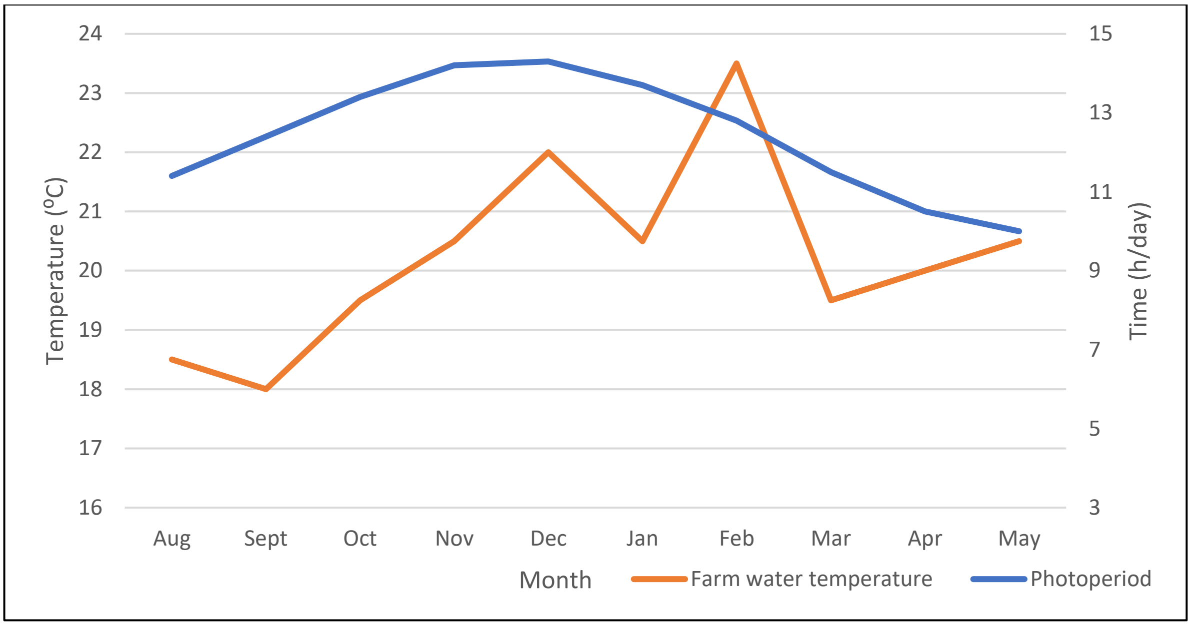


Figure 3.6: Seasonal changes in photoperiod and sea water temperature (on farm) over the 10-month sampling period.

In contrast to the wild sea cucumbers (chapter 2, Figure 2.8), no statistically significant correlation was found between water temperature and gonadosomatic index values for farmed sea cucumbers ($R^2 = 0.27$; $p < 0.05$).

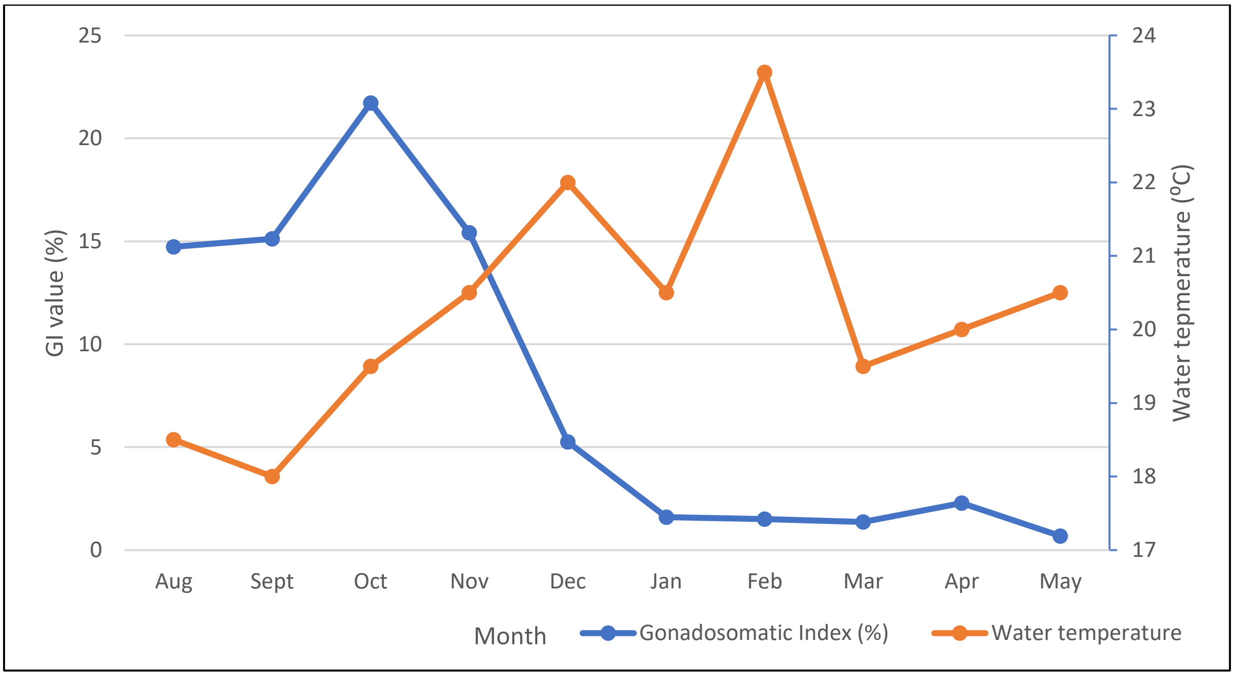


Figure 3.7: A comparison of farmed sea cucumber monthly average Gonadosomatic Index values and fluctuations in sea water temperature on farm.

3.4 Discussion

A similar seasonal pattern of gonadal development was observed in farmed and wild warty sea cucumbers, as evidenced by a strong correlation coefficient in GI data. Gonadal maturation in both groups took place from early winter (starting April) to spring (August - September), after which gonadosomatic index values started to drop (October - December) indicating that spawning occurred throughout the summer months. Some gonads were still found to be mature well into the spawning season. A brief gonad resting period was observed in both groups from January to March, after which GI values increased slightly at the end of autumn (April). The variance between individual GI values within the population was high in the months preceding spawning and less markedly so during the spawning season. A lower degree of GI variation was evident in the resting phase when all animals were either spent or partly spent. Similar observations were reported for the species *Thelenota ananas* (Conand, 1981) and *Isostichopus badionotus* (Agudelo-Martínez and Rodríguez-Forero, 2015) with high variation between individual values during the mature phase of gonadal development.

The farm conditioned sea cucumbers began spawning later, in October – November, compared to their wild counterparts which started spawning in August – September. Several factors could be responsible for this change in reproductive behaviour. As farm water temperature was significantly higher than ambient ocean temperatures, it is thought that the change in temperature could have possibly influenced the reproductive cycle. The loss of sea cucumber weight indicates that a lack of adequate nutrition and/or the presence sediment, most likely influenced the rate of the captive sea cucumbers' gonadal recrudescence. The investigation had to be terminated after 10 months as farmed sea cucumbers lost weight and their condition worsened in the tanks, indicating that the culture environment provided was sub-optimal. Nonetheless, significant insights into sea cucumber reproduction, and additionally, habitat and nutritional requirements, were gained during this short study period.

Despite the sea cucumbers seemingly adapting well to captivity and readily consuming abalone waste food and faeces, the loss of body weight and overall condition pointed to a nutritional deficiency and/ or other environmental stressors. Sea cucumbers, as deposit feeders, consume sediment and extract debris from the sediment for food (Clarke, 1954) as well as aiding in digestion (Watanabe *et al.*, 2012). Onomu *et al.* (2023) found that warty sea cucumbers could survive on abalone waste products alone, however the warty sea cucumbers that had access to sediment showed increased growth rates. A loss of body weight in the sandfish sea cucumber *H. scabra* reared in bare tanks and fed a formulated sea cucumber feed was also reported by Robinson *et al.* (2013), whereas sea cucumbers fed the same diet on a sandy substrate gained weight. Robinson *et al.* (2013) thus concluded that the addition of sand is beneficial to certain sea cucumber species due to the anoxic decomposition of the feed that takes place in the sediment rendering it nutritionally available to the sea cucumbers. This could possibly explain why the experimental on-farm *N. grammatus* lost condition in the tanks as no sand was added, and only abalone faeces and pseudofaeces was made available as a food source.

Later gonad maturation in captivity compared to the wild as observed in the present study, has also been reported on in previous studies performed on *Parastichopus tremulus* (Gunnerus) sea cucumbers. Christopherson *et al.* (2020) found that *P. tremulus* kept in artificial environments spawned several months later than their wild conspecifics. They theorized that holding conditions were suboptimal for gonadal development and had subsequently led to a later spawning event. Food availability is a likely relevant factor contributing to the decline in the sea cucumbers reproductive and overall health. It is unlikely that one single environmental parameter could influence gonadal development, but rather a combination of environmental parameters in unison. Hamel *et al.* (1993) found that a combination of photoperiod, temperature and food availability, during different temporal parts of gonadal development, had significant effects on the gonadal growth of *Psolus fabricii*. It has been suggested factors such as chemical communication (Hamel and Mercier, 1996), illumination intensity (McEuen, 1988) or phytoplankton availability (Hamel *et al.,* 1993) are responsible for gonadal development in several sea cucumber species. It is also plausible that handling stress, such as during tank cleanings, water changes, and the general artificial environment may have had a negative influence on the growth and gonadal development of the farm-conditioned sea cucumbers. These environmental changes need to be investigated to establish reliable and effective growth conditions for *N. grammatus* sea cucumbers held in captivity.

Farm-conditioned females had significantly higher GI values than males ($p<0.04$), whereas no difference was evident between male and female wild sea cucumbers. A similar finding was reported by Dissanayake and Stefansson (2010) in wild *Holothuria atra,* an Aspidochirotid sea cucumber found in Sri Lankan waters. The reason for the size discrepancy is unknown.

3.5 Conclusion

The annual pattern of farmed sea cucumber gonadal maturation followed the seasonal pattern of the wild population's GI cycle, with no significant differences observed between wild and farmed sea cucumber GI values. Even though the on-farm water temperature was significantly higher than the ambient ocean water temperature, temperature did not have a significant effect on gonadal recrudescence, but the farm conditioned animals reached spawning condition later. There was no correlation between photoperiod and on-farm GI values. However, environmental conditions on farm caused a loss of sea cucumber condition suggesting that such as diet or other environmental parameters need to be optimised in order to successfully condition *N. grammatus* broodstock.

Chapter 4: Effect of substrate and temperature on growth and Gonadosomatic Index of *Neostichopus grammatus* sea cucumbers in a farming environment

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4.1 Introduction

When removing animals from their natural environment and placing them in an artificial one, they need to be provided with environmental conditions that will promote growth and gonadal development. The poor condition of the captive sea cucumbers observed in the previous chapter indicated further research was needed to optimise the on-farm environmental and nutritional parameters. The most important environmental requirements for the captive culture of sea cucumbers include diet, water temperature, photoperiod, light intensity, and oxygen content (Morgan, 2000; Ramofafia *et al.,* 2000; Cameron and Fankboner, 1986). All these parameters need to be researched and understood, especially focusing on the effect they have on gonadal development to sexually condition any species of sea cucumber. In the present study, temperature and substrate were manipulated in an attempt to improve the captive culture conditions for *N. grammatus*.

Temperature is the primary controlling factor responsible for gonadal maturation and spawning in several sea cucumber species, including *N. grammatus*. Therefore, temperature control in a farming environment is an important part in conditioning of the broodstock animals (Foster, 1994; Dissanayake and Stefansson, 2013). In Chapter 2, gonadal maturation of wild warty sea cucumbers was observed to take place in colder winter-spring months, with the evidence that the increase in water temperature was an important factor in controlling gonadal maturation (Chapter 2). In Chapter 3, a similar trend was observed for captive sea cucumbers in abalone tanks, but the onset of spawning was delayed. It was thus hypothesised that the significantly warmer on-farm water temperatures might have caused the observed delay in spawning time and possibly contributed poor sea cucumber condition.

The loss of condition of the captive sea cucumbers indicated that they could not effectively utilise the abalone tank detrital waste. In the wild, *Neostichopus grammatus* sea cucumbers live on a sandy substrate under rocks in the infralittoral zone. When searching for specimens, loose rocks needed to be turned over in order to find these animals, as they were usually found on the underside of rocks, clinging to the rock, or partially buried in the sand (Foster, 1994; pers. obs.). When collecting sea cucumbers at Marshstrand, it was observed that the sand under to the sea cucumbers was black, indicating anoxia and the anaerobic breakdown of organic material. Almost all Aspidochirotid Holothurians are deposit-feeding, consuming sand with organic particles as food (Purcell *et al.,* 2016). The warty sea cucumber is no exception, as it has been consistently found during dissections that the gut is full of sand and other small substrate particles (pers. obs.). Sand is an excellent substrate for opportunistic microbes to grow, as detritus and organic particles are found in sand and serve as an energy source for these microbes (Whitman *et al.,* 2014). Furthermore, anaerobic digestion of the organic particles in the sand leads to anoxia in the sediments, releasing nutrients for the sea cucumbers to consume (Robinson *et al.,* 2013). As such, the addition of sand to sea cucumber tanks could potentially create a secondary food source for the sea cucumbers. It was thus hypothesised that warty sea cucumbers would grow better and develop ripe gonads earlier when maintained on a sand substrate. The loss of condition of sea cucumbers under farm conditions indicated the abalone waste food and faeces were not nutritionally adequate. It was therefore hypothesised that the lack of a sand substrate resulted in the inability of the sea cucumbers to utilise the detrital wastes efficiently as a nutritional resource.

The aim of this chapter was to investigate the effect of substrate and water temperature on the growth and gonadal development of warty sea cucumbers. The objectives were to 1) compare the effect of colder (16°C) on-farm water temperature with ambient temperature water (approximately 19 °C -21 °C) on the growth and gonadal development of warty sea cucumbers, and 2) to compare the effect that the presence or absence of sand in sea cucumber tanks has on the growth and gonadal development of warty sea cucumbers.

4.2 Materials and methods

4.2.1 Experimental animals

Sea cucumbers were collected from the infralittoral zone at Marshstrand Beach (32.7602° S, 28.2512° E) several weeks prior to the start of the experiment. The animals were initially held in uninhabited abalone raceways (L 5.0 m x W 2.5 m x H 1.3 m), with outflow pipes positioned at 450 mm from the bottom of the tank, maintaining water depth at 450 mm. Fresh sea water was constantly supplied at 60 L/min. The raceways were covered by shade cloth and the sea cucumbers were provided with refuge in the form of 20 dome-shaped shelters (L 30 cm x W 18 cm x H 9.0 cm). Aeration was supplied via a bottom-suspended polyvinyl chloride (PVC) pipe aeration system into the water. The animals were fed a diet of abalone faeces and pseudofaeces once a week in excess. The tanks were cleaned by removing the animals and shelters and placing them in a bucket with clean water. The tanks were drained and scrubbed, after which they were refilled. The animals were placed back into the clean tanks. Tanks were cleaned weekly.

4.2.2 Experimental system

The experiment was conducted in 14 small square tanks (L 21 cm x W 21 cm x D 30 cm) each with its own water and air supply. The layout of the tanks was two rows of seven tanks. Each tank had two dome-shaped shelters made from PVC pipe cut in half longitudinally (L 20 cm x W 12 cm x H 6.0 cm) to provide darkness and a substrate for the sea cucumbers to cling to.

Figure 4.1: The tank setup for temperature and sediment experiment. Only two of the three rows of tanks were used (n=14 tanks).

Approximately six animals were placed in each tank, with a combined body weight of 46.6g – 51.7g (n = 76). Thus, some tanks contained more than six animals as smaller sea cucumbers weighed less. Animals were purged for three days prior to the start of the experiment. The animals were desiccated for approximately three minutes before the body mass was recorded. The first row of tanks (n=7) received ambient temperature water (fluctuating between 19.0°C and 21.0°C), whereas the other seven tanks received water that was chilled to approximately 16°C (see Fig. 4.2). Three tanks from each row (n=6) were chosen at random to contain sand to enhance feeding, as well as a substrate to bury in to. Approximately 5cm depth of sand was placed in the bottom of the selected tanks. The sand was not aerated. The other eight tanks received only abalone faeces as a food source and the tank bottoms were left bare. Animals were fed once a week with 50ml of abalone faeces sludge per tank and the tanks were cleaned once a week. The sand was replaced every fortnight. Animals were purged for two days prior to each body weight recording. The gonadosomatic index (GI) was recorded for all animals after the two-month experimental period. No GI values could be obtained at the start of the experiment as animals needed to be dissected to obtain gonad weight measurements.

The four treatment groups are described as follows: sand-ambient, sand-cooled, no sand-ambient, and no sand-cooled. These descriptions will be used when referring to treatments in the results section.

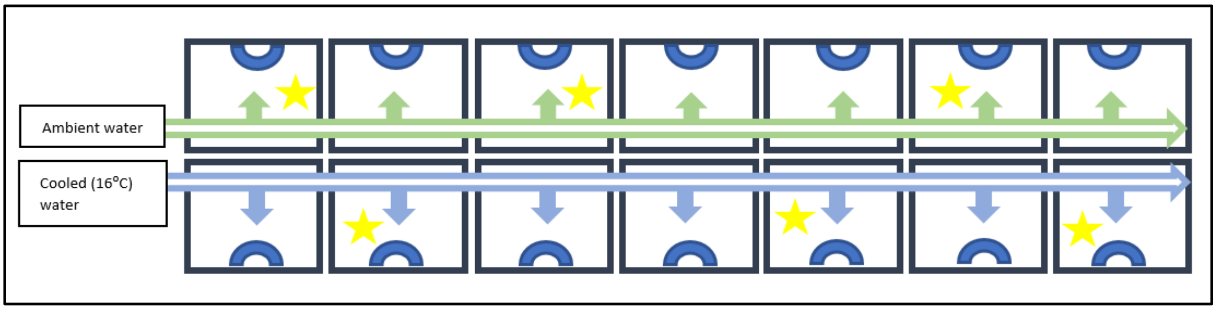

Figure 4.2: Experimental tank layout. Each tank has its own water and air supply and outlet. Dome-shaped shelters are placed in each tank to provide darkness for the sea cucumbers. Sand was added to six tanks, assigned randomly. (Sand present in tank = ★)

4.2.3 Statistical analyses

A multivariate test of significance and a repeated measures ANOVA (p=<0.05) was performed on the initial (WT 0) and final (WT 1) weights of the sea cucumbers to examine the effect of temperature and substrate on body growth over time. A univariate test of significance ANOVA (p=<0.05) was performed on the gonadosomatic index values to highlight the differences caused by the substrate and temperature on the maturation of the gonads.

4.3 Results

Addition of sand to the tanks had a significant positive effect on growth of the sea cucumbers (Figure 4.3). The average body weights of animals in tanks with sand increased by 12.81% over the experimental period, while sea cucumbers placed in bare tanks decreased an average of 13.67%. The effect of the ambient and constant temperature treatments on the growth of the experimental sea cucumbers was not significant (F=4.895, p=0.057).

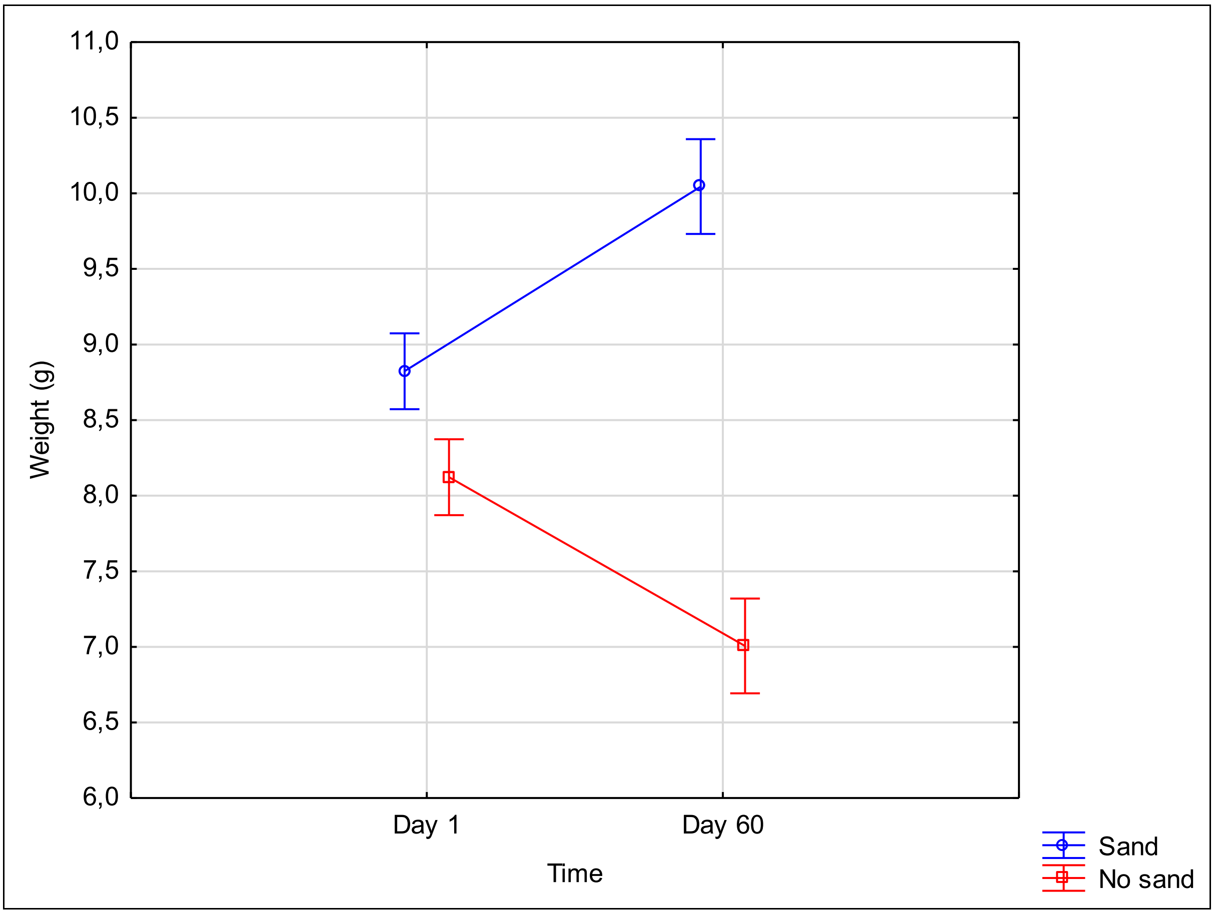

Figure 4.3: The effect of sand on the body growth of sea cucumbers kept in tanks over a two-month sampling period. ANOVA: F= 21.096, p=0.001, error bars =±SE. Sand n=34, no sand n=37.

The sand-ambient treatment group showed the highest weight increase in comparison with the other treatments (Figure 4.4), with an average body weight increase of 1.81g (SE ±0.34) per animal. Positive growth was also recorded in the sand-cold treatment group with an average increase in body weight of 0.63g (SE± 0.41) per specimen. Sea cucumbers in the no-sand treatment groups displayed negative growth, with the no sand-ambient treatment losing an average of 0.58g (SE±0.62) per specimen, and the no sand-cooled treatment group losing on average 1.66g (SE± 0.16) per specimen.

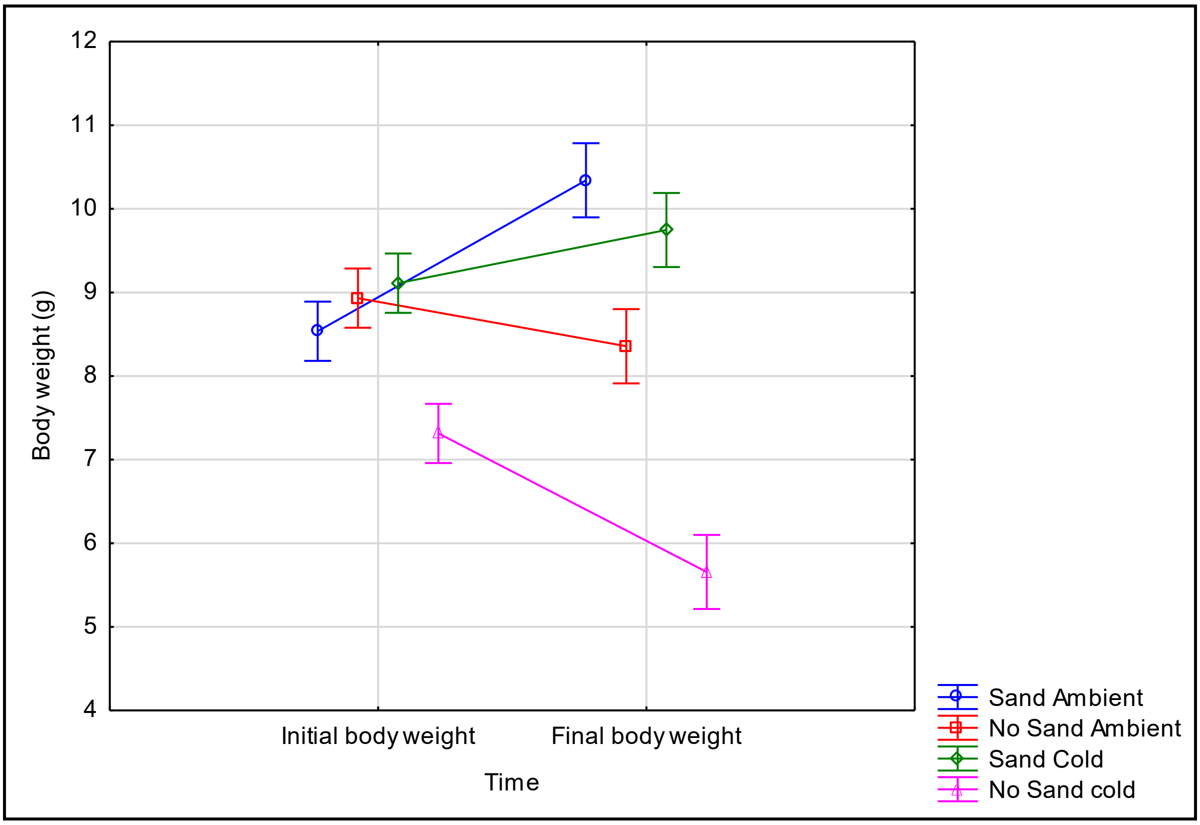

Figure 4.4: Difference in body weight of warty sea cucumbers in four different treatment groups over a two-month sampling period (F=8.666, $p<0.05$, n=76).

The addition of sand to the sea cucumber tanks had a significant positive growth effect on sea cucumber gonads (Figure 4.5). Sea cucumbers supplied with sand in tanks had a final average GI value of 2.99% (SE ±0.56), while sea cucumbers placed in bare tanks had final average GI values of 1.36& (SE ±0.2). The combined effect of water temperature and substrate did not show any significant effect on the GI values of farmed sea cucumbers (F=0.475, p=0.51, Figure 4.4). Temperature alone did not have a significant effect on GI values (F=0.09, p=0.76).

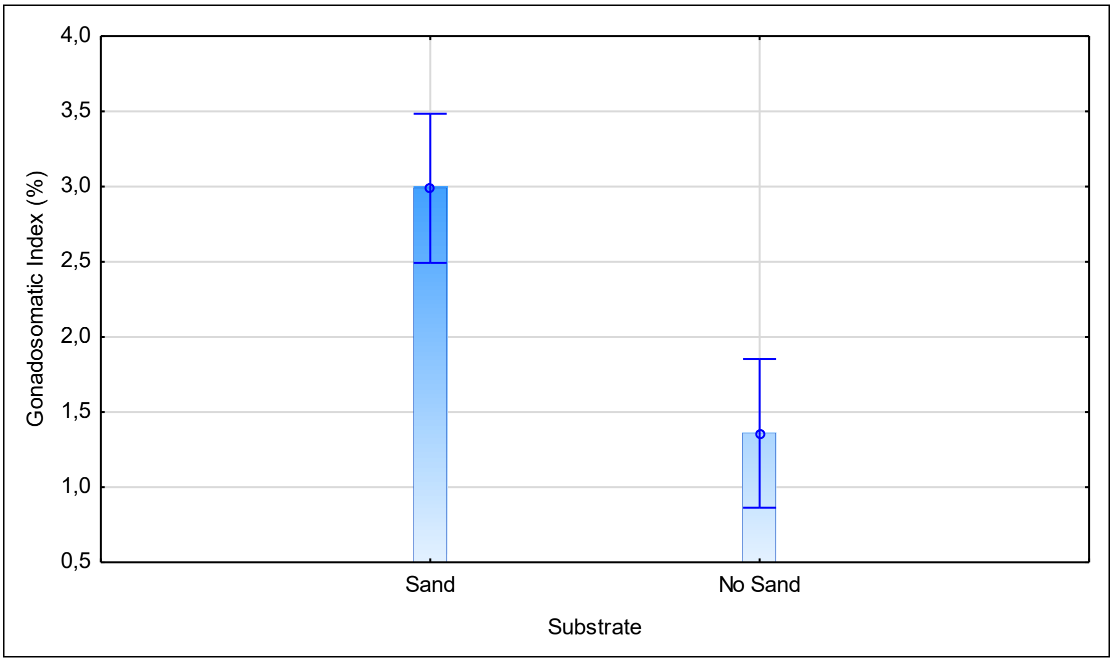

Figure 4.5: The effect of sand on the GI values of farmed sea cucumbers. F=5.41; P<0.05, error bars =±SE.

The highest GI values were recorded in the ambient-sand treatment group with an average value of 3.35% ±1.02 SE (Figure 4.6). Overall, the sea cucumbers supplied with sand had significantly higher GI values than sea cucumbers placed in bare tanks. The two temperature regimes had no significant effect on the GI values.

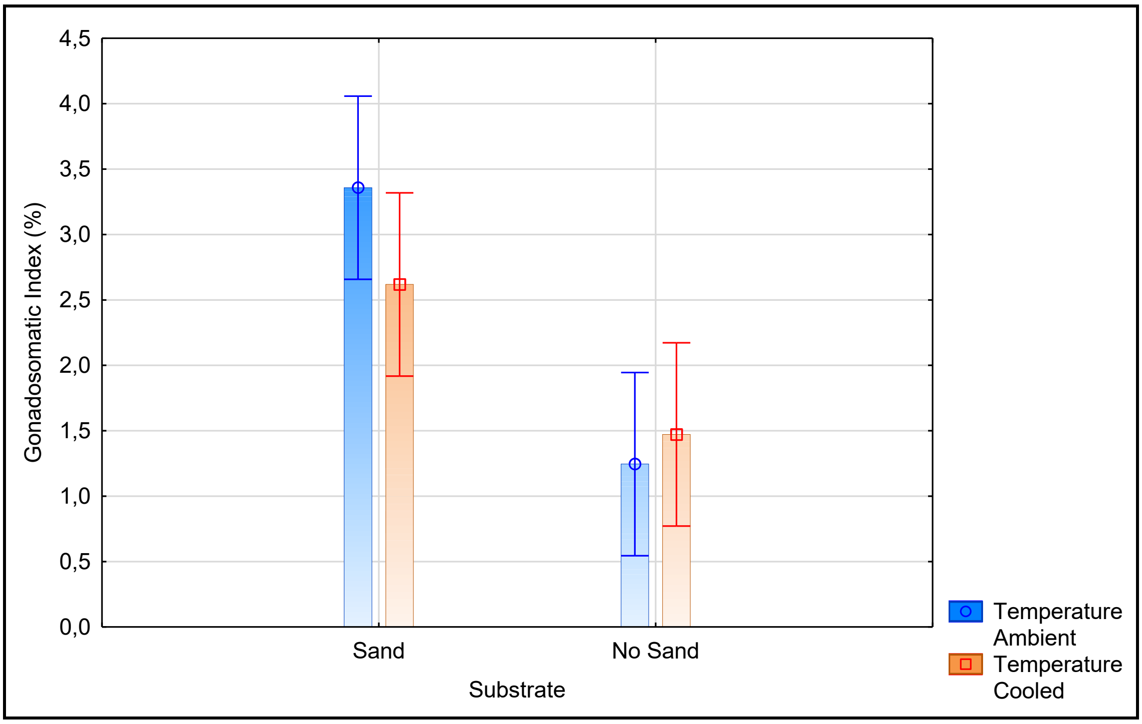

Figure 4.6: The combined effect of substrate and water temperature on the GI values of farmed sea cucumbers over a two-month treatment period. F=0.47, p=0.51. Error bars=±SE.

4.4 Discussion

The addition of sand to the sea cucumber tanks had a significant positive effect on body weight of the experimental sea cucumbers for both the ambient and cooled temperature regimes. The best growth was observed in the sand-ambient group. When cleaning the tanks, the sand was found to be black and foul-smelling, indicating anoxic conditions. It has been well-established that anoxic sand is colonized by bacterial species aiding in the breakdown of organic matter. Clarke (1954) found that holothurians consume substrate as a means to extract organic debris from the substrate. Sand seems to foster anoxic conditions through stratification and the subsequent metabolization of several symbiotic anaerobic bacterial species living in the substrate. Mineralization by different bacterial participants breaks the organic matter down in a slow and stepwise manner, leaving time for the sea cucumbers to consume some of the broken-down organic material (Middelburg *et al.*, 1993; Fenchel *et al.*, 1998). The slow mineralization of nutrients in combination with the colonization of these anaerobic bacterial strains in the guts of the sea cucumbers, appears to make nutrients available for the sea cucumbers to absorb.

The positive effect of anoxic sand on captive warty sea cucumber growth confirms the previous finding of Robinson *et al.* (2013) and Purcell *et al.* (2016) suggesting that it mediates a nutritional benefit probably through anoxic decomposition of the waste organic food supplied. Robinson *et al.* (2015) demonstrated conclusively that *Holothuria scabra* sea cucumbers held over oxic substrate conditions showed negative growth rates whereas sea cucumbers reared over oxic-anoxic substrate conditions displayed positive growth rates. It is thus likely that *N. grammatus* require an anoxic sandy substrate for positive growth to occur in captivity.

The addition of sand also positively affected the gonadal development of the sea cucumbers, probably due to the better nutrition they received. Sea cucumbers supplied with sand in the tanks showed higher GI values than animals kept without sand suggesting that the enhanced nutrition provided by the anoxic substrate benefited their gonadal development. Hamel and Mercier (1996) found that *Cucumaria frondosa* sea cucumbers' gametogenesis increased in correlation with the increase in food availability. Similarly, Gianasi *et al.* (2017) reported that diet had a significant effect on the spawning and gonadal development of adult *C. frondosa* fed diatoms in a captive environment. Sea cucumbers are deposit feeding and are often found to have sand in their digestive tracts (Clarke, 1954; Bonham and Held, 1963; Muthiga, 2006; Muthiga *et al.,* 2007; Robinson *et al.,* 2013). These results indicate that in order to condition and breed with *N. grammatus* in an artificial environment, maintaining them over an anoxic sand substrate is a necessity.

Neither ambient temperature nor cooled (16°C) sea water had a significant effect on the gonadal development of the farm conditioned warty sea cucumbers, suggesting that the available nutrition was the major factor accounting for the observed differences between wild and farm conditioned sea cucumbers. Future research could further examine the effect of anoxic sediments on the growth and gonadal development of the sea cucumbers.

4.5 Conclusion

The addition of sand to sea cucumber tanks had a significant positive effect on the growth and GI of warty sea cucumbers while sea cucumbers reared without sand showed negative growth. Water temperature did not have any significant effect on the body weight and GI of the sea cucumbers over the two-month experimental period. It was concluded that providing a sandy substrate in Neostichopus grammatus sea cucumber tanks is necessary for feeding and conditioning broodstock for breeding and biomass growth purposes.

Chapter 5: General discussion

This study investigated the gonadal cycle of the warty sea cucumber, *Neostichopus grammatus,* with a view to breeding in captivity for IMTA purposes. The study demonstrated the possibility of conditioning wild-caught warty sea cucumbers in land-based systems if suitable environmental conditions are provided.

This baseline knowledge on the reproductive cycle of *N. grammatus* provides a foundation for further research into breeding *N. grammatus* in captivity. The natural gametogenic cycle of the warty sea cucumber was observed to follow an annual cycle with gonadal recrudescence occurring during the winter months followed by spawning from early spring through summer in both the wild and under captive farm-conditioned specimens. A range of maturity assessment techniques were applied in conjunction with gonadosomatic index values to create an array of tools that can be used to determine the sexual maturity of sea cucumbers. These techniques include macroscopic examination of the gonads, microscopic examination of tubules and gametes, and oocyte measurement. The staging of sea cucumber gonads by means of the GI index and macroscopic observations provides a simple and practical technique for monitoring gonad maturity. The results from this study can be used to predict when sexual maturity will be reached, facilitating the planning of captive spawning and rearing.

The study also provided valuable insights into sea cucumbers' environmental and nutritional requirements under farm conditions. *Neostichopus grammatus* held on the bare floor of abalone tanks readily consumed abalone faeces and pseudofaeces and came into spawning condition but gradually lost weight and condition, suggesting a nutritional deficiency. The provision of a sand sediment yielded positive growth and gonadal development, suggesting that the sand makes the abalone waste nutritionally available to the sea cucumbers, by facilitating anoxic decay of the organic matter (Middelburg *et al*., 1993; Fenchel *et al*., 1998; Robinson *et al.,* 2015).

In addition to the results for the main research objectives, valuable practical experience was gained on the inclusion of *N. grammatus* in an IMTA system with South African abalone. The research conducted shed light on where to conduct wild broodstock sampling in the Marshstrand area adjacent to the farm, how to handle the animals to minimise stress, how often to clean tanks, and how much to feed them. A successful spawning event on the farm was observed, confirming that the species will come into spawning condition in a farm environment. It is recommended that spawning trials to close the reproductive cycle are undertaken from early summer (September onwards) through to December, when most individuals have reached maturity. Future research can aim to keep and condition sea cucumbers in a farming environment under different simulated seasonal cycles of temperature and photoperiod to facilitate all-year spawning and production of juveniles. However, a dedicated hatchery and broodstock conditioning setup is necessary so as not to risk the health of abalone broodstock and juveniles. WCA built a multi-species hatchery in 2022, which could be used for sea cucumber broodstock conditioning and larval rearing. A separate settlement structure would be necessary but costly.

A statistically significant correlation between the GI values of wild and farmed sea cucumbers observed during the research trial confirmed that farm-reared sea cucumbers do come into spawning conditions and follow a predictable seasonal gonadal cycle. This finding lays a valuable foundation for future research to close the breeding cycle of *N. grammatus* in captivity. It was, however, observed that the maturation of farm-reared sea cucumbers was delayed by approximately one month. It was hypothesised that the warmer farm water temperatures or inadequate nutrition in the absence of a sand substrate could have delayed gonadal maturation.

The following controlled experiment testing the effects of temperature and substrate indicated that the temperature difference did not affect gonadal maturation and weight gain but that the presence of a sand substrate significantly enhanced growth and gonadal development. This indicates that a sand substrate plays an important role in facilitating the assimilation of organic waste nutrients, probably through the process of anaerobic breakdown. Seeing that no nutritional analysis was performed on the substrate or on the sea cucumbers, it cannot be stated that the addition of sand contributed to increased growth at a nutritional level. It is not clear whether the addition of sand caused an increase in sea cucumber biomass and GI values due to increased dietary supplementation, or if it was caused by a change in the environment, contributing to the well-being of the animals. Nutritional analyses need to be performed on the abalone waste to establish to what extent the diet had an effect on growth and GI values.

The addition of a sandy substrate presents practical challenges for the co-culture of abalone and sea cucumbers. As abalone tanks are cleaned of accumulated organic waste weekly, it is not practical to rear abalone in baskets over sandy sediment that acts as a trap for waste organic matter. Abalone require specific environmental conditions for optimal growth. Sea cucumbers kept below abalone tanks can pose health risks to abalone. The pH, dissolved oxygen, temperature, nitrite levels and salinity levels, among others, need to be monitored and controlled in a commercial farming environment (Burke *et al.,* 2001). The sediment under abalone baskets will likely contain anaerobic and aerobic bacteria, consuming oxygen and producing breakdown products such as ammonia, which may negatively affect abalone health. Furthermore, the bacterial species colonizing the anoxic sand could pose a bio-hazardous risk to abalone. Abalone tanks are cleaned regularly to reduce the risk of bacterial colonization and to keep the environmental parameters within optimal levels. The sediment in sea cucumber tanks should not be exchanged regularly to promote anoxic conditions within the sediments. Anaerobic bacteria in the sediment slowly mineralize the organic matter, providing nutrients for sea cucumbers (Middelburg *et al*., 1993; Fenchel *et al.*, 1998). If sediments are exchanged too often, anoxic bacteria will not be able to colonize the sediments, leaving sea cucumbers with little nutrients to ingest. The fortnightly exchange of sea cucumber sediments and the weekly cleaning of abalone tanks cannot occur together, proving again that placing sea cucumbers under abalone tanks is not feasible and can lead to considerable health risks for the abalone. Further research is required to determine how sea cucumbers reared on a sand substrate could be configured into an IMTA system with abalone. For example, the sea cucumbers could be reared in a system downstream of the abalone tanks and fed with the abalone solid organic waste, i.e., a “daisy-chain” system. In an encouraging study undertaken with the sea cucumber *Cucumaria frondosa,* it was found that animals supplied with effluent water from shore-based salmon holding tanks displayed good growth rates (Sun *et al.,* 2020).

In conclusion, the present research demonstrated that it is possible to keep warty sea cucumbers in a farming environment, on a diet of abalone faeces and pseudofaeces, for extended periods of time and that their gonads develop to spawning condition. This information, combined with the results of the current research, proves that it could be possible to breed *N. grammatus* sea cucumbers in a farming environment, provided its environmental needs are met. Future research should aim to assess the nutritional content of abalone waste being fed to sea cucumbers, to determine the nutritional value to the animals. Extended temperature control experiments can be conducted throughout the gonadal cycle and compared to wild sea cucumber gonadal cycle data to determine the effect of a long-term altered temperature regime on growth and gonadal development. Furthermore, a “daisy-chain” abalone/sea cucumber tank setup can be investigated to determine if an IMTA system can be incorporated in such a way that abalone are not faced with health risks, and sea cucumbers can be supplied with a necessary sandy substrate.

List of Appendices

Appendix A: Stages of gonadal development in wild male, female and indeterminate warty sea cucumbers, indicating average gonadal and body weights and GI values.

		Body weight			Gonad weight			Gonadosomatic Index		
Stage	Value	Female	Male	All	Female	Male	All	Female	Male	All
I	Ave	N/A	N/A	9,26	N/A	N/A	0,01	N/A	N/A	0,14
	SD	N/A	N/A	2,86	N/A	N/A	0,03	N/A	N/A	0,43
	Count	N/A	N/A	14	N/A	N/A	14	N/A	N/A	14
	SE	N/A	N/A	0,76	N/A	N/A	0,01	N/A	N/A	0,12
II	Ave	12,29	13,07	12,67	1,76	1,58	1,67	12,84	11,23	12,06
	SD	5,09	7,43	6,35	1,80	1,77	1,79	9,51	7,91	8,81
	Count	89	84	173	89	84	173	84	86	173
	SE	0,54	0,81	0,48	0,19	0,19	0,14	1,04	0,85	0,67
III	Ave	10,67	10,16	10,52	3,35	3,05	3,27	32,02	29,44	31,26
	SD	5,68	3,08	5,07	1,74	1,18	1,61	6,23	4,73	5,95
	Count	17	7	24	17	7	24	17	7	24
	SE	1,38	1,16	1,03	0,42	0,45	0,33	1,51	1,79	1,21
IV	Ave	11,77	10,96	11,31	1,55	1,50	1,52	12,30	13,22	12,83
	SD	4,04	4,18	4,14	0,96	0,94	0,95	4,08	5,78	5,14
	Count	29	39	68	29	39	68	29	39	68
	SE	0,75	0,67	0,50	0,18	0,15	0,11	0,76	0,93	0,62
V	Ave	10,47	8,60	9,64	0,50	0,21	0,37	4,79	3,24	4,10
	SD	2,72	2,60	2,83	0,42	0,30	0,40	4,27	5,44	4,89
	Count	10	8	18	10	8	18	10	8	18
	SE	0,86	0,92	0,67	0,13	0,10	0,09	1,35	1,92	1,15

Appendix B: Orient beach average sea water temperature over the course of the 16-month study period, including standard deviation, standard error and count.

Month	Average (°C)	SD	SE	Days count
August	17,1	0,79	0,14	31
Sep	17,0	0,94	0,17	30
Oct	17,4	1,10	0,20	31
Nov	18,4	0,89	0,16	30
Dec	18,4	0,71	0,13	31
Jan	19,6	1,31	0,23	31
Feb	19,6	1,51	0,29	28
Mar	19,6	1,52	0,27	31
Apr	17,7	1,42	0,26	30
May	17,9	1,14	0,20	31

Jun	17,3	0,99	0,18	30
Jul	16,6	0,68	0,12	31
Aug	16,4	0,50	0,09	31
Sep	16,7	1,08	0,20	30
Oct	16,9	1,10	0,20	31
Nov	17,4	1,59	0,29	30

Appendix C: Stages of gonadal development in farmed male, female, and indeterminate warty sea cucumbers, indicating average gonadal and body weights, and GI values.

		Body weight			Gonad weight			GI		
Stage	Value	Female	Male	All	Female	Male	All	Female	Male	All
I	Ave	N/A	N/A	4,68	N/A	N/A	0,04	N/A	N/A	0,13
	SD	N/A	N/A	2,38	N/A	N/A	0,05	N/A	N/A	0,39
	Count	N/A	N/A	27	N/A	N/A	27	N/A	N/A	27
	SE	N/A	N/A	0.46	N/A	N/A	0.01	N/A	N/A	0.07
II	Ave	9,02	12,32	10,30	0,89	1,47	1.76	7,61	10,06	8,57
	SD	3,51	5,61	4,73	1,30	1,16	1.25	8,23	5,65	7,43
	Count	33	21	54	33	21	54	33	21	54
	SE	0,61	1,22	0,64	0,23	0,25	0,21	1,43	1,23	1,01
III	Ave	11,48	6,85	10,71	3,72	2,26	3,47	33,37	33,06	33,32
	SD	4,21	0,00	4,21	1,01	0,00	1,07	5,15	0,00	4,71
	Count	5	1	6	5	1	6	5	1	6
	SE	1,88	0,00	1,72	0,45	0,00	0,44	2,31	0,00	1,92
IV	Ave	11,66	8,41	10,68	1,85	1,65	1,79	15,06	19,31	16,33
	SD	4,94	1,01	4,43	1,64	0,54	1,40	8,41	4,51	7,71
	Count	7	3	10	7	3	10	7	3	10
	SE	1,87	0,58	1,40	0,62	0,31	0,44	3,18	2,61	2,44
V	Ave	7,62	8,14	7,97	0,20	0,20	0,20	2,52	2,24	2,33
	SD	1,93	3,24	2,89	0,17	0,20	0,19	1,90	1,94	1,93
	Count	10	21	31	10	21	31	10	21	31
	SE	0,61	0,71	0,52	0,05	0,04	0,03	0,60	0,42	0,35

Appendix D: Initial and final body weight measurements (g) over the two-month sampling period. All body weights listed are average weights compiled for each tank (n=76).

Substrate	Temperature (°C)	Initial BW (g)	Average (g)	SD	SE	Final BW (g)	Average (g)	SD	SE	BW difference (g)
Sand	Ambient	8,592	8,536	0,101	0,058	9,592	10,342	0,592	0,342	1,000
		8,622				11,038				2,416
		8,393				10,395				2,002
	Cooled	8,442	9.277	0.591	0.341	9,66	9.747	0.791	0.457	1,218
		9,698				10,756				1,058
		9,692				8,825				-0,867
No sand	Ambient	8,585	8.931	0.665	0,384	8,855	8.356	0.441	0,255	0,270
		8,347				8,432				0,085
		9,862				7,782				-2,080
	Cooled	8,057	7.314	0.535	0.309	6,527	5.656	0.635	0.366	-1,530
		7,064				5,033				-2,031
		6,820				5,409				-1,411

Appendix E: Average Gonadosomatic Index values of farmed sea cucumbers from all four treatment groups. Listed average GI values are average value per tank (n=76).

Substrate	Temperature (°C)	GI	Average	SD	SE
Sand	Ambient	5,851	3.358	1,774	0,724
		1,872			
		2,350			
	Cooled	3,220	2.619	0.567	0.328
		1,858			
		2,778			
No sand	Ambient	1,418	1,245	0,123	0,05
		1,178			
		1,140			
	Cooled	2,224	1.472	0.664	0.383
		1,583			
		0,609			

References

Adeleke, B., Robertson-Andersson, D., Moodley, G. and Taylor, S., 2020. Aquaculture in Africa: A comparative review of Egypt, Nigeria, and Uganda vis-a-vis South Africa. *Reviews in Fisheries Science & Aquaculture*, *29*(2): pp.167-197.

Agudelo-Martínez V.Y. and Rodríguez-Forero A. 2015. Advances on spontaneous captive breeding and culture conditions of Caribbean sea cucumber *Stichopus* sp. SPC Bêche-de-Mer Information Bulletin 35: pp.50–57.

Agudo, N., 2006. Sandfish hatchery techniques. Canberra, Australia: ACIAR. ISBN 978-982-00-0175-6.

Al-Haj, N.A., Norfarrah, M.A., Shamsudin, M.N., Yusoff, F.M. and Arshad, A., 2009. Novel antibacterial activity of peptide gene extracted from Malaysian sea cucumber. *Research Journal of Biological Sciences*, *4*(4): pp.482-486.

Amosu, A.O., Robertson-Andersson, D., Maneveldt, G., Anderson, R.J. and Bolton, J.J., 2013. South African seaweed aquaculture: a sustainable development example for other African coastal countries.

Anderson, S.C., Flemming, J.M., Watson, R. and Lotze, H.K., 2011^{b}. Serial exploitation of global sea cucumber fisheries. *Fish and Fisheries*, *12*(3): pp.317-339.

Anderson, S.C., Mills Flemming, J., Watson, R. and Lotze, H.K., 2011^{a}. Rapid global expansion of invertebrate fisheries: trends, drivers, and ecosystem effects. *PLOS one*, *6*(3): e14735. https://doi.org/10.1371%2Fjournal.pone.0014735

Banhazi, T. and Rutley, D., 2013. Factors influencing water temperature on farms and the effect of warm drinking water on pig growth. In *Livestock housing: Modern management to ensure optimal health and welfare of farm animals* (pp. 103-122). Wageningen Academic Publishers.

Barnes, R.D., 1987. *Invertebrate zoology* (5). United Kingdom: WB Saunders company.

Battaglene, S.C., Seymour, J.E. and Ramofafia, C., 1999. Survival and growth of cultured juvenile sea cucumbers, *Holothuria scabra*. *Aquaculture*, *178*(3-4): pp.293-322.

Battaglene, S.C., Seymour, J.E., Ramofafia, C. and Lane, I., 2002. Spawning induction of three tropical sea cucumbers, *Holothuria scabra, H. fuscogilva* and *Actinopyga mauritiana*. *Aquaculture*, *207*(1-2): pp.29-47.

Bell, J.D., Rothlisberg, P.C., Munro, J.L., Loneragan, N.R., Nash, W.J., Ward, R.D., Andrew, N.L., 2005. Advances in Marine Biology volume 49: Restocking and stock enhancement of marine invertebrate fisheries. London: Elsevier Academic Press.

Bogatyrenko, E.A. and Buzoleva, L.S., 2016. Characterization of the gut bacterial community of the Japanese sea cucumber *Apostichopus japonicus*. *Microbiology*, *85*: pp.116-123.

Bolton, J.J., Davies-Coleman, M.T. and Coyne, V.E., 2013. Innovative processes and products involving marine organisms in South Africa. *African Journal of Marine Science*, *35*(3): pp. 449-464.

Bolton, J.J., Robertson-Andersson, D.C., Shuuluka, D and Kandjengo, L. 2008. Growing *Ulva* (Chlorophyta) in integrated systems as a commercial crop for abalone feed in South Africa: a SWOT analysis. *Journal of Applied Phycology 21*(5): pp 575-583.

Bonham, K. and Held, E.E., 1963. Ecological observations on the sea cucumbers *Holothuria atra* and *H. leucospilota* at Rongelap Atoll, Marshall Islands. *Pacific Science, 17*(3): pp.305-314.

Boolootian, R.A., 1966. Physiology of Echinodermata. New York: Interscience Publishers.

Britz, P.J., Venter, S. 2016. Aquaculture Review: South Africa. *World Aquaculture,* 47(4): pp.19-28.

Burke, C.M., Harris, J.O., Hindrum, S.M., Edwards, S.J. and Maguire, G.B., 2001. Environmental requirements of abalone. *FRDC Project*, (97/323School).

Cameron, J.L. and Fankboner, P.V., 1986. Reproductive biology of the commercial sea cucumber *Parastichopus californicus* (Stimpson) (Echinodermata: Holothuroidea). I. Reproductive periodicity and spawning behaviour. *Canadian Journal of Zoology*, *64*(1): pp.168-175.

Canene-Adams, K., 2013. Preparation of formalin-fixed paraffin-embedded tissue for immunohistochemistry. *Methods in enzymology,* 533(1): pp. 225-233.

Cayré, P. and Laloë, F., 1986. Review of the gonad index (GI) and an introduction to the concept of its "critical value": application to the skipjack tuna *Katsuwonus pelami*s in the Atlantic Ocean. *Marine Biology*, *90*(3): pp.345-351.

Chao, S.M., Chen, C.P. and Alexander, P.S., 1995. Reproductive cycles of tropical sea cucumbers (Echinodermata: Holothuroidea) in southern Taiwan. *Marine Biology*, *122*: pp.289-295.

Chen, J., 2004. Present status and prospects of sea cucumber industry in China. In: Lovatelli, A., Conand, C., Purcell, S., Uthicke, S., Hamel, J.-F. and Mercier, A., 2004. Advances in Sea Cucumber Aquaculture and Management. Rome: FAO, Fisheries Technical Paper, pp. 25-38.

Christophersen, G., Bjørkevoll, I., Bakke, S. and Kjerstad, M., 2020. Reproductive cycle of the red sea cucumber, *Parastichopus tremulus* (Gunnerus, 1767), from western Norway. *Marine Biology Research*, *16*(6): pp.423-430.

Ciriminna, L., Signa, G., Cilluffo, G., Rakaj, A. and Vizzini, S. 2024. Aquaculture of emerging species in North-Eastern Atlantic and Mediterranean Sea: a systematic review on sea cucumber farming and potential development. *Frontiers in Marine Science*, *11*. https://doi.org/10.3389/fmars.2024.1381836

Clarke, G.L., 1954. *Elements of ecology*. New York: John Wiley and Sons.

Conand, C. 1981. Sexual reproduction of three commercially important holothurian species (Echinodermata) from the lagoon of New Caledonia. *Bulletin of marine science,* 31: pp.523-543.

Conand, C. 2015. About lesser known beche-de-mer markets. *S.P.C. Bêche-de-mer Information Bulletin, 35*: pp.58-59.

Conand, C. and Byrne, M., 1993. A Review of Recent Developments in the world sea cucumber fisheries. *Marine fisheries review*, *55*(4).

Conand, C., 2017. Expansion of global sea cucumber fisheries buoys exports. *Revista de Biología Tropical*, *65*(1): pp. 1-10.

Conand, C., Shea, S. and To, A., 2014. Beche-de-mer trade statistics for Hong Kong in 2012. *S.P.C. Bêche-de-mer Information Bulletin, 34*: pp.43-46.

Cook, P. A. 2023. Worldwide abalone production: an update. *New Zealand Journal of Marine and Freshwater Research*, pp. 1–7. doi: 10.1080/00288330.2023.2261869.

Cook, P.A., 2019. Worldwide abalone production statistics. *Journal of Shellfish Research*, *38*(2): pp.401-404.

Correia, M., Azevedo, I.C., Peres, H., Magalhães, R., Oliva-Teles, A., Almeida, C.M.R. and Guimarães, L., 2020. Integrated multi-trophic aquaculture: a laboratory and hands-on experimental activity to promote environmental sustainability awareness and value of aquaculture products. *Frontiers in Marine Science*, *7*: 10.3389/fmars.2020.00156.

Costello, C., Cao, L., Gelcich, S., Cisneros-Mata, M.Á., Free, C.M., Froehlich, H.E., Golden, C.D., Ishimura, G., Maier, J., Macadam-Somer, I. and Mangin, T., 2020. The future of food from the sea. *Nature*, *588*: pp.95-100.

Costelloe, J., 1985. The annual reproductive cycle of the holothurian *Aslia lefevrei* (Dendrochirota: Echinodermata). *Marine Biology*, *88*: pp.155-165.

DAFF, 2018. Abalone Feasibility Study: Final Report. Department of Agriculture, Forestry and Fisheries, Vlaeberg.

Dissanayake, D.C.T. and Stefansson, G., 2010. Reproductive biology of the commercial sea cucumber *Holothuria atra* (Holothuroidea: Aspidochirotida) in the north-western coastal waters of Sri Lanka. *Invertebrate reproduction & development*, *54*(2): pp.65-76.

Dong, Y., Dong, S., Tian, X., Wang, F., and Zhang, M. 2006. Effects of diel temperature fluctuations on growth, oxygen consumption and proximate body composition in the sea cucumber *Apostichopus japonicus* Selenka. *Aquaculture* 255, 514–521. doi: 10.1016/j.aquaculture.2005.12.013

Duy, N.D.Q., 2012. Large-scale sandfish production from pond culture in Vietnam. In: Hair, C.A., Pickering, T.D. and Mills, D.J., 2011. Asia-pacific Tropical Sea Cucumber Aquaculture: Proceedings of an International Symposium Held in Noumea, New Caledonia, 15–17 February 2011. *Australian Centre for International Agricultural Research (ACIAR)*, pp. 34–39.

Eeckhaut, I., Parmentier, E., Becker, P., Gomez da Silva, S. and Jangoux, M., 2004. Parasites and biotic diseases in field and cultivated sea cucumbers. *Advanced Sea Cucumber Aquaculture Management, 463.*

Eriksson, H. and Byrne, M., 2015. The sea cucumber fishery in Australia's Great Barrier Reef Marine Park follows global patterns of serial exploitation. *Fish and Fisheries*, *16*(2): pp.329-341.

FAO, 2010. The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department, Rome. ISBN 978-92-5-106675-1.

FAO, 2019. Globefish highlights, no. 4, Food and Agriculture Organization of the United Nations, January to June 2019 Statistics, Rome. ISBN 978-92-5-132091-4.

FAO. (2022). *The state of world fisheries and aquaculture 2016 (SOFIA): Towards blue transformation*. Food and Agriculture Organization.

Fenchel, T. and King, G., 1998. Bacterial Biogeochemistry: The Ecophysiology of Mineral Cycling. San Diego: Academic Press. ISBN 0-12-103455-0.

Ferdouse, F., 2004. World markets and trade flows of sea cucumber/beche-de-mer. In: Lovatelli, A., Conand, C., Purcell, S., Uthicke, S., Hamel, J.F. and Mercier, A. (eds). Advances in sea cucumber aquaculture and management. FAO Fisheries Technical Paper No. 463. Rome, FAO.

Fish, J.D., 1967. The Biology of *Cucumaria elongata* (Echinodermata: Holothuroidea). *Journal of the Marine Biological Association of the United Kingdom, 47*(1): pp.129-144.

Foster, G.G. 1994. Aspects of the biology and ecology of some intertidal Holothurians (Echinodermata) along the Eastern Cape coast of South Africa. Master's thesis: Rhodes University.

Foster, G.G. and Hodgson, A.N., 1995. Annual reproductive cycles of three sympatric species of intertidal holothurians (Echinodermata) from the coast of the Eastern Cape Province of South Africa. *Invertebrate reproduction & development*, *27*(1): pp.49-59.

Francour, P., 1997. Predation on holothurians: a literature review. *Invertebrate Biology*, pp.52-60.

Frankic, A. and Hershner, C. 2003. Sustainable aquaculture: developing the promise of aquaculture. *Aquaculture International,* 11(1): pp. 517–530.

Friedman, K., Eriksson, H., Tardy, E. and Pakoa, K., 2011. Management of sea cucumber stocks: patterns of vulnerability and recovery of sea cucumber stocks impacted by fishing. *Fish and Fisheries*, *12*(1): pp.75-93.

Gaudron, S.M., Kohler, S.A. and Conand, C., 2008. Reproduction of the sea cucumber *Holothuria leucospilota* in the Western Indian Ocean: biological and ecological aspects. *Invertebrate Reproduction & Development*, *51*(1): pp.19-31.

Ghobadyan, F., Morovvati, H., Ghazvineh, L. and Tavassolpour, E., 2012. An investigation of the macroscopic and microscopic characteristics of gonadal tubules in the sea cucumber *Holothuria leucospilota* (Persian Gulf, Iran). *S.P.C Bêche-de-mer Information Bulletin*, *32*: pp.6-14.

Gianasi, B.L., Parrish, C.C., Hamel, J.F. and Mercier, A., 2017. Influence of diet on growth, reproduction and lipid and fatty acid composition in the sea cucumber *Cucumaria frondosa*. *Aquaculture research*, *48*(7): pp.3413-3432.

Giese, A.C. and Pearse, J., 1974. Reproduction of Marine Invertebrates, Volume I: Acoelomate and pseudocoelomate metazoans (Eds) New York: Academic Press.

Giese, A.C., 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. *Annual review of physiology*, *21*(1): pp.547-576.

Grahame, J. and Branch, G.M., 1985. Reproductive patterns of marine invertebrates. *Oceanography and Marine Biology: Annual Review* (23): pp.373-398.

Granada, L., Sousa, N., Lopes, S. and Lemos, M.F., 2016. Is integrated multitrophic aquaculture the solution to the sectors' major challenges? –a review. *Reviews in Aquaculture*, *8*(3): pp.283-300.

Grosso, L., Rakaj, A., Fianchini, A., Morroni, L., Cataudella, S. and Scardi, M., 2021. Integrated multi-trophic aquaculture (IMTA) system combining the sea urchin *Paracentrotus lividus*, as primary species, and the sea cucumber *Holothuria tubulosa* as extractive species. *Aquaculture*, *534*: 736268. https://doi.org/10.1016/j.aquaculture.2020.736268

Hair, C.A., 2012. Sandfish (*Holothuria scabra*) production and sea-ranching trial in Fiji. In: Hair, C.A., Pickering, T.D., Mills, D.J. (Eds.), Asia-pacific Tropical Sea Cucumber Aquaculture: Proceedings of an International Symposium Held in Noumea, New Caledonia, 15–17 February 2011. Australian Centre for International Agricultural Research (ACIAR), pp. 129–141.

Hamel, J.F. and Mercier, A., 1996. Studies on the reproductive biology of the Atlantic sea cucumber *Cucumaria frondosa*. *SPC bêche-de-mer Information Bulletin*, *8*: pp.22-33.

Hamel, J.F., Conand, C., Pawson, D.L. and Mercier, A., 2001. The sea cucumber *Holothuria scabra* (Holothuroidea: Echinodermata): its biology and exploitation as bêche-de-mer. *Advances in Marine Biology, 41:* pp.131-293.

Hamel, J.F., Himmelman, J.H. and Dufresne, L., 1993. Gametogenesis and spawning of the sea cucumber *Psolus fabricii* (Duben and Koren). *The Biological Bulletin*, *184*(2): pp.125-143.

Han, Q., Keesing, J.K. and Liu, D., 2016. A review of sea cucumber aquaculture, ranching, and stock enhancement in China. *Reviews in Fisheries Science & Aquaculture*, *24*(4): pp.326-341.

Hasan, M.H., 2019. Destruction of sea cucumber populations due to overfishing at Abu Ghosoun area, Red Sea. *The Journal of Basic and Applied Zoology*, *80*: pp.1-10.

Herrero-Pérezrul M.D., Bonilla H.R., García-Domínguez F. and Cintra-Buenrostro C.E. 1999. Reproduction and growth of *Isostichopus fuscus* (Echinodermata: Holothuroidea) in the southern Gulf of California, Mexico. *Marine Biology, 135*(3): pp.521–532.

Hopper, D.R., Hunter, C.L. and Richmond, R.H., 1998. Sexual reproduction of the tropical sea cucumber, *Actinopyga mauritiana* (Echinodermata: Holothuroidea), in Guam. *Bulletin of Marine Science*, *63*(1): pp.1-9.

Huo, D., Su, F., Cui, W., Liu, S., Zhang, L., Yang, H. and Sun, L., 2022. Heat stress and evisceration caused lipid metabolism and neural transduction changes in sea cucumber: Evidence from metabolomics. *Marine Pollution Bulletin*, *182*: 113993. https://doi.org/10.1016/j.marpolbul.2022.113993

Ito, S. and Kitamura, H., 1997. Induction of larval metamorphosis in the sea cucumber *Stichopus japonicus* by periphytic diatoms. *Hydrobiologia*, *358*(1): pp.281-284.

Ivy, G. and Giraspy, D.A.B., 2006. Development of large-scale hatchery production techniques for the commercially important sea cucumber *Holothuria scabra versicolor* (Conand, 1986) in Queensland, Australia. *Bêche-de-Mer information bulletin*, *24*: pp.28-34.

Jangoux, M. and Kinne, O., 1990. Diseases of Echinodermata. In: Kinne, O. (Ed.), 1990. Diseases of marine animals, Vol.3. Hamburg: Biologische Anstalt Helgoland. ISBN 3980081818.

Juinio-Meñez, M.A., Paña, M.A.S., Peralta, G.M.D., Catbagan, T.O., Olavides, R.D.D., Edullantes, C.M.A. and Rodriguez, B.D.D., 2012. Establishment and management of communal sandfish (*Holothuria scabra*) sea ranching in the Philippines. In: Hair, C.A., Pickering, T.D., Mills, D.J. (Eds.), Asia-pacific Tropical Sea Cucumber Aquaculture: Proceedings of an International Symposium Held in Noumea, New Caledonia, 15–17 February 2011. Australian Centre for International Agricultural Research (ACIAR), pp. 121–128.

Kang, K.H., Kwon, J.Y. and Kim, Y.M., 2003. A beneficial coculture: charm abalone *Haliotis discus hannai* and sea cucumber Stichopus japonicus. *Aquaculture*, *216*(1-4): pp.87-93.

Kemp, J.O.G., Britz, P.J. and Agüero, P.T., 2015. The effect of macroalgal, formulated and combination diets on growth, survival and feed utilisation in the red abalone *Haliotis rufescens*. *Aquaculture*, *448*: pp.306-314.

Kim, Y.D., Park, M.S., Min, B.H., Jeong, S.J., Kim, H.C., Yoo, H.I., Lee, W.C. and Choi, J.S., 2014. Study on growth characteristics of *Sargassum fulvellum* in the integrated multi-trophic aquaculture (IMTA) system. *Journal of Environmental Science International*, *23*(10): pp.1703-1718.

Kristensen, E., 2001. Impact of polychaetes (*Nereis* spp. and *Arenicola marina*) on carbon biogeochemistry in coastal marine sediments. *Geochemical Transactions*, *2*(12): pp.92-103.

Largo, D.B., Diola, A.G. and Marababol, M.S., 2016. Development of an integrated multi-trophic aquaculture (IMTA) system for tropical marine species in southern Cebu, Central Philippines. *Aquaculture Reports*, 3: pp. 67-76.

Lohrer, A.M., Thrush, S.F. and Gibbs, M.M., 2004. Bioturbators enhance ecosystem function through complex biogeochemical interactions. *Nature*, *431*: pp.1092-1095. https://doi.org/10.1038/nature03042

Lucas, J.S., Southgate, P.C. and Tucker, C.S., 2019. *Aquaculture: Farming Aquatic Animals and Plants*. 3rd ed. New Jersey: Wiley Blackwell. ISBN 13 9781119230861.

Lutz, C.G., 2003. Principles of polyculture. *Aquaculture Magazine Arkansas*, *29*(2): pp.34-39.

MacTavish, T., Stenton-Dozey, J., Vopel, K. and Savage, C., 2012. Deposit-feeding sea cucumbers enhance mineralization and nutrient cycling in organically enriched coastal sediments. *PloS one*, *7*(11): e50031. https://doi.org/10.1371/journal.pone.0050031

Maxwell, K.H., Gardner, J.P. and Heath, P.L., 2009. The effect of diet on the energy budget of the brown sea cucumber, *Stichopus mollis* (Hutton). *Journal of the World Aquaculture Society*, *40*(2): pp.157-170.

McClanahan, T.R., 1988. Seasonality in East Africa's coastal waters. *Marine ecology progress series, 44*(2): pp.191-199.

McEuen, F.S., 1988. Spawning behaviours of the northeast Pacific sea cucumbers (Holothuroidea: Echinodermata). *Marine Biology*, (98): pp.565-585.

McNevin, A.A. 2020. The path to sustainability for the increasingly important aquaculture-based source of global food production. *In* Boyd, C.E., D'Abramo, L.R., Glencross, B.D., Huyben, D.C., Juarez, L.M., Lockwood, G.S., McNevin, A.A., Tacon, A.G., Teletchea, F., Tomasso Jr, J.R. and Tucker, C.S., 2020. Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. *Journal of the World Aquaculture Society*, *51*(3): pp.578-633.

Mercier, A. and Hamel, J.F., 2013. Sea cucumber aquaculture: hatchery production, juvenile growth and industry challenges. In *Advances in aquaculture hatchery technology*. Woodhead Publishing, pp.431-454.

Mercier, A., Battaglene, S.C. and Hamel, J.F., 1999. Daily burrowing cycle and feeding activity of juvenile sea cucumbers *Holothuria scabra* in response to environmental factors. *Journal of Experimental Marine Biology and Ecology*, *239*(1): pp.125-156.

Mgaya, Y. and Mmbaga, T., 2007. Sea cucumbers in Tanzania. In: Conand, C. and Muthiga, N., 2007. Commercial sea cucumbers: a review for the Western Indian Ocean. *WIOMSA Book Series*, (5): pp.51-56.

Middelburg, J.J., Vlug, T., Jaco, F. and Van der Nat, W.A., 1993. Organic matter mineralization in marine systems. *Global and Planetary change*, *8*(1-2): pp.47-58.

Milstein, A., 1992. Ecological aspects of fish species interactions in polyculture ponds. *Hydrobiologia*, *231*(3): pp.177-186.

Morgan, A.D. 2000. Aspects of the reproductive cycle of the sea cucumber *Holothuria scabra* (Echinodermata: Holothuroidea). *Bulletin of Marine Science, 66(1):* pp.47-57.

Morgan, A.D., 2009a. Assessment of egg and larval quality during hatchery production of the temperate sea cucumber, *Australostichopus mollis* (Levin). *Journal of the World Aquaculture Society*, *40*(5): pp.629-642.

Morgan, A.D., 2009b. Spawning of the temperate sea cucumber, *Australostichopus mollis* (Levin). *Journal of the World Aquaculture Society*, *40*(3): pp.363-373.

Msangi, S and Batka, M., 2014. The rise of aquaculture: The role of fish in global food security. Chapter 8. In*:* 2014-2015 Global Food Policy Report (IFPRI), pp.61-72.

Muthiga, N.A. and Conand, C., 2013. Sea cucumbers, a Poorly Understood but Important Coastal Resource: A Regional Analysis to Improve Management. *WIOMSA Book Series,* (13).

Muthiga, N.A. and Kawaka, J., 2008. The effects of temperature and light on the gametogenesis and spawning of four sea urchin and one sea cucumber species on coral reefs in Kenya. In: Proceedings of the 11th international coral reef symposium, Florida: pp.356-360.

Muthiga, N.A. and Kawaka, J.A., 2009. The breeding pattern and variations in timing and reproductive output of the commercial sea cucumber *Holothuria fuscogilva* in Kenya. *Western Indian Ocean Journal of Marine Science*, *8*(2). 10.4314/wiojms.v8i2.56978.

Muthiga, N.A., 2006. The reproductive biology of a new species of sea cucumber, *Holothuria (Mertensiothuria) arenacava* in a Kenyan marine protected area: the possible role of light and temperature on gametogenesis and spawning. *Marine Biology*, *149*: pp.585-593.

Muthiga, N.A., Ochiewo, J., and Kawaka, J. 2007. Sea cucumbers in Kenya. In: Conand, C., Muthiga, N.A. (Eds), 2007. Commercial Sea cucumbers: a review for the Western Indian Ocean. *WIOMSA Book Series*, (5): pp.8-20.

Navarro, P.G., Garcia-Sanz, S. and Tuya, F. 2012. Reproductive biology of the sea cucumber *Holothuria sanctori* (Echinodermata: Holothuroidea). *Scientia Marina*, *76*(4), pp.741-752.

Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigel, M. and Yarish, C., 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture*, *231*(1-4): pp.361-391.

Olive, P.J., Porter, J.S., Sandeman, N.J., Wright, N.H. and Bentley, M.G., 1997. Variable spawning success of *Nephtys hombergi* (Annelida: Polychaeta) in response to environmental variation: a life history homeostasis? *Journal of Experimental Marine Biology and Ecology*, *215*(2): pp.247-268.

Ong Che, R.G., 1990. Reproductive cycle of *Holothuria leucospilota* Brandt (Echinodermata: Holothuroidea) in Hong Kong and the role of body tissues in reproduction. *Asian Marine Biology*, *7*: pp.115-132.

Onomu, A.J., Slater, M.J. and Vine, N.G., 2023. Feeding indicators and bioremediation ability of warty sea cucumber *Neostichopus grammatus* fed potential wastes from abalone *Haliotis midae* farming. *Aquaculture Environment Interactions*, *15*: pp.45-57.

Pasquini, V., Porcu, C., Marongiu, M.F., Follesa, M.C., Giglioli, A.A. and Addis, P. 2022. New insights upon the reproductive biology of the sea cucumber *Holothuria tubulosa* (Echinodermata, holothuroidea) in the Mediterranean: Implications for management and domestication. *Frontiers in Marine Science*, *9*, p.1029147.

Pearse, J.S. and Cameron, R.A., 1991. Echinodermata Echinoidea. In: Giese, A.C. and Pearse, J.S. (Eds), 1991. Reproduction of Marine Invertebrates, Vol. 6. pp. 514-663. ISBN 094016809X.

Pennington, J.T., Rumrill, S.S. and Chia, F.S., 1986. Stage-specific predation upon embryos and larvae of the Pacific sand dollar, *Dendraster excentricus*, by 11 species of common zooplanktonic predators. *Bulletin of Marine Science*, *39*(2): pp.234-240.

Preston, G.L., 1993. Beche-de-mer. In: Wright, A. and Hill, L. (Eds), 1993. Nearshore marine resources of the South Pacific. Honiara: Forum Fisheries Agency Institute of Pacific Studies. pp.371-401. ISBN 9820200822.

Purcell, S.W. and Eriksson, H., 2015. Echinoderms piggybacking on sea cucumbers: benign effects on sediment turnover and movement of hosts. *Marine Biology Research*, *11*(6): pp.666-670.

Purcell, S.W., Conand, C., Uthicke, S. and Byrne, M., 2016. Ecological Roles of Exploited Sea Cucumbers. *Oceanography and Marine Biology*, (54): pp.367-386.

Purcell, S.W., Hair, C.A. and Mills, D.J., 2012. Sea cucumber culture, farming, and sea ranching in the tropics: Progress, problems and opportunities. *Aquaculture* (368): pp.68-81.

Purcell, S.W., Mercier, A., Conand, C., Hamel, J.F., Toral-Granda, M.V., Lovatelli, A. and Uthicke, S., 2013. Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. *Fish and fisheries*, *14*(1): pp.34-59.

Purwati, P. and Luong-van, J.T., 2003. Sexual reproduction in a fissiparous holothurian species, *Holothuria leucospilota* Clark 1920 (Echinodermata: Holothuroidea). *Bêche-de-Mer Information Bulletin*, (12): pp.11-14.

Qi, Z., Wang, J., Mao, Y., Liu, H., Fang, J. 2013. Feasibility of offshore co-culture of Abalone *Haliotis discus hannai* Ino, and Sea Cucumber *Apostichopus japonicus*, in a Temperate zone. *Journal of the World Aquaculture Society, 44*(4): pp.565-573.

Raison, C.M., 2009. Advances in sea cucumber aquaculture and prospects for commercial culture of *Holothuria scabra*. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. pp.3-15.

Ramofafia, C., Battaglene, S.C., Bell, J.D. and Byrne, M., 2000. Reproductive biology of the commercial sea cucumber *Holothuria fuscogilva* in the Solomon Islands. *Marine Biology*, (136): pp.1045-1056.

Ramofafia, C., Byrne, M. and Battaglene, C., 2003. Reproduction of the commercial sea cucumber *Holothuria scabra* (Echinodermata: Holothuroidea) in the Solomon Islands. *Marine Biology*, (142): pp.281-288.

Ramofafia, C., Byrne, M. and Battaglene, S., 2001. Reproductive biology of the intertidal sea cucumber *Actinopyga mauritiana* in the Solomon Islands. *Journal of the Marine Biological Association of the United Kingdom*, *81*(3): pp.523-531.

Reichenbach, N., 1999. Ecology and fishery biology of *Holothuria fuscogilva* (Echinodermata: Holothuroidea) in the Maldives, Indian Ocean. *Bulletin of marine Science*, *64*(1): pp.103-114.

Reise, K., 2002. Sediment mediated species interactions in coastal waters. *Journal of Sea Research*, *48*(2): pp.127-141.

Ren, Y., Zhang, J., Wang, Y., Chen, J., Liang, C., Li, R. and Li, Q., 2020. Non-specific immune factors differences in coelomic fluid from polian vesicle and coelom of *Apostichopus japonicus*, and their early response after evisceration. *Fish & shellfish immunology*, (98): pp.160-166.

Robertson-Andersson, D.V., Potgieter, M., Hansen, J., Bolton, J.J., Troell, M., Anderson, R.J., Halling, C. and Probyn, T., 2009. Integrated seaweed cultivation on an abalone farm in South Africa. *Journal of Applied Phycology, 20*(5): pp.579-595.

Robinson, G., Caldwell, G.S., Jones, C.L., Slater, M.J. and Stead, S.M., 2015. Redox stratification drives enhanced growth in a deposit-feeding invertebrate: implications for aquaculture bioremediation. *Aquaculture Environment Interactions*, (8): pp.1-13.

Robinson, G., Slater, M.J., Jones, C.L.W., Stead, S.M. 2013. Role of sand as substrate and dietary component for juvenile sea cucumber *Holothuria scabra*. *Aquaculture* (392): pp.23-25.

Rokop, F.J., 1974. Reproductive patterns in the deep-sea benthos. *Science*, *186*(4165): pp.743-745.

Ru, X., Zhang, L., Li, X., Liu, S. and Yang, H. 2019. Development strategies for the sea cucumber industry in China. *Journal of Oceanology and Limnology*, *37*(1), pp.300-312.

Samyn, Y. and Massin, C., 2003. The holothurian subgenus *Mertensiothuria* (Aspidochirotida: Holothuriidae) revisited. *Journal of Natural History*, *37*(20): pp.2487-2519.

Santos, R., Dias, S., Pinteus, S., Silva, J., Alves, C., Tecelao, C., Pedrosa, R. and Pombo, A., 2016. Sea cucumber *Holothuria forskali*, a new resource for aquaculture? Reproductive biology and nutraceutical approach. *Aquaculture research*, *47*(7): pp.2307-2323.

Schagerström, E., 2003. A study of Juvenile *Holothuria scabra* Jaeger (Echinodermata) on Different Types of Sediment at Bolinao, Pangasinan, Philippines. Master's Thesis, University of Kalmar.

Schneider, K., Silverman, J., Kravitz, B., Rivlin, T., Schneider-Mor, A., Barbosa, S., Byrne, M. and Caldeira, K., 2013. Inorganic carbon turnover caused by digestion of carbonate sands and metabolic activity of holothurians. *Estuarine, Coastal and Shelf Science*, (133): pp.217-223.

Schneider, K., Silverman, J., Woolsey, E., Eriksson, H., Byrne, M. and Caldeira, K., 2011. Potential influence of sea cucumbers on coral reef CaCO3 budget: A case study at One Tree Reef. *Journal of Geophysical Research: Biogeosciences*, *116*(G4). https://doi.org/10.1029/2011JG001755

Sewell, M.A. and Levitan, D.R., 1992. Fertilization success during a natural spawning of the dendrochirote sea cucumber *Cucumaria miniata*. *Bulletin of Marine Science*, *51*(2): pp.161-166.

Shiell, G.R. and Uthicke, S., 2006. Reproduction of the commercial sea cucumber *Holothuria whitmaei* (Holothuroidea: Aspidochirotida) in the Indian and Pacific Ocean regions of Australia. *Marine Biology*, (148): pp.973-986.

Slater, J. and James, P. 2023. Low trophic species in aquaculture – growth and research challenges. *Journal of the World Aquaculture Society,* 54 (1): pp.4-6.

Slater, M.J., 2006. Polyculture of the sea cucumber *Stichopus mollis* with green-lipped mussels. Doctoral dissertation, University of Auckland.

Smiley, S. and Cloney, R.A., 1985. Ovulation and the fine structure of the *Stichopus californicus* (Echinodermata: Holothuroidea) fecund ovarian tubules. *The Biological Bulletin*, *169*(2): pp.342-364.

Smiley, S., McEuen, F.S., Chaffee, C. and Krishan, S., 1991. Echinodermata: Holothuroidea. In: Giese, A.C. and Pearse, J.S. (Eds), 1991. Reproduction of Marine Invertebrates, Vol. 6. pp. 514-663. ISBN 094016809X.

Smirnov, A.V., 2012. System of the class Holothuroidea. *Paleontological Journal*, *46*(8): pp.793-832.

Solan, M., Cardinale, B.J., Downing, A.L., Engelhardt, K.A., Ruesink, J.L. and Srivastava, D.S., 2004. Extinction and ecosystem function in the marine benthos. *Science*, *306*(5699): pp.1177-1180.

Sun, J., Hamel, J.F., Gianasi, B.L., Graham, M. and Mercier, A., 2020. Growth, health and biochemical composition of the sea cucumber *Cucumaria frondosa* after multi-year holding in effluent waters of land-based salmon culture. *Aquaculture Environment Interactions*, *12*: pp.139-151.

Thandar, A.S., 1987. The southern African stichopodid holothurians, with notes on the changes in spicule composition with age in the endemic *Neostichopus grammatus* (HL Clark). *African Zoology*, *22*(4): pp.278-286.

Thandar, A.S., 2015. Biodiversity and distribution of the southern African sea cucumbers (Echinodermata: Holothuroidea). *Zootaxa*, *4058*(3): pp.341-361.

Tolon, T. 2017. Effect of salinity on growth and survival of the juvenile sea cucumbers *Holothuria tubulosa* (Gmelin 1788) and *Holothuria poli* (Delle chiaje 1923). *FRESENIUS Environmental Bulletin* 26, 163–171. doi: 10.11598/btb.2019.26.3.1041

Toral-Granda, V., Lovatelli, A. and Vasconcellos, M., 2008. Sea cucumbers. *A global review on fishery and trade. Bêche-de-mer Information Bulletin,* (27): pp.4-6.

Tyler, P.A. and Gage, J.D., 1983. The reproductive biology of *Ypsilothuria talismani* (Holothuroidea: Dendrochirota) from the NE Atlantic. *Journal of the Marine Biological Association of the United Kingdom*, *63*(3): pp.609-616.

Uthicke, S., 1999. Sediment bioturbation and impact of feeding activity of *Holothuria* (Halodeima) *atra* and *Stichopus chloronotus*, two sediment feeding holothurians, at Lizard Island, Great Barrier Reef. *Bulletin of Marine Science*, *64*(1): pp.129-141.

Wang, H., Wu, G., Ma, T., Zhao, Z., Tian, R., Yu, Y., Huang, X., Ding, P., Wang, Q. and Zhao, C. 2023. Crevice is beneficial to fitness-related behaviors of small sea cucumbers *Apostichopus japonicus* exposed to handling stress. *Aquaculture Reports*, *31*, p.101642.

Warf, B. (Ed), 2010. Encyclopaedia of Geography Vol 3: Nature and Society. Thousand Oaks, CA: SAGE Publications. ISBN 9871412956970.

Watanabe, S., Kodama, M., Zarate, J.M., Lebata-Ramos, M.J.H., Nievales, M.F.J., 2012. Ability of sandfish (*Holothuria scabra*) to utilise organic matter in black tiger shrimp ponds. In: Hair, C.A., Pickering, T.D., Mills, D.J. (Eds.), Asia-pacific Tropical Sea Cucumber Aquaculture: Proceedings of an International Symposium Held in Noumea, New Caledonia, 15–17 February 2011. Australian Centre for International Agricultural Research (ACIAR), pp. 113–120.

Whitman, R.L., Harwood, V.J., Edge, T.A., Nevers, M.B., Byappanahalli, M., Vijayavel, K., Brandão, J., Sadowsky, M.J., Alm, E.W., Crowe, A. and Ferguson, D., 2014. Microbes in beach sands: integrating environment, ecology, and public health. *Reviews in Environmental Science and Bio/Technology*, (13): pp.329-368.

Woo, S.P., 2018. Systematic Studies on Sea Cucumbers of the Family Stichopodidae (Echinodermata: Holothuroidea). Doctoral dissertation, Beihai University (北海道大学).

Xia, B., Ren, Y., Wang, J., Sun, Y. and Zhang, Z., 2017. Effects of feeding frequency and density on growth, energy budget and physiological performance of sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture*, (466): pp.26-32.

Yanagisawa, T., 1998. Aspects of the biology and culture of the sea cucumber. In: Da Silva, S. (Ed), 1998. Tropical mariculture. London: Elsevier Academic Press. ISBN 9700122108457.

Yang, H., Hamel, J.F. and Mercier, A. (Eds), 2015. The sea cucumber *Apostichopus japonicus*: history, biology and aquaculture. London: Elsevier Academic Press. ISBN 9780127999531.

Yang, H., Yuan, X., Zhou, Y., Mao, Y., Zhang, T., and Liu, Y. 2005. Effects of body size and water temperature on food consumption and growth in the sea cucumber *Apostichopus japonicus* (Selenka) with special reference to aestivation. *Aquac. Res.* 36, 1085–1092. doi: 10.1111/j.1365-2109.2005.01325.x

Zamora, L.N., Maxwell, K.H. and Jeffs, A.G., 2017. Growth of hatchery-reared juveniles of the Australasian Sea cucumber, *Australostichopus mollis*, fed with artificial and natural diets. *Journal of the World Aquaculture Society*, *48*(4): pp.643-655.

Zamora, L.N., Yuan, X., Carton, A.G. and Slater, M.J., 2016. Role of deposit-feeding sea cucumbers in integrated multitrophic aquaculture: progress, problems, potential and future challenges. *Reviews in Aquaculture* (10)1: pp.57-74.

Zhang, C., Yu, Z., Xue, Z., Li, H., Zhu, J., Wang, L. and Song, L., 2021. The temporal dynamics of bacteria in the coelomic fluid of sea cucumber *Apostichopus japonicus* after evisceration. *Invertebrate Survival Journal*, *18*(1): pp.46-55.

Zhao, Y., Wang, H., Wang, H., Pi, Y., and Chen, M. 2022. Metabolic response of the sea cucumber *Apostichopus japonicus* during the estivation-arousal cycles. *Frontiers in Marine Science*, 9. doi: 10.3389/fmars.2022.980221

Zhou, Y., Yang, H., Liu, S., Yuan, X., Mao, Y., Liu, Y., Xu, X. and Zhang, F., 2006. Feeding and growth on bivalve biodeposits by the deposit feeder *Stichopus japonicus* Selenka (Echinodermata: Holothuroidea) co-cultured in lantern nets. *Aquaculture*, *256*(1-4): pp.510-520.