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Nature of contribution by PhD candidate	Research and writing up the manuscript.
Extent of contribution by PhD candidate (%)	95%

CO-AUTHORS

Name	Nature of Contribution
Andrew Jeffs	Comment and editing of manuscript.

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CO-AUTHORS

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Andrew Jeffs	Supervision, discussing experimental design and comment on manuscript.

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Advances for the Aquaculture of the Australasian Sea Cucumber *Australostichopus mollis*

By

Leonardo Nicolás Zamora Allendes

A thesis submitted in fulfilment of the requirements for the degree of
PhD in Marine Sciences

Leigh Marine Laboratory
University of Auckland, New Zealand
January 2014

Figure 0.0. Juveniles (from 8 to 50 g wet weight) of the Australasian sea cucumber *Australostichopus mollis*.

Abstract

The Australasian sea cucumber, *Australostichopus mollis*, is a deposit-feeding echinoderm that has attracted increasing interest for commercial fishing and aquaculture in New Zealand. According to an extensive literature review most of the information available for this species deals with the ecology and biology of adults and larvae, with only a few studies on the feeding biology and ecology of juveniles. Although this existing information can be used for aquaculture purposes in this species, there is still a need to better understand how the juveniles will respond to changes of extrinsic variables. Therefore, in this thesis the response of wild juveniles to changes in food availability and temperature was evaluated in the laboratory and used as a proxy for hatchery and grow-out scenarios. The effect of food availability in terms of organic matter content (1, 4, 12 and 20% TOM) was evaluated in terms of feeding behaviour (diurnal variation of ingestion rate and digestion), feeding biology (TOM selection from the food and absorption along the digestive tract) and growth. The effect of temperature (15, 18, 21 and 24 °C) was evaluated through changes in their metabolism (oxygen consumption and ammonia excretion), feeding behaviour, growth and macronutrient utilization (lipid, protein, and carbohydrate selection from the food and absorption along the digestive tract), with the determination of an energy budget for each temperature treatment. Finally, the effect of temperature and the presence/absence of seawater during the transport of juvenile sea cucumbers were also evaluated by measuring their subsequent feeding activity and overall well-being (i.e., skin lesions, evisceration and survival) after exposure to the different transport conditions. According to the results, the juvenile sea cucumbers feed more actively during night than day without changing their digestive activity. They are able to cope with a wide variation of TOM in their diet (from 4 to 20% TOM) in order to generate similar growth rates. The juveniles were able to compensate for the lower nutrient content and absorption in the 4% TOM treatment by increasing their selective feeding behaviour and ingestion rate. However, this was not observed in the juveniles fed with the 1% TOM treatment for which their body weight declined during the course of the experiment. Temperature was also found to be an important factor for the juveniles of this species. Temperature of around 24 °C turned out to be lethal for the juveniles, and the sea cucumbers responded negatively to increasing holding temperatures from 15 °C to 21 °C, which resulted in increased metabolism, reduced food intake and energy available for growth. The metabolism data showed that the juveniles rely more on protein as an energy source than lipid and carbohydrates regardless of the temperature. In terms of macronutrient selection and absorption, the juvenile sea cucumbers select and absorb lipid

more efficiently than carbohydrate and protein at all experimental temperatures. Although selection and absorption of ingested nutrients are negatively affected by an increase of holding temperature from 15 °C to 21 °C, carbohydrate and protein remained as the dominant dietary macronutrients that are absorbed by the juveniles. Higher temperatures (i.e., above 17 °C) also proved to be negative for the successful transportation of juveniles due to an increase in their metabolism. However, lower temperatures (i.e., below 10 °C) are also detrimental for the feeding activity, increasing the appearance of skin lesions, evisceration and mortality and therefore also affect transportation success. Nonetheless, the results obtained indicate that the juveniles can be transported for short periods of time without seawater if desiccation is avoided. Transport of juveniles for longer periods of time (i.e., more than 8 hours) is possible provided they are kept with seawater and temperature is maintained within a suitable range (i.e., 12 - 15 °C). Overall, the results of the research presented in this thesis provide useful new information for the development of the aquaculture of this species, including improved methods for the transportation of sea cucumber juveniles from the hatchery to grow-out locations. Providing a better understanding of the feeding biology and growth of the juveniles and how they are affected by extrinsic factors, will help not only to improve the holding conditions of juveniles in the hatchery and nursery stage, but also help the selection of suitable aquaculture locations for grow-out, management of stocking densities, feeding regimes and the development of artificial diets.

Acknowledgements

I want to use this space in my thesis to thank all the people that directly or indirectly (even without knowing) helped me during the development of this research.

I would like to thank Dr. Andrew Jeffs for all his support and help throughout my entire research, not only for the development of my project but also during the writing process. Another important person during my early days in the lab was Dr. Matthew Slater, my predecessor on the sea cucumber research, which without his help this project wouldn't have started so well. Also, I would like to give a big thank to all the Leigh Marine Laboratory staff and students for making this time such a great one. Special thanks go to Peter Browne, Murray Birch and John Atkins for their technical support in the various holding arrangements for the sea cucumbers. Also to all the volunteer divers that endured one of the best diving spots in northern New Zealand (the Mahurangi Estuary), without the help of Matt S., Charlie B., Peter B., Brady D., Bhakti P., Pippa K., Kate H., Oliver T., Matt P., Andrew J., and Paul C., I would not have gotten my hands into the juveniles that I used for my research. When I needed sediment samples from underneath a mussel farm also got help from Jenni S., Paul C., Oliver T., Brady D., and Murray B., so thanks to them again.

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Thesis Structure and Research Aims

Overall objective

The objective of the research presented in this thesis was to evaluate important aspects of the feeding biology, growth and handling of juvenile sea cucumber *Australostichopus mollis* as they relate to their potential development for aquaculture in New Zealand. As outlined in the review of previous research on this species (Chapter One), information in the areas targeted by the research presented in this thesis is currently lacking and is required for assisting in the development of the aquaculture of this species.

Specific aims

Chapter One

A review was undertaken of previous research on the biology of *A. mollis* as it relates to development for aquaculture production as a mean of identifying knowledge gaps that could begin to be addressed in the research undertaken for this thesis. This literature review forms the basis of the Chapter One, the General Introduction to the thesis. The contents of this chapter have been published as:

Zamora, L. N., Jeffs, A. G., 2013. A review of the research on the Australasian sea cucumber *Australostichopus mollis* (Echinodermata: Holothuroidea) (Hutton, 1872) with emphasis on aquaculture. Journal of Shellfish Research 32 (3), 613-627.

Chapter Two

The feeding behaviour of juveniles of the sea cucumber *A. mollis* is poorly understood and it is not clear how they cope with natural variation of food in seafloor sediments. Therefore, the aim of the experiments conducted in Chapter Two were to evaluate the feeding behaviour, food particle selectivity, digestion, absorption and faecal production of juvenile *A. mollis* when feeding with different levels of organic matter in the food (i.e., 1, 4, 12, and 20% TOM). These results will be useful for understanding the feeding ecology of this species, and for the development of artificial feeds for aquaculture. The contents of this chapter have been published as:

Zamora, L. N., Jeffs, A. G., 2011. Feeding, selection, digestion and absorption of the organic matter from mussel waste by juveniles of the deposit-feeding sea cucumber, *Australostichopus mollis*. Aquaculture 317, 223-228.

Chapter Three

There is a lack of knowledge about how changes in the foraging behaviour and nutrient acquisition of juveniles of *A. mollis*, as examined by the research presented in Chapter Two, may in turn influence their growth. Therefore, in Chapter Three the feeding behaviour and growth of juvenile *A. mollis* was experimentally evaluated in relation to changes in the organic matter in the feed (i.e., 1, 4, 12, and 20% TOM) under simulated aquaculture conditions. These results will help to improve the understanding of how juveniles of this species respond under aquaculture conditions in which food availability changes spatio-temporally. The contents of this chapter have been published as:

Zamora, L. N., Jeffs, A. G., 2012. The ability of the deposit-feeding sea cucumber *Australostichopus mollis* to use natural variation in the biodeposits beneath mussel farms. Aquaculture 326-329, 116-122.

Chapter Four

It is unclear how seawater temperature affects the feeding behaviour, metabolism, and ultimately the growth of the sea cucumber *A. mollis*. Therefore, in Chapter Four the response of juvenile *A. mollis* to different seawater temperatures (i.e., 15, 18, 21, and 24 °C) was experimentally evaluated. The response of juvenile sea cucumbers to seawater temperatures was evaluated in terms of feeding, ammonia excretion, oxygen consumption and growth. The results of this research will assist in the selection of suitable locations for coastal aquaculture of these sea cucumbers, and begin to define the ideal operating parameters of land-based aquaculture facilities where manipulation of temperature is possible. The contents of this chapter have been published as:

Zamora, L. N., Jeffs, A. G., 2012. Feeding, metabolism and growth in response to temperature in juveniles of the Australasian sea cucumber, *Australostichopus mollis*. Aquaculture 358-359, 92-97.

Chapter Five

There is very limited information about the natural feeding biology and nutrient requirements of juvenile *A. mollis*. Therefore, in Chapter Five the specific macronutrient (i.e., lipid, protein, carbohydrate) selection and absorption in juvenile *A. mollis* were determined experimentally using mussel waste as an effective diet. In addition, the role of seawater temperature (i.e., 15, 18, and 21 °C) in influencing these processes and its consequences for energy intake and growth in

juvenile sea cucumbers were examined. This information will contribute to the initial development of more effective artificial diets for raising juvenile sea cucumbers of this species. The contents of this chapter have been accepted for publication as:

Zamora, L. N., Jeffs, A. G., (*in press*). Macronutrient selection, absorption and enerrgy Budget of juveniles of the Australasian sea cucumber, *Australostichopus mollis*, feeding on mussel biodeposits at different temperaturas. Aquaculture Nutrition, doi: 10.111/anu.12144.

Chapter Six

The transport of live juvenile sea cucumbers, especially in large numbers, between hatchery and grow out locations has proven to be problematic for many species of sea cucumber, including *A. mollis*. Therefore, the research presented in Chapter Six aimed to determine a more effective method of transporting juvenile *A. mollis* by experimentally comparing different transportation conditions (i.e., with and without seawater at different temperatures and exposure times). The success of the experimental transportation treatments were evaluated by monitoring evisceration, skin lesions, and subsequent feeding activity and survival of juvenile sea cucumbers. The results of this research will help to increase the efficiency of long and short-term transportation of juvenile sea cucumbers for aquaculture. The contents of this chapter have been accepted for publication as:

Zamora, L. N., Jeffs, A. G., (*in press*). Evaluation of transportation methods of juveniles of the Australasian sea cucumber, *Australostichopus mollis*. Aquculture Research.

Chapter Seven

Together, the information resulting from the research addressing the above aims provides greatly increased understanding of the physiology, ecology, feeding behaviour, nutrient absorption and utilization as well as growth of juvenile *A. mollis* as it relates to the development of this species for aquaculture. This information will be especially relevant for the nursery stage production of this species, and for the grow-out of juveniles either in ponds, sea ranching, or as a part of integrated multi-trophic aquaculture. The relevance of the collective results of the research presented in the thesis is discussed in Chapter Seven, the General Discussion.

Chapter One

A review of the research on the Australasian sea cucumber *Australostichopus mollis* (Echinodermata: Holothuroidea) (Hutton, 1872) with emphasis on aquaculture.

Abstract

The Australasian sea cucumber, *Australostichopus mollis*, has an extensive distribution, being found along the coast of southern Australia and throughout New Zealand's coastal waters. This species is very similar in appearance to the highly-prized Japanese sea cucumber, *Apostichopus japonicus*, and as a consequence has attracted increasing interest for commercial fishing and aquaculture. Australostichopus mollis currently supports a small commercial fishery in New Zealand of 10 - 20 t per annum. A review of the research on this sea cucumber indicates that the development of aquaculture for this species has been impeded by a general lack of background biological knowledge. Future research needs to be targeted toward resolving the constraints that the aquaculture industry is facing for this species, including reliable methods for broodstock conditioning, mass larval rearing, juvenile nutrition and husbandry, as well as development of effective grow-out technology and identification of suitab;le farming sites.

1.1. Introduction

The commercial aquaculture of sea cucumbers has begun in relatively recent times in response to a constrained supply from wild stocks, and increasing market demand, leading to a rise in prices (Toral-Granda et al., 2008). Most of the sea cucumbers harvested worldwide are exported to Asian markets, especially China, where for centuries they have been regarded as a nutritious food with health-giving properties (Choo, 2008). Sea cucumbers can be traded fresh or alive, but they are usually sold gutted and dried. The dried sea cucumber is often known as "beche-de-mer", "trepang" or "haishen". There is a huge diversity of species of sea cucumbers (66 species) that are commercially harvested and traded around the world, with the majority of these species belonging to the Order Aspidochirotida, and only a few to the Order Dendrochirotida (Purcell et

al., 2010). Most of these species come from the multi-species sea cucumber fisheries that are found throughout the tropical regions of the world (Kinch et al., 2008). In higher latitudes with temperate waters the sea cucumber fisheries tend to be monospecific (Hamel and Mercier, 2008).

The price of sea cucumbers varies greatly among species and also within species depending on the size of the animals and processing methods (Ferdouse, 2004; Purcell et al., 2012a). Therefore, large scale commercial aquaculture has only been developed for a small number of the most valuable species; the temperate Japanese sea cucumber, *Apostichopus japonicus*, and the tropical sandfish, *Holothuria scabra* (Renbo and Yuan, 2004; Xiyin et al., 2004; Huiling et al., 2004; Gamboa et al., 2012; Duy, 2012). Both species can fetch more than US$ 300 kg-1 (dried) at retail markets for large and well-processed specimens (Purcell et al., 2010). Aquaculture technology (hatchery and grow-out technology) is also being developed for several other temperate and tropical sea cucumber species around the world, such as *Isostichopus fuscus*, *Athyonidium chilensis*, *Cucumaria frondosa*, *Parastichopus californicus*, *Stichopus horrens*, *Holothuria fuscogilva*, *Actynopiga* spp. (Paltzat et al., 2008; Guisado et al., 2012; Mercier et al., 2012; Jimmy et al., 2012; Nelson et al., 2012). Another such species is the Australasian sea cucumber, *Australostichopus mollis*. This is an aspidochirote sea cucumber that is very similar in appearance to the valuable Japanese species, *A. japonicus*, and as a consequence, can be sold into the same markets at good prices.

Despite the strong commercial interest in the development of fisheries, sea ranching and aquaculture of the Australasian sea cucumber, progress has been slow because of a lack of comprehensive knowledge about the biology of this species. Although a considerable amount of research has been conducted on this sea cucumber, much of this information is widely dispersed and contained in unpublished reports and research theses coming mainly from New Zealand, with very few studies in Australia. Therefore, the aim of this review is to collate the available information on *A. mollis*, and identify those knowledge gaps creating difficulties for the commercial aquaculture development of this species.

1.2. Taxonomy

Australostichopus mollis is the most conspicuous sea cucumber species in shallow waters along the coastlines of New Zealand and southern Australia. It is commonly known as the brown sea

cucumber, due to its colouration, or as the Australasian sea cucumber, due to its distributional range. This species was first described as *Holothuria mollis* (Hutton, 1872). Subsequently, other specific names were applied such as, *Holothuria robsoni* (Hutton, 1878), Stichopus sordidus (Theel, 1886), *Holothuria victoriae* (Bell, 1887), *Stichopus mollis* (Dendy, 1896), and *Stichopus simulans* (Dendy and Hindle, 1907) with the assignment of the various names based mostly on comparisons of the morphology of ossicles in the body wall. Of all these names, *Stichopus mollis* has been most widely accepted and used for this species. Recently, this species was reclassified as *Australostichopus mollis* due to differences in morphology, including ossicles, and chemical composition, a taxonomic distinction that has subsequently been supported by analyses of genetic markers (Moraes et al., 2004; Byrne et al., 2010).

1.3. Biology and Ecology

1.3.1. Distribution and Habitat

Australostichopus mollis can be found all along the coast of New Zealand, including The Snares Islands and Chatham Rise, as well as along the southern coasts of Australia, including all of Tasmania (Fig. 1.1) (Joshua, 1914; Clark, 1922; Hickman, 1962; Pawson, 1970; Fenwick and Horning, 1980; Lawler, 1998; Cohen et al., 2000; Fromont et al., 2005; Shears and Babcock, 2007). However, there is some doubt whether the individuals found in the south-western coast of Australia correspond to *A. mollis* or not (Maria Byrne, The University of Sydney, Australia, *pers. comm.*).

Australostichopus mollis are subtidal animals that can be typically found in shallow waters of sheltered coastlines, and occasionally in intertidal pools, but they have also been recorded at depths of up to 1,000 m (Dawbin, 1949a, 1950; Pawson, 1970; Sewell, 1990). They can inhabit a wide range of substrates such as rocky reef, biogenic reefs (beds of mussels, polychaetes and bryozoans), as well as sediments of a range of grain size from gravel to mud (Dawbin, 1949a, 1950; Pawson, 1970; Probert et al., 1979; Sewell, 1990; Smith et al., 2005; Wing et al., 2008; Slater et al., 2010; Morgan, 2011). Juveniles and adults generally occupy different habitats. This pattern of distribution is possibly related to the cryptic behaviour of the juveniles which are often found under boulders in shallow waters, while adults tend to be found out in the open, and in deeper waters than juveniles (Joshua, 1914; Sewell, 1990). Occasionally juveniles and adults can

be found together in high numbers in habitats where suitable juvenile shelter is available within adult habitat, such as where large curved shell fragments from horse mussels (*Atrina zelandica*) are overlying areas of organically enriched mud substrate (Slater et al., 2010).

1.3.2. External Appearance

Australostichopus mollis is a medium-sized species of sea cucumber, reaching up to 300 g in wet weight and up to 25 cm in length (Dawbin, 1949a; Sewell, 1990). This species has the typical cylindrical shape of sea cucumbers, with the mouth located anteriorly facing the seafloor and the anus positioned at the posterior end. The dorsal surface of this species is characterized by a series of irregularly positioned papillae of different sizes and shapes (Dendy, 1896; Sewell, 1987). Whereas the ventral surface is smoother, with the presence of ambulacral podia, or tube feet, which are used for locomotion (Dendy, 1896; Sewell, 1987). The colouration of this species varies significantly among individuals; they can be either a single colour (from dark brown to white), or have a combination of different brown, yellow and cream-white tones, but usually dominated by the brown tones (Dendy, 1896; Sewell, 1987) (Fig. 1.2). Some individuals are mottled, but in most the colouration of the ventral and dorsal surfaces are different, with the colour of the ambulacral podia varying widely among individuals (Dendy, 1896; Sewell, 1987). The highly individual patterns of colouration permits the use of reliable photo-identification of individual sea cucumbers for research in this species as they are difficult to tag using conventional physical marking methods (Raj, 1998b; Stenton-Dozey, 2007b).

1.3.3. Internal Anatomy

The internal anatomy has been described as being similar to other sea cucumbers of the *Stichopus* genus, with a large and undivided coelom filled with fluid (Sewell, 1987). The coelom volume of this species can change by as much as 5% due to the exchange of seawater in the coelom, facilitated by cloacal pulsations used to ventilate the respiratory trees (Robertson, 1972). The circulation of oxygen and nutrients is achieved by the perivisceral coelomic fluid, as well as the haemal and water vascular systems which are associated with the digestive system and the major sites of oxygen uptake in the respiratory trees, body wall and podia (Fig. 1.3) (Robertson, 1972; Lawrence, 1987). Oxygen transportation is facilitated by haemoglobin in dendrochirotid and molpadiid sea cucumbers, however, the presence of any such respiratory pigment is yet to be

described in *A. mollis* (Baker and Terwilliger, 1993; Hoffmann et al., 2012). The water vascular system of this species is comprised of a circular canal as well as five radial canals, canals to the ambulacra, oral tentacles with their ampullae, a stone canal, and a madreporite (Fig. 1.3) (Sewell, 1987). This species has a single polian vesicle, an elongated digestive system (folded twice to fit inside the coelom), a pair of well-developed respiratory trees, gonads (consisting of branched tubes divided in two parts), and no cuverian organs (Fig. 1.3) (Sewell, 1987). Most of these internal organs are suspended in the coelom by mesenteries (Sewell, 1987). Under situations of extreme stress, rarely encountered in their natural environment, this species can undergo autotomy and evisceration of their digestive tract and respiratory trees (Dawbin, 1949a, 1949b; Sewell, 1990). Both small juveniles (around 8 g) and large adults (up to 300 g) are able to recover from this, and in adequate conditions it will take around 110 days to recommence feeding, and more than 145 days to regenerate the full extent of the alimentary canal, while the full recovery of the respiratory trees takes longer (Dawbin, 1949a).

1.3.4. Reproduction and early development

Australostichopus mollis is dioecious and reaches sexual maturity at > 75 g wet weight (Sewell, 1987; Raj, 1998a). They have an annual reproductive cycle, spawning synchronously during the austral summer (Sewell, 1990, 1992; Sewell and Bergquist, 1990; Archer, 1996; Raj, 1998a). Individuals from different latitudes have shown different patterns of gonad maturation, with populations in northern New Zealand completely reabsorbing their gonads after spawning, whereas individuals in southern parts of the country do not completely reabsorb the gonad, instead having a progressive gonadal maturation during winter time (Sewell and Bergquist, 1990; Sewell, 1992; Archer, 1996; Sewell et al., 1997; Raj, 1998a). *Australostichopus mollis* has a nocturnal spawning behaviour, which has been observed in the wild, with individuals reaching their anterior end up into the water column while releasing their gametes (Archer, 1996). This species has indirect development with more than one larval stage, starting with a swimming and feeding auricularia larva, which after around two weeks develops into a non-feeding doliolaria larva (Table 1.1) (Archer, 1996; Stenton-Dozey and Heath, 2009). Then the doliolaria metamorphoses into a pentactula larva which settles to the seafloor at around 25 days after fertilization, and starts deposit-feeding as a competent juvenile sea cucumber (Table 1.1) (Archer, 1996; Stenton-Dozey and Heath, 2009). There is no clear evidence whether there is any preferred substrate for settling juveniles in the wild, as newly settled individuals are very cryptic

and are rarely found (Joshua, 1914; Sewell, 1990). However, juvenile recruitment appears to be highly localized and is thought to be related to patterns of larval settlement, rather than restricted availability of suitable juvenile habitat (Slater and Jeffs, 2010).

1.3.5. Feeding Behaviour

Australostichopus mollis is a deposit-feeding sea cucumber that uses its oral tentacles to feed on the organic particles accumulated on the seafloor, by gathering the particles with shield-shaped tentacles and bringing them inside the mouth as the animal moves forward along the seafloor (Roberts et al., 2000). This species has the capability of exploiting different food sources in different habitats, by ingesting inorganic particles of a wide range of sizes and by actively selecting the associated organic material, including live microorganisms, decaying material of plant and animal origin, and the faeces of other marine organisms (Roberts et al., 2000; Wing et al., 2008; Slater et al., 2009; Slater and Jeffs, 2010; Slater et al., 2011a; Zamora and Jeffs, 2011; MacTavish et al., 2012). On sediments with relatively low levels of organic food availability (around 1%) individuals will actively seek out patches with higher organic content, however, at higher organic levels (around 3%) this behaviour is greatly diminished (Slater et al., 2011a). Most individuals of *A. mollis* are nocturnally active feeders, although feeding during day has been observed on occasions for both juveniles and adults (Slater, 2006; Zamora and Jeffs, 2011).

1.3.6. Predators, Parasites and Commensals

Predation of *A. mollis* in the wild has been poorly documented, with only a small number of predation events being reported by the asteroid *Luidia varia* (Sewell, 1990), and by the giant boarfish *Paristiopterus labiosus* (Russell, 1983). Under laboratory holding conditions, the omnivorous crab *Notomithrax ursus* has also been observed preying on *A. mollis* (Woods, 1993). *Australostichopus mollis* can serve as host for symbiotic organisms such as a group of subcuticular bacteria living between the epidermal cells and the outer cuticle (Lawrence et al., 2010), an isopod that can be found on the skin of the sea cucumber (Menzies and Miller, 1955), a platyhelminth that lives in the coelom (Cannon, 1990) and turbellarian parasites that live in their intestine (Hickman, 1955; Jondelius, 1996).

1.4. Fisheries

1.4.1. Fishing History in Australasia

There is no history of traditional or recreational fishing of *A. mollis* in New Zealand or Australia. In more recent years these sea cucumbers have been increasingly harvested by Asian immigrants at coastal locations around major urban centers in New Zealand, a localised activity which is poorly represented in recreational fishing surveys (Ministry of Fisheries, 2011). *Australostichopus mollis* is the only sea cucumber commercially fished in New Zealand, and despite being present in significant quantities in southern parts of Australia it is not commercially harvested. Inquiries with Australian state fisheries management agencies indicate they do not intend to allow the commercial fishing of this species in their waters, due to lack of commercial interest and concerns about the difficulty of managing new fisheries of this kind (Kim Evans, Department of Primary Industries, Parks, Water and Environment, Tasmania. *pers. comm.*).

Most of the commercial landings of sea cucumbers in New Zealand are a result of by-catch in other fisheries, such as scallop dredging and bottom trawling (Ministry of Fisheries, 2011). The annual commercial landings have been relatively low (under 10 t) since landing records began in 1991, when the value of this species in export markets was first recognized (Fig. 1.4) (Ministry of Fisheries, 2011). In the early 2000's there was a significant increase in total annual landings of these sea cucumbers before they were included in the quota management system (QMS) for fisheries in 2004, after which time the annual landings fluctuated (Fig. 1.4) (Ministry of Fisheries, 2011).

1.4.2. Stock Assessment and Management

There has only ever been one systematic attempt at stock assessment in south-western New Zealand, in four fiords (Thompson Sound, Bradshaw Sound, Charles Sound and Doubtful Sound) (Mladenov and Guerring, 1997; Mladenov and Campbell, 1998). This assessment resulted in an estimate of an average of 1,574 kg of sea cucumbers per kilometre of coastline within the surveyed fiords. On this basis the estimate for the total sea cucumber biomass present within in a depth range of 0 - 20 m all of Fiordland was at around 1,950 t (Mladenov and Guerring, 1997; Mladenov and Campbell, 1998). There has been only one attempt to measure

population parameters such as size at age, growth and mortality of a small population in shallow coastal waters in northern New Zealand (Morgan, 2012).

Due to the lack of abundance and distribution information of wild stocks in New Zealand, fisheries managers established conservative harvesting limits when sea cucumbers where incorporated into the QMS (Ministry of Fisheries, 2011). A total allowable commercial catch (TACC) of 35 t was established, which is comparatively small when considering the magnitude of the landings for *A. japonicus* (1,000 – 6,000 t for Japan and Republic of Korea respectively) and *Parastichopus californicus* (around 400 t for Canada, British Columbia) (Choo, 2008; Hamel and Mercier, 2008). This 35 t TACC was allocated into 15 arbitrary fishery management areas in which sea cucumbers can only be commercially harvested by free diving (Fig. 1.5) (Ministry of Fisheries, 2011). Of these fishery management areas, the north- and south-eastern New Zealand areas, together with the Cook Strait area (SSC 1B, 3 and 7A respectively) consistently have the highest reported annual landings in recent years, with typically over 2 t each (Fig. 1.5) (Ministry of Fisheries, 2011). However, there are no further harvesting controls on the commercial fisheries of this species, such as minimum legal size, fishing season or protected nursery zones (Ministry of Fisheries, 2011). There are currently no controls on the recreational harvesting of sea cucumbers. The effect of marine protected areas on sea cucumber populations cannot be assessed due to the low fishing pressure in non-protected areas, as seen in a study which included *A. mollis* in Tasmania (Barrett et al., 2009).

1.4.3. Processing and Market Value

Most of the sea cucumbers harvested in New Zealand are processed locally and exported to Asia. Usually the sea cucumbers are gutted, boiled for up to 20 min in salt water, and then air dried while covered in salt (Andrew Jeffs, University of Auckland, *pers. comm.*). The highest prices are paid for dried sea cucumbers that are larger in size, evenly dark brown in colour, and with large and erect papillae. There is considerable individual variability in terms of the final appearance of dried sea cucumbers following processing, which is addressed through careful grading. The dry weight recovery of sea cucumbers of this species is around 9% of the wet weight of the freshly landed sea cucumbers, depending on the extent of drying and the addition of salt during processing (Zamora, *unpublished data*).

This sea cucumber is considered as a medium to high value species, however, it can reach market values over US$ 275 kg^{-1} dry weight (Purcell et al., 2012a). There is the potential to add further value to this species through the extraction of bioactive compounds for manufacturing high value biopharmaceuticals or human health supplements (Kelly, 2005; Bordbar et al., 2011). Some of the bioactive compounds that have been described from *A. mollis* include long chain fatty acids, collagens, complex carbohydrates, and triterpene glycosides (Freeman and Simon, 1964; Baker, 1998; Moraes et al., 2004, 2005; Liu, 2010; Yibmantasiri et al., 2012). There is also an opportunity to develop additional high-value products from the viscera (i.e., digestive tract, respiratory trees and gonads) that is usually discarded during processing (Morgan and Archer, 1999; Morgan, 2000b).

1.5. Aquaculture Advances, Constrains and Future Directions

Interest in the aquaculture of *A. mollis* started in the early 2000's due to the high value of the species in Asian markets, and the discovery of the possibility of co-culturing with other aquaculture species in New Zealand, such as the green-lipped mussel, *Perna canaliculus*, and the native abalone, *Haliotis iris* (Morgan and Archer, 1999; Morgan, 2000a, 2000b, 2000c, 2001, 2009c; Maxwell, 2006; Stenton-Dozey, 2007a). Information useful for the development of the aquaculture of this species is scarce, especially in relation to effective hatchery technology and the biology and ecology of post settlement juveniles (i.e., nursery). Therefore, in the following sections the available information is summarized, with emphasis on identifying knowledge gaps for the aquaculture of *A. mollis*.

1.5.1. Hatchery and Nursery Technology Development

Using current hatchery and nursery culture methods for this sea cucumber it can take almost a year after larval settlement to raise small *A. mollis* juveniles with a mean weight of 0.05 g, and with only a few individuals reaching 2 - 5 g (Stenton-Dozey and Heath, 2009; Heath et al., 2014). The efficiency of production of juvenile *A. mollis* could be greatly improved through the development of more cost effective technology customized for this species (Morgan, 2002, 2004a, 2005a, 2005b, 2008b). This becomes evident when considering that the nursery production of *A. japonicus* and *H. scabra* requires only a few months after settling, and 1 or 2 years of subsequently grow-out to reach commercial sizes of around 300 g in China and Vietnam

respectively (Renbo and Yuan, 2004; Pitt and Duy, 2004). For these species the reduction in the nursery production time came after many years of research and development to establish the efficient hatchery and nursery techniques used today. This research and development has occurred since the 1950's for *A. japonicus* and since the 1990's for *H. scabra* (James et al., 1994; Renbo and Yuan, 2004; Xiyin et al., 2004; Pitt and Duy, 2004; Mills et al., 2012; Juinio-Meñez et al., 2012a; Duy, 2012). These improvements allowed the production of large numbers of juveniles in a simplified and cost effective manner, stabilizing the production and allowing the expansion of the commercial grow-out of these species. The development of broodstock management, larval rearing, nursery culture, and techniques for transporting juveniles, were key to this successful commercial aquaculture development of *A. japonicus* and *H. scabra*, hence will require marked improvement for *A. mollis* and are discussed further below.

1.5.1.1. Broodstock management

Australostichopus mollis broodstock are currently obtained from the wild during their natural spawning season (Sewell, 1990, 1992; Sewell and Bergquist, 1990; Archer, 1996; Morgan 2003). Spawning, is enhanced by collecting individuals within one week after the full moon and exposing them to seawater temperatures of around 3 - 5 °C above ambient (Archer, 1996; Morgan, 2007b, 2009a; Maxwell, 2006). This approach to broodstock management is unreliable, because not all individuals have mature gonads or respond to the spawning stimulus, affecting the number and quality of the gametes obtained (Battaglene et al., 2002; Hamel and Mercier, 2004; Eeckhaut et al., 2012). The use of wild broodstock is also a limitation in terms of introducing effective selective breeding through the careful broodstock selection from superior performing aquaculture stock. Being confined to the short natural breeding season is also inefficient in terms of the effective utilisation of hatchery and nursery infrastructure. Therefore, there is a need for a more reliable method for obtaining good quality female and male gametes throughout the year in large numbers (Morgan, 2005c). This can be achieved through conditioning captive broodstock by manipulating diet and holding conditions in order to obtain mature gonads with high quality gametes, and by developing more reliable spawning methods (Hamel and Mercier, 2004; Morgan, 2004b; Agudo, 2006; Leonet et al., 2009; Gamboa et al., 2012). An alternative to unreliable forced spawning induction is in vitro fertilization which has been tested successfully in *H. scabra*, however, its effectiveness in *A. mollis* has to be proven (Eeckhaut et al., 2012).

Retaining individual broodstock that respond well to spawning stimuli and yield large numbers of good quality gametes with the highest fertilization rates has been shown to be a rapid route for improving broodstock performance in *A. mollis* (Morgan, 2007a, 2007b, 2007c, 2009d). The development of complete control over broodstock will allow for selective breeding, the application of which has considerable potential to improve the value of this species. Selective breeding could improve traits related to production and market appeal, such as fast growth and dark body colouration with large papillae. However, it will be necessary to firstly determine the extent that these commercially important traits are genetically determined in *A. mollis*.

1.5.1.2. Larval rearing

The food requirements of the planktotrophic larvae of *A. mollis* is poorly understood, despite it being critical to effective hatchery production because larvae in poor nutritional condition fail to complete metamorphosis to post-settled juvenile (Archer, 1996; Morgan, 2007c, 2007d). Researchers have raised *A. mollis* larvae (auricularia) with mono- and mixed-cultures of microalgal species including, *Dunaliella* sp., *Phaedoctylum* sp., *Nannochloropsis oculata*, *Isochrysis* (T-iso) and *Chaetoceros muelleri* at different concentrations, with the best results obtained with a mixture of microalgae (*N. oculata*, *Isochrysis* (T-iso) and *C. muelleri*) supplied at concentrations between 3,000 and 5,000 cells ml^{-1} (Archer, 1996; Maxwell, 2006, Morgan, 2008a; Heath et al., 2014). In order to reduce costs and use cultured microalgal feeds efficiently, the feeding rate of the auricularia should be adjusted carefully as they grow by checking the presence of microalgae in the digestive tract of the larvae (Renbo and Yuan, 2004). More efficient larval production for *A. mollis* may also be possible by using one species of cultured microalgae, or commercially available algal pastes, which have been shown to be effective in the larviculture of *H. scabra* (Hair et al., 2011; Duy, 2012; Gamboa et al., 2012). Resolving these larviculture issues should be relatively straightforward, since it appears that the timing of larval development is associated with the nutritional status of the larvae, which can be indirectly evaluated by checking the readily visible hyaline spheres in the transparent larvae of *A. mollis* (Archer, 1996; Morgan, 2008a, 2009b, 2010; Stenton-Dozey and Heath, 2009; Peters-Didier and Sewell, 2012). In addition, to determine optimum larval feeding regimes, a variety of other standard larviculture husbandry parameters also need to be optimised, such as larval stocking densities, seawater exchange, temperature, oxygen, salinity and pH levels as these are poorly understood in this sea cucumber species, but have been shown to be critical in the larviculture of

other commercially important sea cucumber species (Battaglene et al., 1999; Kashenko, 2000; Renbo and Yuan, 2004; Li and Li, 2010; Duy, 2012).

Induction of larval settlement in *A. mollis* has been achieved successfully using methods that are identical to those used for *A. japonicus*. This involves the use of polycarbonate and polyethylene sheets pre-conditioned in seawater in order to develop a natural biofilm that promotes larval settlement in the sea cucumbers (Xiyin et al., 2004; Heath et al., 2014). However, simplified methods for larval settlement that have been described for *H. scabra* and *A. japonicus*, could also be tested in order to reduce operational costs and the chances of introducing harmful animals, such as copepods (Xilin, 2004; Mills et al., 2012; Juinio-Meñez et al., 2012a). One of these methods consists of using clean settlement plates, and then providing food for the sea cucumbers after they have settled to the plates (Xilin, 2004). Alternatively the settlement plates could be covered with a paste of *Spirulina* sp. that induces settlement and serves as an initial food for the settled individuals (Mills et al., 2012; Juinio-Meñez et al., 2012a).

1.5.1.3. Nursery culture of post-settled juveniles

The nursery culture of post-settled juveniles of *A. mollis* is a significant bottleneck to the development of commercial aquaculture of this species. Only one study reports nursery-rearing large numbers of early juveniles (Heath et al., 2014). In this study, the post-settlement juveniles were taken from the settlement plates and placed into tanks where they were fed with an artificial diet consisting of fine sediment mixed with powdered macroalgae and benthic diatoms (Heath et al., 2014). However, the growth rate of these juveniles was very poor using these nursery culture methods (Heath et al., 2014). Therefore, alternative ways to culture the post-settlement juveniles should be tested to improve the nursery production. The early juveniles could be held in tanks with or without sediment (Xilin, 2004; Xiyin et al., 2004; Pitt and Duy, 2004), or they could be placed in earthen ponds or at sea (e.g., inside floating or bottom-set fine-mesh cages “hapas”, or in bottom cages or in pens), all methods which have proven successful in other species of sea cucumber (Pitt and Duy, 2004; Lavitra et al., 2010; Duy, 2012; Juinio-Meñez et al., 2012a). Depending of the system used, it may also be necessary to supply additional food for the juvenile sea cucumbers (Xilin, 2004; Xiyin et al., 2004; Pitt and Duy, 2004). The lack of availability of earthen ponds in New Zealand and Australia that would be suitable for culturing early juvenile *A. mollis*, means that the early nursery culture will need to be developed in indoor tanks as it is in

A. japonicus (Xilin, 2004; Xiyin et al., 2004). However, the potential for nursery culture in ponds or in the sea should not be dismissed as these culture methods could substantially reduce production costs, especially in terms of the costs of supplying food, provided adequate nursery locations are selected (Pitt and Duy, 2004; Duy, 2012; Juinio-Meñez et al., 2012a). Alternatively, finding more effective feeds, either natural or artificial, for the early juveniles in nursery culture is also important as it is likely to greatly accelerate growth rates and reduce production times (Huiling et al., 2004; Watanabe et al., 2012). For example, early post settlement juvenile *A. mollis* of around 100 mg increased their weight on average by three-fold over six weeks when feeding on mussel waste, compared to barely any growth on artificial diets (Zamora et al., *in preparation*). While effective feed is the primary determinant for improving the performance of nursery cultured *A. mollis*, a range of other husbandry variables also warrant further research, including the determination of optimum stocking densities and seawater parameters (Pitt and Duy, 2004; Renbo and Yuan, 2004; Xiyin et al., 2004; Agudo, 2006; Mercier et al., 2012). The development of adequate grading techniques to separate the fast growing juveniles, and releasing the growth potential of smaller individuals is also needed (Battaglene and Seymour, 1998; Yin-Geng et al., 2004).

1.5.1.4. Transportation of juveniles for grow-out

The transport of juvenile sea cucumbers from nursery facilities to grow-out locations has proved problematic in other species of sea cucumbers (Purcell et al., 2006a; Sun et al., 2006). Transportation of juvenile *A. mollis* can be done with or without seawater for different amount of times (Zamora and Jeffs, *in press* b). The juveniles can be transported without seawater only if desiccation is avoided and for short periods of time (up to 8 h), whereas transportation with seawater can be done for longer periods of time, although careful monitoring of oxygen and pH levels is required (Zamora and Jeffs, *in press* b). However, it is still not known whether or not hatchery reared juveniles of this species of sea cucumbers require acclimation prior to release into grow-out sites (Dance et al., 2003; Purcell et al., 2006a; Purcell and Simutoga, 2008). In this regard, the size of *A. mollis* juveniles used for stocking grow-out sites is critical because in other species of sea cucumbers, larger juveniles have consistently proven to have better chances of survival, growing faster than smaller ones (Renbo and Yuan, 2004; Purcell, 2004, 2012; Purcell and Simutoga, 2008; Mills et al., 2012).

1.5.2. Alternatives for Grow-out of Sea Cucumbers

Sea cucumbers produced in hatchery and nursery facilities can be grown-out in a variety of different ways, such as releasing them into the wild in order to restore the depleted spawning biomass of a wild population (i.e., restocking), or to increase the fishery yield of overfished populations of sea cucumbers (i.e., stock enhancement) (Purcell, 2004, 2012; Bell et al., 2005). Currently, this is not the case for *A. mollis*, but it may be necessary if demand and commercial awareness of the market potential of this species increases, leading to a reduction of the natural stocks through increased commercial harvesting (Bell and Nash, 2004). The most commonly used grow-out systems for sea cucumbers are earthen ponds, sea pens, sea cages, and the release into delimited areas of seabed (i.e., sea ranching) (Renbo and Yuan, 2004; Chen, 2004; Yaqing et al., 2004; Purcell and Simutoga, 2008; Agudo, 2012; Duy, 2012; Mills et al., 2012). An alternative grow-out method that has recently gained interest is the feasibility of using sea cucumbers as a critical component of integrated multi-trophic aquaculture (IMTA) systems by utilising their capacity to feed on organic waste generated by the aquaculture of other species, such as shellfish or fish (Ahlgren, 1998; Kang et al., 2003; Zhou et al., 2006; Slater and Carton, 2007; Bell et al., 2007; Paltzat et al., 2008; Maxwell et al., 2009; Ren et al., 2012; Yokoyama, 2013).

All the above methods of grow-out could potentially be used in *A. mollis*, however, further research is needed regarding the optimal seawater parameters for the growth of this species. So far it is known that the growth and feeding activity of juveniles of *A. mollis* from northern New Zealand become negatively affected when temperature increases from 15 to 21 °C, and that temperatures around 24 °C are lethal (Zamora and Jeffs, 2012b). In southern populations negative effects of seawater temperature manifest around 18 °C, which suggests that there are latitudinal differences in temperature tolerance in this species (Maxwell et al., 2009). Salinity also affects feeding activity and survival, with juvenile sea cucumbers found to decrease their feeding activity as salinity decreased from 34 to 24 ppt, and dying after 5 days of exposure to seawater at 28 ppt (Zamora, *unpublished data*). Oxygen levels may also become a problem under confined culture situations (e.g., earthen ponds) when sea cucumbers are cultured at higher densities, despite *A. mollis* having a low metabolic rate and corresponding oxygen demand (Robertson, 1972; Maxwell et al., 2009; Zamora and Jeffs, 2012b). However, in these situations organic loading may place additional pressures on oxygen demand at the sediment-seawater

interface where sea cucumbers dwell (Renbo and Yuan, 2004). The effect of pH has not been researched in *A. mollis*, but research in *A. japonicus* suggests that pH levels should not go below 7.9 pH units (Renbo and Yuan, 2004). Overall, increasing the knowledge of how *A. mollis* responds to environmental variables in the laboratory and under culture conditions is essential for the development of the aquaculture of this species. This information will be particularly important for the selection of the most suitable grow-out locations and culture system to be used.

1.5.2.1. Pond culture

Pond culture of sea cucumbers is common practice in China (over 7,000 ha) as well as in other tropical regions of the world since abandoned shrimp ponds can often be adapted for growing sea cucumbers, thus saving in construction costs (Yaqing et al., 2004; Renbo and Yuan, 2004; Agudo, 2012; Duy, 2012). However, not all sea cucumbers perform well under pond culture conditions, with some species found to lose weight and die because the culture conditions do not resemble their natural habitat (Purcell et al., 2012b; Mercier et al., 2012). The muddy floor of earthen ponds should not be problematic for *A. mollis* since this species can be found naturally in high numbers in shallow waters with fine sediment (Slater et al., 2010). However, since typically there is no continuous water flow in pond culture, the sea cucumbers may be exposed to water stratification and even to daily and seasonal fluctuations in seawater temperature and salinity which may impact their growth and survival (Yaqing et al., 2004; Renbo and Yuan, 2004; Pitt and Duy, 2004; Bell et al., 2007; Yuan et al., 2010; Agudo, 2012; Bowman, 2012). Usually in ponds there is some natural supply of food for the sea cucumbers, however, depending on the pond conditions and stocking densities, the supply of additional food may be needed (Renbo and Yuan, 2004; Qin et al., 2009; Ren et al., 2010; Sun et al., 2013). Stocking densities of ponds could also be increased by adding artificial substrates in order to increase the surface area (Renbo and Yuan, 2004). Determining the relative importance of all these variables would be necessary for advancing the pond culture of *A. mollis*, and may involve adjustments at different growing latitudes (Purcell et al., 2012b).

1.5.2.2. Sea pens and cages culture

A common method for growing out sea cucumbers is in the sea, inside cages or pens, which have been used in China and tropical regions of the world (Chen, 2004; Renbo and Yuan, 2004; Mills

et al., 2012). The use of these confinement structures prevents the sea cucumbers migrating to other places, and helps to designate ownership of stocks and prevent theft of the cultured sea cucumbers (Purcell et al., 2012b). There is a need to determine effective sea culture systems (sea pens, bottom cages or floating cages) in order to enhance growth, reduce mortality and escapes (Chen, 2004; Renbo and Yuan, 2004; Purcell and Simutoga, 2008; Hair, 2012; Robinson and Pascal, 2012). *Australostichopus mollis* grows well when enclosed in small plastic mesh cages fixed to the seafloor at relatively high densities, provided the cages are placed where there is a high ongoing organic food input, such as beneath shellfish farms (Slater and Carton, 2007; Slater and Jeffs, 2010; Zamora and Jeffs, 2012a). However, using methods of containment for juvenile sea cucumbers with more extensive benthic area, such as sea pens and ponds, would provide the greater surface area for the animals to forage (Purcell and Simutoga, 2008; Robinson and Pascal, 2012). The use of sea pens and sea cages, despite ensuring confinement of the sea cucumbers, can be expensive to establish and maintain, and also constrain the stocking density (Purcell et al., 2012b).

1.5.2.3. Sea ranching

The release of juvenile sea cucumbers without any confinement into a specific coastal location with the intention of subsequent harvesting has been researched recently for a number of sea cucumber species (Purcell and Simutoga, 2008; Purcell, 2012; Fleming, 2012; Juinio-Meñez et al., 2012b; Bowman, 2012). These research show that successful sea ranching of sea cucumber relies on the selection of an area of adequate size and with the correct environmental conditions for the sea cucumbers to survive and grow, and thereby reducing mortality and migration. It has been shown in *A. mollis*, as well as in *H. scabra*, that if the habitat conditions are adequate at the site where the juvenile sea cucumbers are released, they will not move great distances before they are harvested years later (Mercier et al., 2000; Purcell and Kirby, 2006; Slater and Carton, 2010). Survival of the released juveniles is a major concern since the majority of them do not survive to a market size in the wild (Purcell and Simutoga, 2008; Juinio-Meñez et al., 2012b). Survival could be increased by increasing the size of the released animals, improving transport conditions, and better acclimation of the juveniles to the conditions at the site of their release (Pitt and Duy, 2004; Purcell et al., 2006a; Purcell and Simutoga, 2008; Robinson and Pascal, 2012). Predation of juveniles at the release site is also important and should be mitigated if possible by providing refuges or through timing the release at periods when low predation risk is

expected (Purcell et al., 2012b). However, this would require further research given the lack of information regarding natural predation in *A. mollis* (Sewell, 1990).

In addition, before applying this kind of sea ranching system, care should be taken with the possible genetic pollution and transfer of diseases from hatchery-produced individuals to the local wild population (Purcell, 2004, 2012). The released juveniles should ideally be tagged in order to distinguish them from the wild conspecifics, providing a proof of ownership and a means to evaluate the effectiveness of the release (Purcell, 2012). Several tagging methods have been tested in *A. mollis*, such as the use of freeze branding, micro-sand blasting, pit-tagging, T-bars and visible implant fluorescent elastomer (VIFE) (Archer, 1996; Stenton-Dozey, 2007b). Of these methods the VIFE had the highest retention (87%) after three months (Stenton-Dozey, 2007b). However, other cost-effective methods such as fluorochromes, which mark the sea cucumbers ossicles for a longer period, should not be dismissed as being a potentially useful marking method (Purcell et al., 2006b; Purcell and Blockmans, 2009).

1.5.2.4. IMTA systmems

Integrated multi-trophic aquaculture is the culture of two or more compatible species together, that maximizes production by using organisms that occupy different trophic levels and niches (Bardach, 1986). Culture of *A. mollis* with other economically important aquaculture species shows some considerable promise for further advancement toward commercial production as it has been revealed in other sea cucumber species (Ahlgren, 1998; Kang et al., 2003; Zhou et al., 2006; Paltzat et al., 2008; Bell et al., 2007; Ren et al., 2012; Yokoyama, 2013).

The culture of *A. mollis* together with the New Zealand abalone, *Haliotis iris*, in land-based aquaculture systems revealed that the waste from cultured abalone has sufficient energy to support growth in juvenile sea cucumbers, but not for adults (Maxwell et al., 2009). Culture of *A. mollis* with oysters appears to have promise as it has been shown to be effective in A. japonicus and *P. californicus* (Zhou et al., 2006; Paltzat et al., 2008). Caged juvenile *A. mollis* can grow well in sites nearby oyster farms compared with sites further away from the farms (Slater and Jeffs, 2010). Initial experiments co-culturing juvenile *A. mollis* in cages beneath oyster farms has shown their growth depends on a combination of stocking density, food availability and seawater temperature (Zamora and Jeffs, *in review*). The possibility for culturing *A. mollis* beneath salmon

farms in Australasia should also be evaluated considering the extensive sea cage salmonid production in the region, and given that other sea cucumbers species have performed well in co-culture with finfish (Ahlgren, 1998; Yokoyama, 2013). Co-culturing *A. mollis* with other aquaculture species, such as scallops and crustaceans, should also be considered (Bell et al., 2007; Zhou et al., 2006; Ren et al., 2012).

The most promising and therefore most studied co-culture option for *A. mollis* is with mussels (*Perna canaliculus* and *Mytilus galloprovincialis*) of which there is extensive production in New Zealand and Australian waters (Slater, 2006; Slater and Carton, 2007, 2009, 2010; Slater et al., 2009; Zamora and Jeffs, 2011, 2012a, 2012b; MacTavish et al., 2012). *Australostichopus mollis* has been observed to occur naturally in high numbers beneath some mussel farms in New Zealand, presumably attracted by the organic enrichment of the seabed (Gribben and Bell, 2000, 2001). Co-culturing sea cucumbers beneath mussel farms will not only provide a second crop for the mussel industry but also will help to reduce the impact of the organic loading on the sediments beneath the farms (Slater and Carton, 2009; MacTavish et al., 2012). Caged adult sea cucumbers can survive and grow at high densities when caged on the sediments under mussel farms, however, as the sea cucumbers grow, the food availability in the cages becomes a limiting factor (Slater and Carton, 2007), possibly because the distribution of mussel waste under mussel farms is patchy (Zamora and Jeffs, 2012a). A possible solution would be to place the sea cucumbers under the mussel farms without cages, allowing them to freely forage, and being retained by their behavioural attraction to the organically enriched seabed beneath the mussel farm (Slater and Carton, 2010). However, the success of this type of co-culture will depend on the abundance of mussel waste in the sediment, as well as the seawater temperature and salinity, which together can greatly influence the sea cucumbers feeding behaviour and growth (Slater et al., 2009; Zamora and Jeffs, 2012a, 2012b; Zamora, *unpublished data*). Therefore, to optimise growth and survival of the sea cucumbers cultured beneath mussel farms it is useful to know the temperature and salinity of the seawater to which the mussel farm is exposed throughout the year, and estimates of mussel waste biodeposition can be used to help manage the stocking densities of *A. mollis*.

1.5.3. Artificial Diet Development

The development of an artificial diet for the culture of *A. mollis* has been slow, mainly because of limited information about the nutrient requirements and digestive capabilities of this species, which are likely to involve differences between nursery and grow-out stages of production (Maxwell et al., 2009; Slater et al., 2009, 2010, 2011b; Zamora and Jeffs, 2012b). Consequently, artificial diet development has been a specific focus for recent research. In terms of nutrients, protein and lipid are the most important structural and energy reserve components for adult *A. mollis* (Liu, 2010). However, juveniles of this species appear to rely more on protein than both lipid and carbohydrate as a source of metabolic energy (Gay and Simon, 1964; Zamora and Jeffs, 2012b). When feeding on mussel waste, juvenile *A. mollis* absorb lipid more efficiently than protein and carbohydrate, however, the total quantity of carbohydrate and protein absorbed from this food source are substantially higher (Zamora and Jeffs, *in press* a). Juveniles of this species are able to digest, absorb and grow on a wide variety of artificial sources of protein and carbohydrate, which is an important prerequisite for developing artificial feeds (Slater, 2010; Slater et al., 2011b). Several potential artificial diet ingredients such as fishmeal, mussel meal, dried seaweed (*Sargassum policystum*), *Spirulina* sp., fish oil, and even artificial diets formulated for abalone and carnivorous fish, have proved to be palatable to the juveniles of *A. mollis* when offered in the correct proportions with sand or diatomaceous earth (Maxwell et al., 2009; Slater et al., 2009, 2011b; Slater, 2010; Zamora, *unpublished data*). However, further research is needed in terms of the selection of adequate feed ingredients, feed attractants, ingestion stimulants, and feed presentation in order to develop an effective artificial diet for *A. mollis* (Lawrence et al., 2007). For example, in *A. japonicus* several artificial feed ingredients have been evaluated, and detailed nutrient requirements are available, even to the level of specific amino-acid requirements (Huiling et al., 2004; Yuan et al., 2006; Liu et al., 2009; Okorie et al., 2008, 2011; Seo and Lee, 2011; Xia et al. 2013). Improved artificial diets can enhance growth as well as giving the sea cucumbers greater tolerance to survive stressful conditions (Qin et al., 2009; Wang et al., 2009; Zhang et al., 2010; Seo et al., 2011; Ma et al., 2013).

1.6. Conclusions

Considering the high value and the growing market demand for sea cucumbers, wild populations of *A. mollis* may face increasing fishing pressure, and further commercial interest in the development of their aquaculture production. Considering the current state of the fisheries for this species throughout its natural range, it is unlikely that *A. mollis* could face overexploitation

in the near term. However, stock identification and biomass estimations together with more biological and ecological data are required for a better management of this species. Aquaculture development seems to be a more likely route for increasing the supply of this sea cucumber to meet international market demand. However, further information is needed in some key areas to provide a reliable basis for proceeding with sustainable commercial aquaculture at any scale. Major research priorities for improving the development prospects of the commercial aquaculture of this species are improving hatchery and nursery technology (e.g., broodstock management, larval rearing, post-settlement culture) and identifying the most suitable grow-out systems and locations (e.g., pond culture, sea pens and cages, sea ranching, IMTA). Initial research has indicated that broodstock of this species do have the potential to be manipulated into reproductive condition (Morgan *pers. comm.*), but reliable broodstock conditioning techniques need to be further researched and developed for commercial application. Methods used for commercial hatchery-rearing larvae of *A. japonicus* in China have recently been proven to be effective for *A. mollis* on a pilot commercial scale conducted by a company in New Zealand (Heath et al., 2014; Maxwell, *pers. comm.*). However, weaning and early nursery methods have proved problematic because of a lack of knowledge of the types of suitable foods and the manner of their presentation. This is an area in great need of research if effective nursery production methods for *A. mollis* are to be established to support commercial scale production of juveniles. Research to date shows that *A. mollis* can tolerate a variety of grow-out conditions and regimes. However, their growth and survival varies enormously in response to ambient conditions and is frequently sub-optimal, especially with poor quality or low availability of food. Therefore, defining optimal growing conditions for *A. mollis* under aquaculture conditions is now critical for advancing the commercial grow-out of this species.

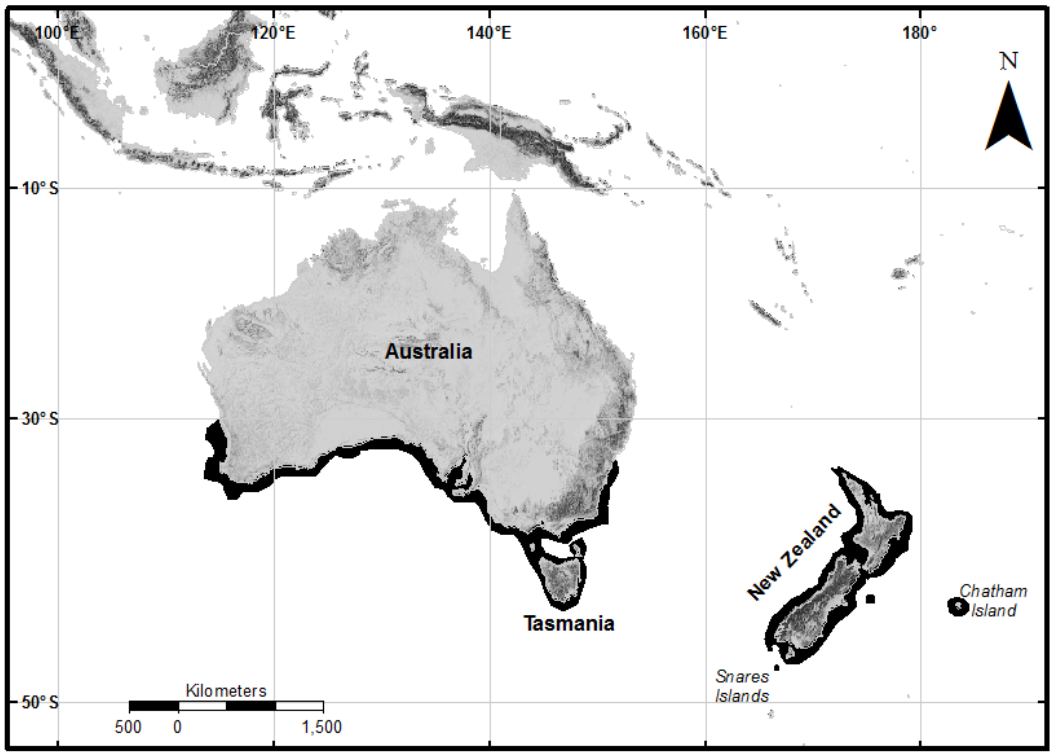

Figure 1.1. Distribution of *Australostichopus mollis* throughout coastal region of New Zealand and southern Australia – dark colour on coastal margin indicates the natural range of this species.

Figure 1.2. Adults (over 90 g wet weight) of the Australasian sea cucumber *Australostichopus mollis* showing clear differences in colouration.

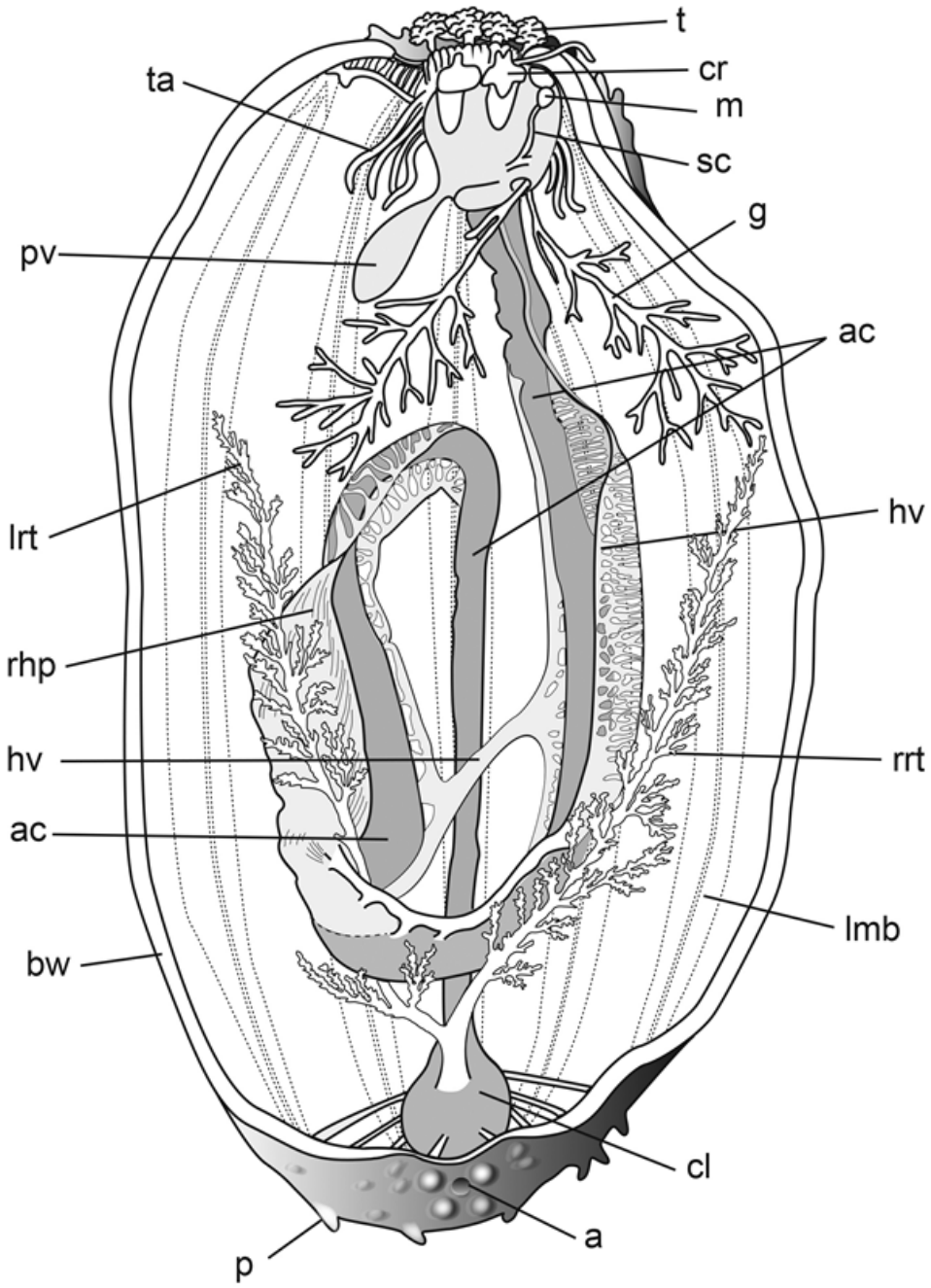

Figure 1.3. Representation of the general internal anatomy of a specimen of *Australostichopus mollis* opened along the dorsal interambulacrum (Modified with permission from Sewell, 1987). Where, t: oral tentacle, cr: calcareus ring, m: madreporite, sc: stone canal, g: gonad, ac: alimentary canal, hv: haemal vessel, rrt: right respiratory tree, lmb: longitudinal muscle band, cl: cloaca, a: anus, p: external body wall papillae, bw: body wall, rhp: respiratory-haemal plexus, lrt: left respiratory tree, pv: polian vesicle, and ta: tentacular ampullae.

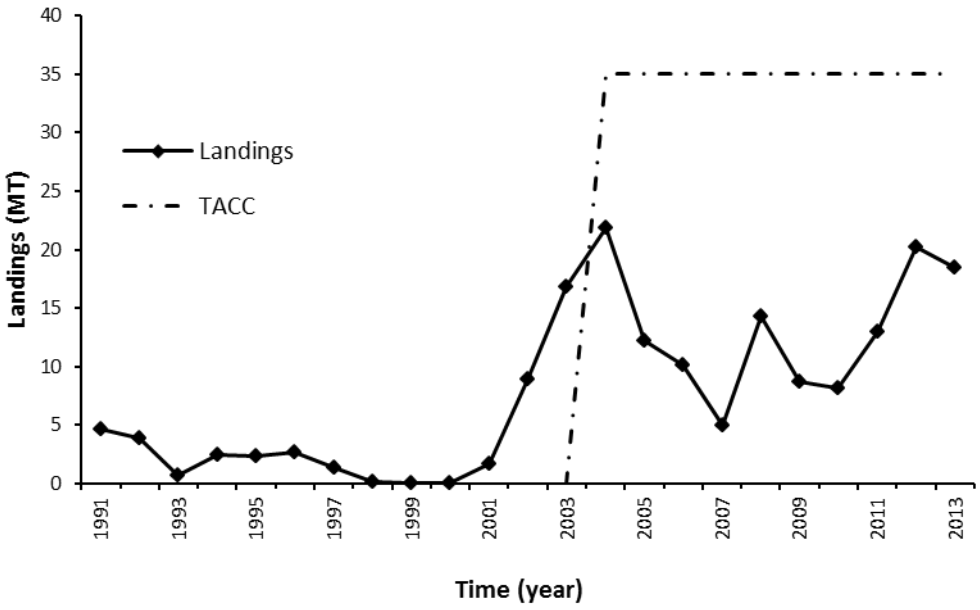

Figure 1.4. Annual reported commercial landings in metric tonnes (MT) of *Australostichopus mollis* in New Zealand in relation to the total allowable commercial catch (TACC). Data taken from: Ministry of Fisheries (2011).

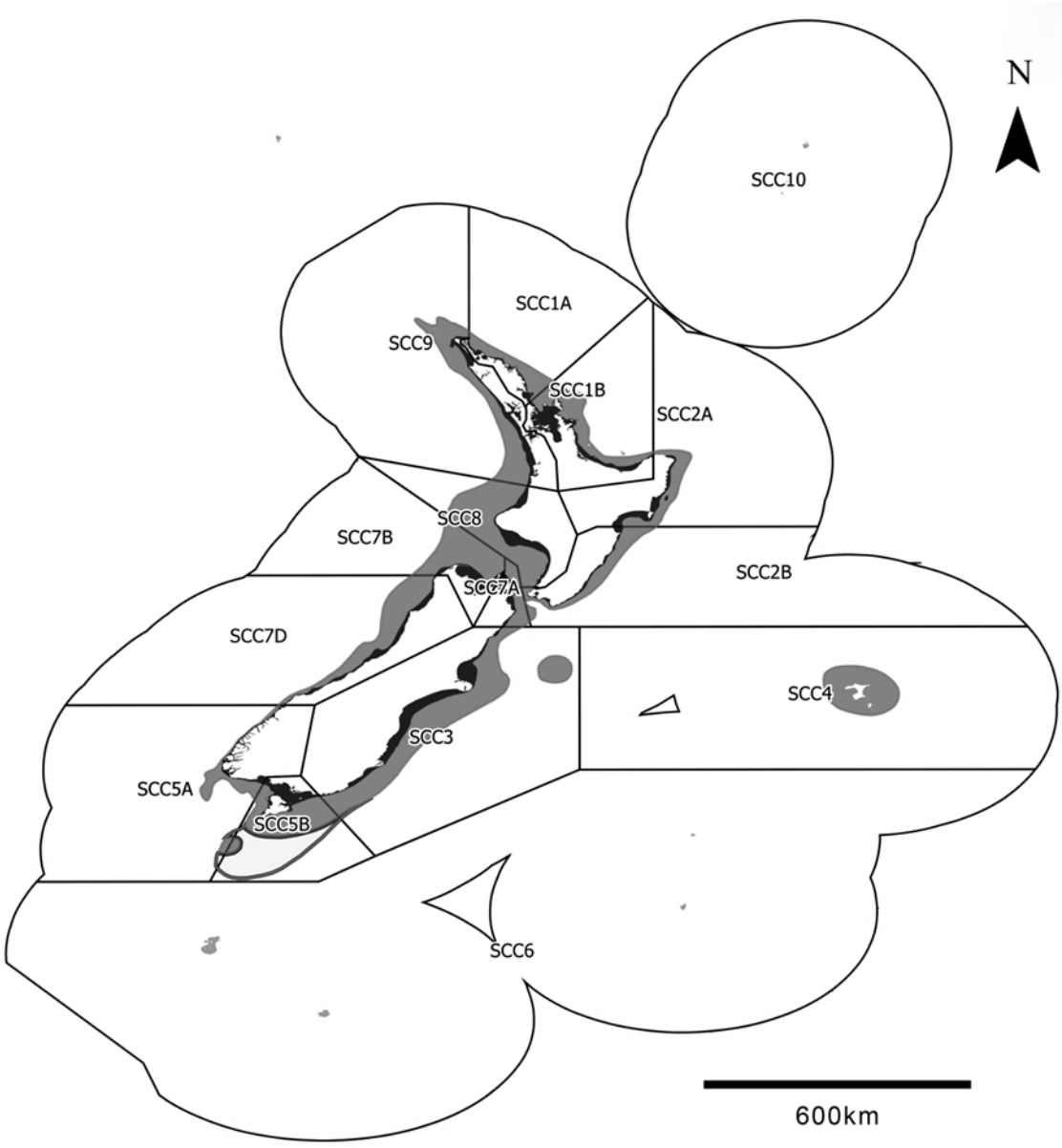

Figure 1.5. Fishing marine areas designated for the management of the commercial sea cucumber (SCC) *Australostichopus mollis* fishery in New Zealand. The majority of the commercial landings are currently from areas SCC1B, SCC3, and SCC7A. Different grey tones indicate apparent relative abundance within New Zealand, darker being higher abundance. Map source: Ministry of Primary Industries, NABIS crown copyright reserved (Modified).

Table 1.1. Timetable of embryonic and larval development of *Australostichopus mollis*, coming from two different studies (Archer, 1996; Stenton-Dozey and Heath, 2009). In the first study, fertilized eggs were obtained from a naturally occurring spawning event of broodstock in northeastern New Zealand and incubated, at 19 ± 0.5 °C (Archer, 1996). In the second study, fertilized eggs from artificially induced spawning events in broodstock collected from Wellington Harbour and Marlbourough Sounds in southern New Zealand were incubated at 18 °C (Stenton–Dozey and Heath 2009).

	Archer, 1996		**Stenton-Dozey and Heath, 2009**	
Developmental stage	**Time**	**Size (µm ± S.D.)**	**Time**	**Size (µm)**
Release (polar bodies)	<65 min	--	20-30 min	140-145
First cleavage	90 min	--	40-60 min	140-145
Second cleavage	140 min	185±6	--	--
Third cleavage	225 min	188±13	--	--
Fourth cleavage	290 min	195±11	--	--
Blastula	6 hour	189±11	5-6 hour	140-150
Initiating gastrulation	9 hour	154±10	--	--
Gastrula	14 hour	171±11	25-36 hour	150-200
Very small auricularia	--	--	56-60 hour	350-380
Early auricularia	1 day	404±40	--	--
Small auricularia	--	--	4-5 days	450-500
Medium auricularia	--	--	8-11 days	600-700
Late auricularia	17 days	686±72	--	--
Large auricularia	--	--	12-16 days	800-980
Early doliolaria	22 days	454±50	--	--
Doliolaria	--	450±69	18-20 days	330-500
Early pentactula	23 days	--	--	--
Pentactula	--	344±68	21-23 days	320-200
Settlement	25 days	--	24-27 days	165-280

Chapter Two

Feeding, selection, digestion and absorption of the organic matter from mussel waste by juveniles of the deposit-feeding sea cucumber *Australostichopus mollis*

Abstract

An understanding of the feeding and digestive capabilities of juvenile sea cucumbers is an important step for their aquaculture development. The feeding behaviour, food selectivity, digestion and absorption of total organic matter (TOM) were determined for juveniles (35 - 40 g wet weight) of the Australasian brown sea cucumber, *Australostichopus mollis*, kept in circular plastic tanks (9 l; 6 replicate tanks per feed treatment). Sea cucumbers were fed with feeds containing different levels of TOM (1, 4, 12, and 20%) prepared from sand mixed with the biodeposits of aquacultured green-lipped mussel, *Perna canaliculus*. No significant diurnal patterns in digestion were detected in all feed treatments. As the TOM level in the feed increased, the selection of organic particles decreased and most importantly overall nutrient absorption increased, which is likely to lead to improved growth rates. However, absorption efficiency declined as organic matter content of the feed increased. This study suggests that palatable-artificial diets rich in organic matter can be used to increase nutrient uptake and potentially facilitate increased stocking densities of sea cucumbers under controlled conditions.

2.1. Introduction

Despite the commercial importance of sea cucumbers, in both fisheries and aquaculture, information about the feeding behaviour and digestive capabilities of juvenile sea cucumbers is only available for a few species. Most of this information comes from studies on the Japanese sea cucumber, *Apostichopus japonicus* (Liu et al., 2004; Renbo and Yuan, 2004; Xiyin, 2004, Huiling et al., 2004; Dong et al., 2008a; Yuan et al., 2006; Dong et al., 2008b; Okorie et al., 2008, Liu et al., 2009) and the sandfish, *Holothuria scabra* (Battaglene et al., 1999; Mercier et al., 1999; Hamel et al., 2001; James, 2004; Giraspy and Ivy, 2008). Very little is known about

juveniles of the Australasian brown sea cucumber, *Australostichopus mollis*, which has a great commercial potential. This species is the current focus of commercial aquaculture development such as the improvement of hatchery techniques and the development of co-culture methods with other aquaculture species in New Zealand (Morgan and Archer, 1999; Slater and Carton, 2007; Morgan, 2009a; Morgan, 2009b; Stenton-Dozey and Heath, 2009; Maxwell et al., 2009; Slater and Carton, 2010).

Australostichopus mollis is an aspidochirotid sea cucumber that is common in the shallow coastal waters of New Zealand and can be found in many parts of southern Australia in a wide range of habitats from shallow rocky reef to sandy bottoms and mudflats (Pawson, 1970). In New Zealand this species can also be found in large numbers under green-lipped mussel (*Perna canaliculus*) farms (Gribben and Bell, 2000; 2001). The great variety of habitats where this species can be found could be explained by its ability to exploit different food sources. Although juveniles can be found together with the adults in several types of habitats, settlement seems to be restricted to specific areas (Slater and Jeffs, 2010; Slater et al., 2010). As an aspidochirotid, *A. mollis* moves along the seafloor collecting particles (i.e., a mix of minerals, live organisms, decaying material, including faeces of other marine organisms) by extending peltate (shield-shaped) tentacles that surround the mouth. The tentacles trap particles and then take them into the mouth (Roberts et al., 2000). Once inside the mouth the particles are compressed and transported by peristalsis, without further mixing, along a simple tubular digestive system that ends in the anus located in the posterior part of the animal (Feral and Massin, 1982; Penry, 1989). In previous studies, green-lipped mussel biodeposits (faeces and pseudofaeces) have proved to be highly palatable and good for juvenile *A. mollis* growth (Slater and Carton, 2007; Slater et al., 2009). These biodeposits can be used to obtain basic information about the feeding biology of this species which in turn is useful for the development of the aquaculture of sea cucumbers.

Deposit-feeding sea cucumbers, such as *A. mollis*, can only digest and utilize the organic component removed from the sediment (Roberts et al., 2000). Typically the naturally occurring organic content in marine sediments, measured as total organic matter (TOM), is very low. Consequently, the foraging behaviour and digestive capabilities of sea cucumbers appears to be modified to improve the intake of nutrients from the organic component in the sediments that are needed for their growth and survival (Roberts et al., 2000). For example, some sea cucumber

species search for patches of sediment with higher nutritive value on which to feed (Uthicke and Karez, 1999; Mercier et al., 1999; Slater, 2010), or they actively pick out organic rich particles from the sediment (Massin, 1982; Moriarty, 1982; Hammond, 1983; Rainer and Herndl, 1991; Paltzat et al., 2008). They can also alter their ingestion rate to suit their nutrient requirements (Huiling et al., 2004; Yuan et al., 2006; Liu et al., 2009; Slater et al., 2009; Maxwell et al., 2009), or they can digest and absorb particles with different efficiencies depending on the food that is being consumed (Sibuet et al., 1982; Hammond, 1983; Huiling et al., 2004, Yuan et al., 2006; Liu et al., 2009; Slater et al., 2009, 2011a; Maxwell et al., 2009).

Due to the fact that the utilization of the particles within the sediment by sea cucumbers is a result of the interaction of biotic and abiotic factors, a better understanding of the feeding and digestive capabilities of a deposit-feeding sea cucumber species is an important step for their aquaculture development. However, currently this knowledge is lacking for *A. mollis*. From studies in natural habitats it is known that juvenile *A. mollis* can be found on sediments with a TOM content between 5.5 and 7%, but they can also survive on sediments with a TOM content as low as 4% (Slater and Jeffs, 2010). However, this species is also capable of consuming sediments with a higher TOM content, such as biodeposits from green-lipped mussel with 25% TOM (Slater et al., 2009) and uneaten formulated abalone food (*Laminaria japonica* kelp flakes) and abalone faeces with 76% and 55% TOM content respectively (Maxwell et al., 2009).

The aim of the current study is to obtain information useful for understanding the feeding biology of *A. mollis* to assist in aquaculture development. The research evaluated how the feeding behaviour, food particle selectivity, digestion, absorption and faecal production of the sea cucumber *A. mollis* changed in response to being fed different levels of organic matter under controlled conditions.

2.2. Materials and Methods

Two experiments were undertaken. The first experiment aimed to determine how different levels of TOM in the feed influenced feeding and digestion in juveniles of *A. mollis*. The second experiment examined the ability of juvenile sea cucumbers to select and absorb organic particles from sediments with different levels of TOM. For both experiments, biodeposits derived from green-lipped mussel aquaculture were mixed with sand in different proportions to produce the

experimental feeds. Biodeposits from this mussel contain 19.6% carbohydrate, 5.1% protein, 1.0% lipid and 72.3% ash (Slater et al., 2009).

2.2.1. Experimental Animals

Juvenile sea cucumbers were collected from the Mahurangi Harbour in northern New Zealand by divers in September 2009 at a depth of 12 m. The sample site is characterised by a substratum dominated by silt/mud with large shell fragments (Slater and Jeffs, 2010). The sea cucumbers were transferred to the nearby Leigh Marine Laboratory and held in tanks with flowing 100 µm filtered seawater at ambient temperature. The sea cucumbers were unfed for 48 h to ensure that the gut contents were fully evacuated. The sea cucumbers were then weighed to the nearest gram, after the excess water from the respiratory tree was removed by gently squeezing the posterior half of each animal and the external water was blot dried (Sewell, 1990). Only sea cucumbers of similar weight were used in the experiments: 37.56 g (± 2.51 S.E.) for the feeding and digestion experiment, and 38.24 g (± 2.08 S.E.) for the food selectivity and absorption experiment.

For both experiments the sea cucumbers were maintained in circular plastic tanks (9 l), which had a floor surface area of 0.03 m^2 (water depth of 23 cm) and were supplied with ambient filtered seawater (50 µm) at a rate of 14 l h^{-1}. A natural light cycle was maintained during the experiment, although shaded from direct sunlight (L: 0.2 - 0.3 µE m^{-2} s^{-1}). For the feeding and digestion experiment a single gut-evacuated juvenile sea cucumber was randomly allocated into each of 24 tanks. For the food selectivity and absorption experiment, two gut-evacuated juvenile sea cucumbers were randomly allocated to each tank in a second set of 24 tanks. In both experiments six replicate tanks were randomly selected for each of four feed treatments and the sea cucumbers were allowed to acclimate to the respective diet for six days. The amount of feed supplied for each treatment was equivalent to 30% of the wet body weight of the sea cucumbers per day (Slater et al., 2009).

2.2.2. Experimental Feed Treatments

Four feed treatments were used in both experiments. The feeds were prepared using acid washed sand (Ajax Finechem Pty Ltd), with a grain size between 250 and 500 µm, mixed in different

proportions with homogeneous biodeposits, collected from beneath green-lipped mussel (*P. canaliculus*) cultured in tanks on raw ambient seawater. To avoid any size class stratification of grains in the feed treatments, the ingredients were mixed thoroughly to a thick paste then spread to form a thin sheet and rapidly frozen at -80 °C. Each feed treatment contained a different percentage of TOM, nominally 1, 4, 12, and 20% of the dry weight of sediment that was made by mixing the proportions of sand to biodeposits 95.8:4.2, 83.3:16.7, 50:50, 16.7:83.3 respectively.

Three random samples of each prepared feed treatment were analysed to determine the actual TOM of the four feed treatments by a variation of the combustion method recommended by Byers et al. (1978). The samples were oven dried at 60 °C for 48 h, weighed and then placed in a furnace for 6 h at 500 °C to ensure complete combustion of organic matter, and then re-weighed. The percentage of TOM by dry weight was calculated by sample weight lost after combustion.

2.2.3. Feeding and Digestion

Feed was supplied frozen on Petri dishes (57 cm^2), which were placed in the centre of the circular tank floor. During the experimental acclimation period the feed was changed every 48 h. From a pilot experiment it was determined that the TOM content of all feed treatments did not significantly change in a period of 48 h, either to leaching or bacterial action. After the acclimation period the feed was replaced and the faeces produced by each sea cucumber were carefully collected from the floor of each tank with a plastic pipette every 3 h for a period of 48 h. At each sampling interval the number of sea cucumbers that had produced faeces per treatment was recorded, as well as the number of sea cucumbers located on the floor or on the wall of the tanks. The presence of faeces in a tank was an indirect indicator of food consumption and if a sea cucumber was on the walls of the tank it was an indicator that it was not feeding on the feed provided, which was only available on the floor of the tank. Tanks walls were kept clean and incoming sea water was filtered so it was not possible that the sea cucumbers were feeding on other sources of organic material, such as thick bacterial films.

Faecal samples were stored in small plastic containers and rapidly frozen to -80 °C and then transferred to a -20 °C freezer for storage. The samples were thawed and then filtered on a pre-burnt glass microfiber filter (47 mm diameter). During the filtering procedure the faeces were

gently rinsed with deionised water to remove salt residue. Then TOM content was determined as described before, but with a correction for the filter weight, in order to determine if there are changes in the digestion over time expressed as changes in the TOM content of the faeces.

The faeces produced by each sea cucumber in each treatment were pooled together but separated by the faeces produced during day and night in order to estimate the faecal production rate (FPR) and to be able to identify differences in the feeding intensity expressed in changes in the FPR for day and night. The FPR was calculated as per Maxwell et al. (2009):

$$FPR = (W_{fa}/ W_{sc})/t$$

Where W_{fa}, is the dry weight of the faeces (mg); W_{sc}, is the wet weight of the sea cucumber in the tank (g), and t, is the time (h).

At the end of the experiment, the remaining feed from each replicate tank was fully recovered with a plastic pipette, and dried to a constant weight to quantify consumption by measuring the ingestion rate (IR). The IR was calculated as per Maxwell et al. (2009):

$$IR = ((W_o\text{-}W_u)/W_{sc})/t$$

Where W_o, is the dried weight of the offered food (mg); W_u, is the dried weight of the remaining food (mg); W_{sc}, is the wet weight of the sea cucumber in the tank (g), and t, is the duration of the experiment (h).

2.2.4. Food Selectivity and Absorption

Feed was supplied frozen in the centre of the circular tank floor. During the experimental acclimation period the feed was changed every 48 h. After acclimation, fresh feed was supplied to every tank and samples of freshly evacuated faeces were collected 9 h later to measure their TOM content. In the early morning of the following day the sea cucumbers were removed at 01:00 h, when they are known to most likely have a full gut (Slater, 2006). The sea cucumbers were immediately placed inside plastic bags and into iced seawater to slow down their metabolism and to stop the activity of the digestive enzymes. Each sea cucumber was then

dissected and the alimentary canal was extracted, laid out, measured from mouth to anus and divided in three equal parts by length (Foregut: pharyngeal bulb and mainly the anterior descending intestine; Midgut: mainly the anterior ascending intestine; Hindgut: mainly the posterior descending intestine and the cloaca). From each part of the gut, the digesta (i.e., mixture of feed and digestive juices) was completely removed and stored in an Eppendorf at -80 °C immediately for the subsequent measurement of the TOM.

To determine TOM in the frozen digesta samples, they were firstly thawed, then gently washed with deionised water and centrifuged at 10,000 rpm for 5 min to remove digestive enzymes, and then the remaining pellet analysed for TOM to determine changes in the TOM due to digestive processes sequentially along the gut.

The selection efficiency of the feeding sea cucumbers was determined as the mean difference between the mean TOM available in the supplied food and in the foregut of sea cucumbers for each treatment expressed as a percentage of the TOM available to the feeding sea cucumber in the supplied food.

Absorption efficiency, defined as the mean percentage of ingested organic material moving across the gut wall, was calculated as the observed mean percentage of TOM loss between the foregut and the faeces. The absorption occurring in each sequential part of the gut was calculated as the mean difference in TOM between that part of the gut and the subsequent part of the gut. The absorption within the hindgut was calculated as the mean difference in TOM between the hindgut and the faeces.

2.2.5. Statistical Analyses

Data of continuous variables were tested for normality using a Shapiro-Wilk's test and for homogeneity of variance using a Levene's test. When data conformed to the above assumptions for ANOVA, this method of analyses was used. When an overall experimental effect was determined, significant differences were then identified with pairwise comparisons of treatment means using the Tukey's test (Quinn and Keough, 2002).

When data were non-parametric, comparisons were made using a Kruskall-Wallis test and if an overall experimental effect was observed, then significant differences among treatments were identified with pairwise comparisons using the Tukey's test (Quinn and Keough, 2002).

The mean wet weights of the sea cucumbers for each treatment at the commencement of the experiment were compared using a one-way ANOVA to confirm there were no differences among treatments that could bias the results of the experiment ($F_{(3, 20)} = 1.3$, $P = 0.3$ for the feeding and digestion experiment and $F_{(3, 44)} = 0.2$, $P = 0.9$ for the selection and absorption experiment).

2.3. Results

2.3.1. Experimental Feed Treatments

The measured TOM content of the four feed treatments (i.e., 1, 4, 12, and 20% TOM by dry weight) was very close to the nominal values and varied little between replicate samples, i.e., 1.0% (± 0.1 S.E.), 3.9% (± 0.1 S.E.), 11.6% (± 0.4 S.E.), 19.8% (± 0.3 S.E.) respectively.

2.3.2. Feeding and Digestion

2.3.2.1. Sea cucumber position in the tank

The position of the sea cucumbers in the tanks differed among treatments (Kruskal-Wallis, H = 25.5, d.f.= 3, $P < 0.001$). In particular, sea cucumbers were more frequently observed on the floor of the tanks or near the food in the treatments with 12 and 20% of TOM than in the treatments with 1 and 4% of TOM (Tukey, $P < 0.05$).

2.3.2.2. Ingestion rate

The mean IR among treatments was not significantly different (ANOVA, $F_{(3, 20)} = 0.8$, $P = 0.5$). The mean IR was 2.6 mg g $ww^{-1}h^{-1}$ (± 0.7 S.E.); 4.4 mg g $ww^{-1}h^{-1}$ (± 1.6 S.E.); 4.3 mg g $ww^{-1}h^{-1}$ (± 0.9 S.E.) and 4.7 mg g $ww^{-1}h^{-1}$ (± 0.4 S.E.) for the 1, 4, 12, and 20% TOM feed treatment respectively. However, there was a considerable variability in the measured food intake of

individual sea cucumbers and the extent of this variability was lower in the 20% TOM feed treatment (Fig. 2.1).

2.3.2.3. Faecal production rate

During the 48 h of the experiment there was intermittent faecal production in all the treatments as not all sea cucumbers produced faeces all of the time. Overall, sea cucumbers in the treatments with 12 and 20% of TOM produced faeces more frequently than in the treatment with 1% of TOM (Kruskal-Wallis, H = 20.8, d.f. = 3, $P < 0.001$; Tukey, $P < 0.05$).

The mean FPR among treatments was not significantly different (ANOVA, $F_{(3, 20)} = 0.35$, $P = 0.79$). The mean FPR was 2.5 mg g $ww^{-1}h^{-1}$ (± 0.8 S.E.); 3.8 mg g $ww^{-1}h^{-1}$ (± 1.6 S.E.); 3.4 mg g $ww^{-1}h^{-1}$ (± 0.8 S.E.) and 3.0 mg g $ww^{-1}h^{-1}$ (± 0.4 S.E.) for the 1, 4, 12, and 20% TOM feed treatment respectively. Within each treatment there were not significant differences among the day and night FPR with a $P > 0.05$. However, there was a great deal of variability in the FPR data for both day and night (Fig. 2.2).

2.3.2.4. Organic matter variation over time of the faeces

The TOM content of the faeces increased significantly as the TOM of the feed treatment increased (ANOVA, $F_{(3, 20)} = 45.7$, $P < 0.001$; Tukey, $P < 0.05$ for all pairwise comparisons of treatment means) (Fig. 2.3). However, the organic content of the sea cucumber faeces were not directly proportion to the level of TOM of the feeds. The TOM of the faeces of the 1 and 4% TOM feed treatments were 110% and 26% richer in TOM than their respective feed treatments (Kruskal-Wallis, H = 9.5, d.f.= 1, $P < 0.01$; Kruskal-Wallis, H = 9.5, d.f.= 1, $P < 0.01$ respectively). Whereas, in the 12% TOM feed treatment, the TOM of the faeces was similar to that of the feed (Kruskal-Wallis, H = 0.0001, d.f.= 1, $P = 1.0$) and for the sea cucumbers fed with 20% of TOM, the TOM of the faeces were 14% lower in TOM than their feed (Kruskal-Wallis, H = 9.5, d.f.= 1, $P = 0.002$) (Fig. 2.3).

The TOM of the faeces was not significantly different between day and night for any of the four feed treatments (Kruskal-Wallis, H = 0.6, d.f.= 1, $P = 0.43$; Kruskal-Wallis, H = 0.1, d.f.= 1, $P =$

0.79; ANOVA, $F_{(1, 10)}$ = 2.2, *P* = 0.14; Kruskal-Wallis, H = 0.3, d.f.= 1, *P* = 0.58 for the feeds with 1, 4, 12, and 20% of TOM respectively) (Fig. 2.3).

2.3.3. Food Selectivity and Absorption

2.3.3.1. Organic matter selection from the sediment

The TOM content of the foregut digesta was always higher than the TOM in the experimental food ($\%TOM_{foregut}$-$\%TOM_{food}$) in all feed treatments. In the 1% feed treatment, TOM was 1.7% (± 0.1 S.E.) higher in the foregut than in the sediment (Kruskal-Wallis, H = 19.7, d.f. = 1, *P* < 0.001), while in the 20, 12, and 4% TOM feed treatments the TOM was 3.5% (± 0.2 S.E.) (Kruskal-Wallis, H = 19.7, d.f. = 1, *P* < 0.001), 3.8% (± 0.3 S.E.) (Kruskal-Wallis, H = 19.7, d.f.= 1, *P* < 0.001) and 4.6% (± 0.3 S.E.) (Kruskal-Wallis, H = 19.7, d.f. = 1, *P* < 0.001) higher respectively (Fig. 2.4).

The selection efficiency of organic matter by feeding sea cucumbers was significantly different among treatments (Kruskal-Wallis, H = 19.7, d.f. = 3, *P* < 0.001). There were no significant differences in selection efficiency between the 20% and 12% TOM feed treatments (17.6 ± 0.8% S.E., 32.2 ± 2.2% S.E., respectively) and the feed treatments with 4% and 1% TOM (115.6 ± 8.2% S.E., and 166.7 ± 8.5% S.E., respectively) (Tukey, *P* > 0.05 in all cases) (Fig. 2.5).

2.3.3.2. Organic matter absorption along the gut

Absorption of organic matter in the gut of the sea cucumbers was detected in all feed treatments as a significant reduction in the TOM content of the digesta between the foregut and the hindgut (Tukey, *P* < 0.05 for all feed treatments) (Fig. 2.4). Furthermore, the mean TOM of the hindgut and the mean TOM of the faeces were not significantly different in all the feed treatments (Tukey, *P* > 0.05 for all TOM treatments) (Fig. 2.4). Therefore, the majority of TOM absorption occurred between the foregut and the midgut. This was on average 62.1% (± 6.7 S.E.) of the total absorption in the 20% TOM feed treatment and 77.1% (± 13.2 S.E.), 68.9% (± 8.2 S.E.) and a 100% in the 12, 4, and 1% TOM feed treatments respectively.

The mean amount of TOM absorbed over the entire length of the gut ($\%TOM_{foregut}$-$\%TOM_{faeces}$) increased significantly from 0.6% (± 0.2 S.E.) in the 1% TOM treatment to 6.4% (± 0.2 S.E.) in the 20% feed treatment (Kruskal-Wallis, H = 39.6, d.f. = 3, $P < 0.001$) (Fig. 2.4). However, in terms of absorption efficiency, the treatment with 4% TOM presented the highest absorption efficiency (42.2 ± 2.4% S.E.) when compared with the other feed treatments (ANOVA, $F_{(3, 44)} = 33.1$, $P < 0.001$) (Fig. 2.6).

2.4. Discussion

In this study the feeding biology of juvenile *A. mollis* was studied to elucidate how this species responded to feed treatments with different amounts of organic composition. Successful juvenile feeding is important for the development of the aquaculture of this species. In sea cucumbers the use of the adequate feed, when other variables such as oxygen level, temperature and salinity are controlled; can be critical for the growth of the animals (Battaglene et al., 1999; James, 2004; Liu et al., 2004; Renbo and Yuan, 2004; Xiyin, 2004, Huiling et al., 2004; Yuan et al., 2006; Okorie et al., 2008; Giraspy and Ivy, 2008; Liu et al., 2009).

2.4.1. Differential Organic Matter Selection

Under poor quality feeding conditions, as often encounter in the wild, this species greatly increase TOM selection and intake. This is thought to be the result of more active particle selection helped by the presence of chemosensory receptors and/or the selection of smaller-organic rich particles which may be easier to capture and retain in the tentacles than bigger particles (Moriarty, 1982; Hammond, 1983; Roberts., et al., 2000). According to the results of Slater (2010), *A. mollis* has no grain size selection when feeding on natural sediments in the wild or when feeding with natural sediments enriched with green-lipped mussel biodeposits in the laboratory. Therefore, this TOM selection could be due to the sea cucumbers capability to distinguish between particles of the same size but with different nutrient content. The TOM selection was significantly greater in the feed treatments with lower TOM in which the TOM in the foregut more than doubled the TOM levels in the feed. The extent of this selection behaviour is similar to that detected in tropical sea cucumbers for organic carbon and nitrogen when fed with natural sediments with poor organic content (Hammond, 1983). However, an even greater increase has been reported in *Parastichopus californicus* in which the mean TOM content in the

foregut was around four times higher than in the sediment beneath oyster farms (Paltzat et al., 2008). In contrast, in some sea cucumber species a lack of organic particle selectivity has been reported, e.g., when *Parastichopus parvimensis* are fed with low TOM content sediments (Yingst, 1976).

2.4.2. Ingestion Rate Compensation

In the current study the ingestion rate of *A. mollis* varied little, although not significantly, with the level of organic matter in the feed, showing a tendency to decrease with increasing organic content of the feed which is thought to be a compensatory feeding mechanism in deposit-feeding animals (Lopez and Levinton, 1987). This pattern of decreasing ingestion rate in response to increasing organic matter availability is also found in *A. japonicus* when fed with diets with a TOM content ranging between 8% and 74% (Yuan et al., 2006; Liu et al., 2009).In the current study there was a great deal of variability in terms of feeding behaviour (location in the tanks, ingestion rate). Especially in the 1, 4, and 12% TOM feed treatments which may indicate the expression of a variety of feeding responses by individual sea cucumbers, or intermittent feeding behaviour in these feed treatments, which could be in response to the holding conditions.

2.4.3. Differential Organic Matter Absorption

Once the ingested material enters the digestive tract of sea cucumbers, it is mixed with digestive enzymes and compacted into a plug which moves throughout the gut following a plug-flow reactor model without radial and axial mixing of the sediment (Feral and Massin, 1982; Penry, 1989). As this plug moves from mouth to anus in *A. mollis*, absorption of TOM was found to occur all along the gut for three feed treatments. Only in the 1% TOM feed treatment did all the detected absorption of organic matter occur within the foregut. In the remaining feed treatments more than 62% of the TOM absorption took place within the foregut and midgut. The net absorption of organic matter increased as the organic content of the feed increased, most likely due to an increase of the organic particles available for digestion and the proportionate reduction of inorganic particles travelling through the gut (Yingst, 1976; Lopez and Levinton, 1987; Rainer and Herndl, 1991; Roberts et al., 2000). However, their absorption efficiency did not follow a similar trend and was at a maximum in the 4% feed treatment (42.2% absorption). This feed treatment, although low, was the closest one to the TOM levels that they can found in the natural

habitat of this species (Slater and Jeffs, 2010). This high value of absorption efficiency is similar to those detected in other sea cucumbers species feeding on sediments with a low content of organic matter (Hammond, 1983; Mercier et al., 1999; Paltzat et al., 2008). Conversely, lower absorption efficiencies have also been reported for sea cucumbers that are feeding in low organic content sediments (Yingst, 1976; Zhou et al., 2006).

The TOM content of the faeces of juvenile *A. mollis* presented no diurnal variation in all feed treatments, regardless of the timing of food ingestion and the gut residence time. Therefore, there would be no advantage in manipulating the timing of the introduction of artificial feeds for improving the nutrient utilization of this species. Although, in the current study sea cucumbers appeared to feed both during day and at night with similar intensity, there is no strong evidence to contradict the pronounced nocturnal feeding behaviour described before in captive juveniles of *A. mollis* (Slater, 2006). These differences may be due to differences in the holding conditions between the two studies.

2.5. Conclusions

Overall, the results of this study in *A. mollis* showed that their feeding behaviour allows them to take advantage of the low TOM content in their natural habitats as seen in the responses in the 4% TOM feed treatment. Nevertheless, increasing the availability of organic matter in the available food (by up to 20% TOM) will reduce particle selection, most likely to reduce ingestion rates and will increase overall nutrient absorption by juvenile sea cucumbers under controlled conditions; which is likely to lead to increased growth rate. Therefore, the use of palatable organic-rich diets, like those used in *A. japonicus* aquaculture, could be an option to increase the growth rates and sustain higher densities of *A. mollis* juveniles. However, the use of organically rich feeds for commercial culture of *A. mollis* juveniles will require careful husbandry in terms of closely managed feeding regimes and cleaning in order to avoid excessive loss of food material and crop losses due to the decomposition of food and faeces.

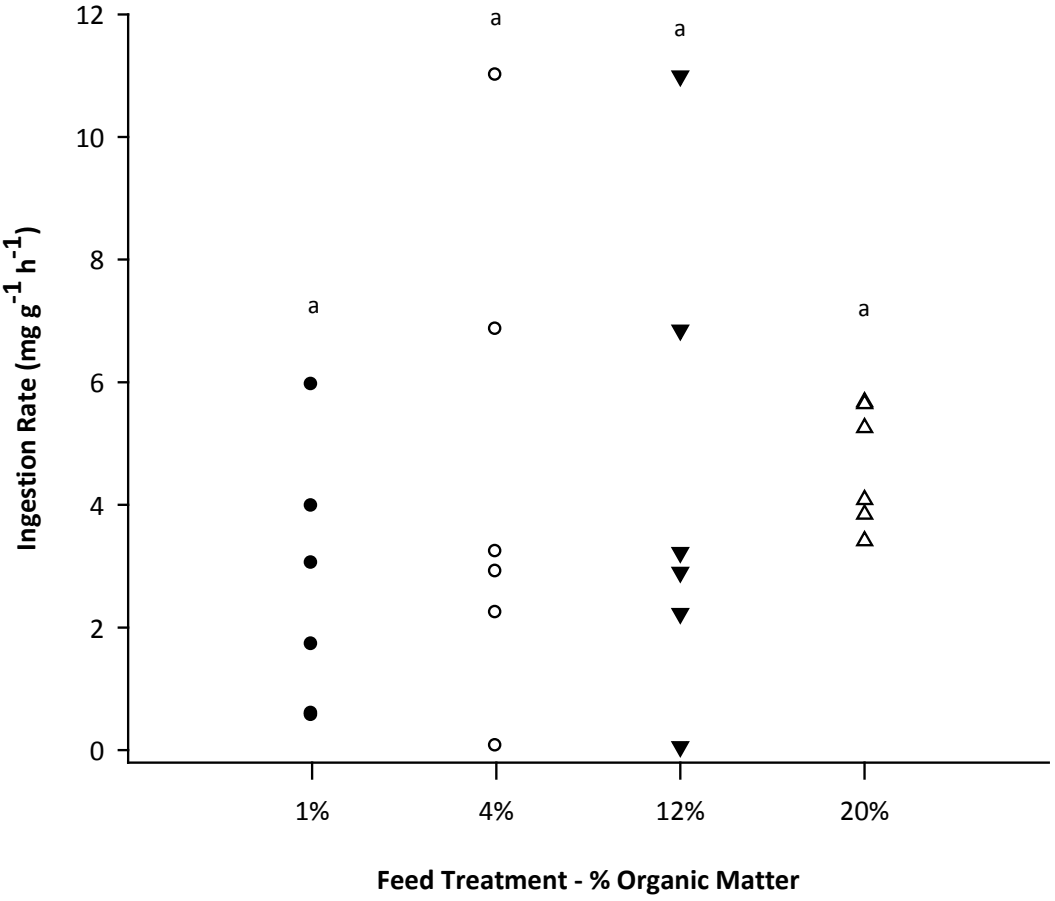

Figure 2.1. Ingestion rate of juvenile *Australostichopus mollis* for four feed treatments, containing 1, 4, 12, and 20% TOM. Different letters within each treatment indicate significant differences ($P < 0.05$).

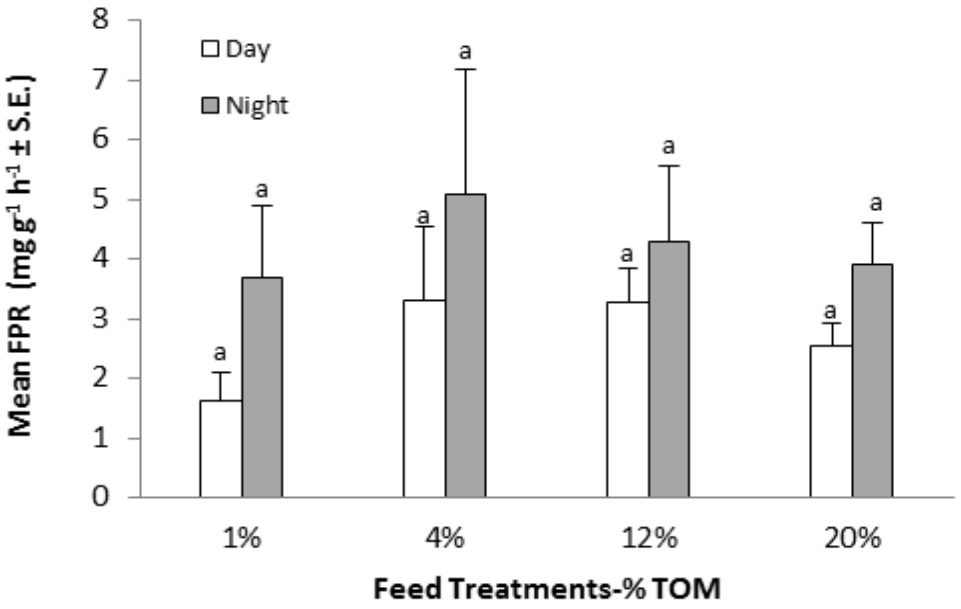

Figure 2.2. Mean night and day faecal production rate (± S.E.) in juvenile *Australostichopus mollis* for four feed treatments, containing 1, 4, 12, and 20% TOM. Different letters within each treatment indicate significant differences ($P < 0.05$).

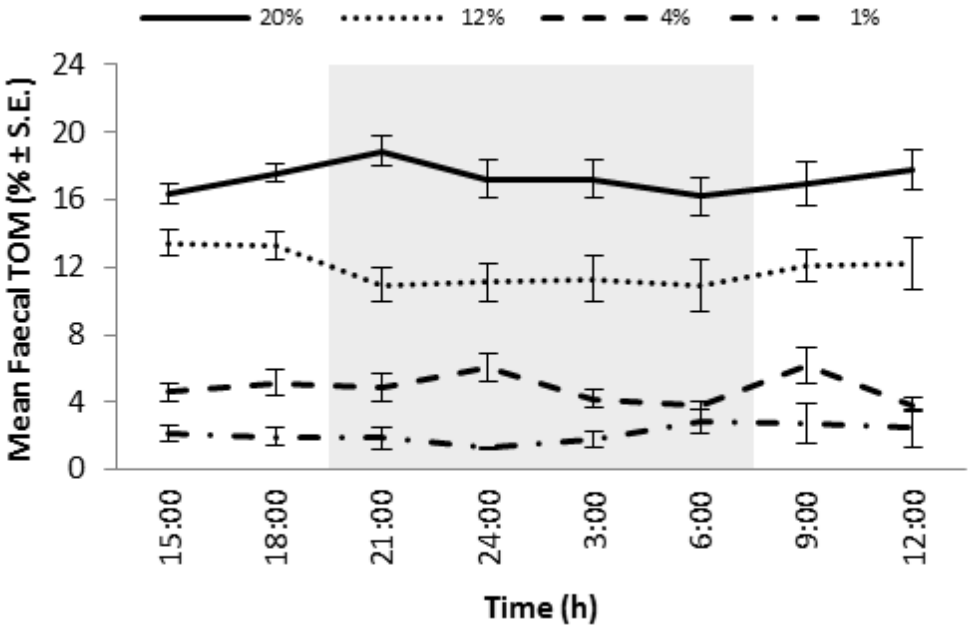

Figure 2.3. Mean TOM (± S.E.) of the faeces produced by juvenile *Australostichopus mollis* at 3 h intervals over 24 h in four feed treatments with different levels of dietary TOM, containing 1, 4, 12, and 20% TOM. The grey shading corresponds to dark hours.

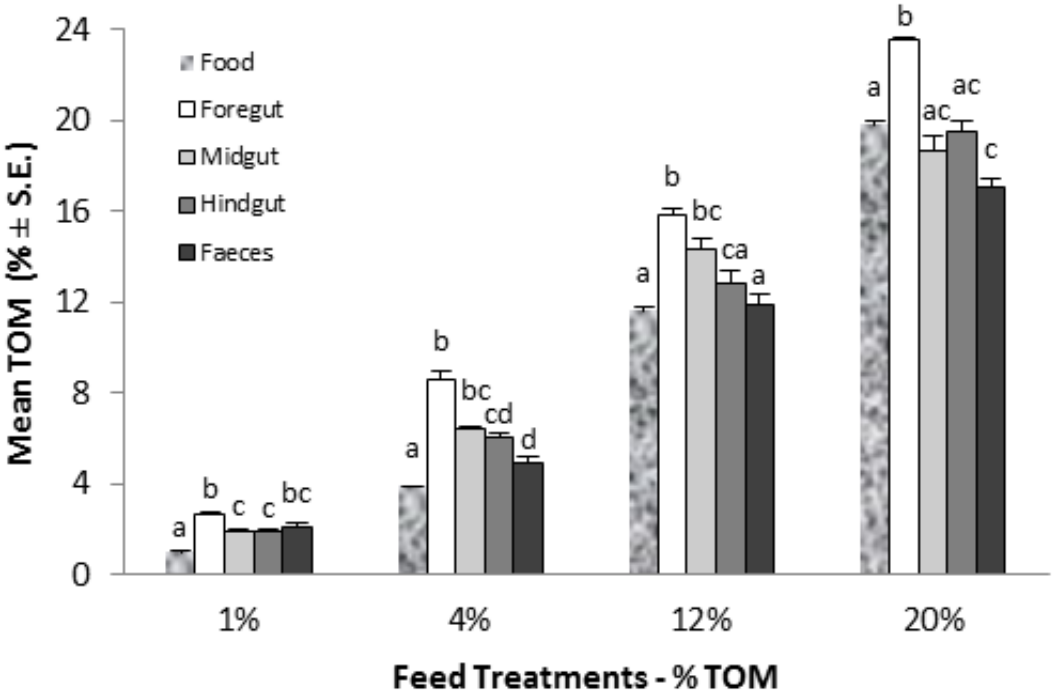

Figure 2.4. Mean TOM (± S.E.) in the food offered to juvenile *Australostichopus mollis*, and in the digesta of the different sections of the sea cucumber's gut (foregut, midgut and hindgut) and in the faeces of sea cucumbers for four feed treatments, containing 1, 4, 12, and 20% TOM. Different letters within each treatment indicate significant differences ($P < 0.05$).

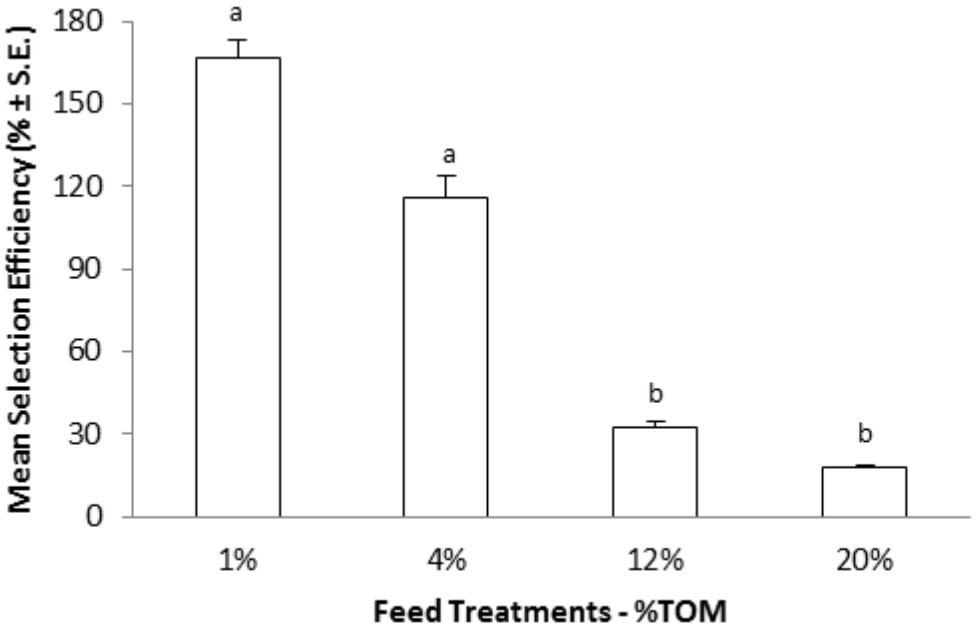

Figure 2.5. Mean TOM selection efficiency (± S.E.) in juvenile *Australostichopus mollis* for four feed treatments, containing 1, 4, 12, and 20% TOM. Different letters among treatments indicate significant differences ($P < 0.05$).

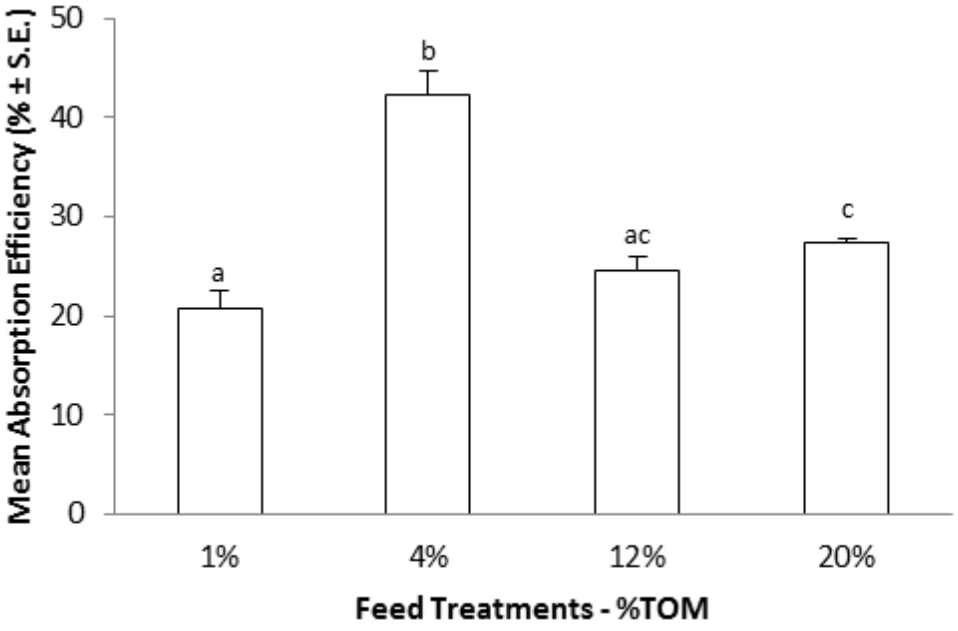

Figure 2.6. Mean TOM absorption efficiency (± S.E.) in juvenile *Australostichopus mollis* for four feed treatments, containing 1, 4, 12, and 20% TOM. Different letters among treatments indicate significant differences ($P < 0.05$).

Chapter Three

The ability of the deposit-feeding sea cucumber *Australostichopus mollis* to use natural variation in the biodeposits beneath mussel farms

Abstract

The feeding biology and ecology of juveniles of the Australasian sea cucumber, *Australostichopus mollis* are not well understood. A better understanding may provide useful information for the development of the aquaculture of this species. Currently, *A. mollis* is being co-cultured beneath mussel farms and it is not clear if it can effectively use the wide natural variation in the food availability typically found under mussel farms. In this study the feeding and growth of juvenile *A. mollis*, was examined on feeds containing different levels of mussel, *Perna canaliculus*, biodeposits, resulting in different levels of total organic matter (TOM) (1, 4, 12, and 20%) covering a range similar to that found beneath a typical mussel farm in New Zealand. As the TOM content increased in the food from 4% to 20%, the ingestion rate of sea cucumbers decreased and both the apparent absorption and food conversion efficiencies increased. There was no significant growth of sea cucumbers in the 1% TOM feed treatment, while sea cucumbers in the remaining treatments had similar final wet weights with a combined mean daily specific growth rate of 0.6% d^{-1}. These results demonstrate the ability of this species to use different levels of TOM to generate similar growth rates mainly by changes in their feeding behaviour and digestive physiology. These changes in their feeding biology together with the spatial and temporal variation of food availability in the sediments impacted by the mussel farm need to be taken into account when selecting suitable mussel farms for co-culture as well as when defining the initial stocking biomass of sea cucumbers.

3.1. Introduction

Studies on the growth and aquaculture of juvenile sea cucumbers have become more frequent as the demand for this valuable seafood product grows internationally, accelerating the depletion of

many wild sea cucumber populations (Ferdouse, 2004; Toral-Granda et al., 2008). Most of this research has focused on juveniles of the more commercially valuable sea cucumbers species, the temperate, *Apostichopus japonicus*, and the tropical, *Holothuria scabra* (Yanagisawa, 1998; Battaglene et al., 1999; Pitt and Duy, 2004; Huiling et al., 2004; Yuan et al., 2006, 2010; An et al., 2007; Dong et al., 2008a, 2008b; Giraspy and Ivy, 2008; Liu et al., 2009). These studies provide valuable information for the culture of those species, however in sea cucumbers species that have recently become target for aquaculture development, their specific requirements need to be determined. This is the case for the Australasian sea cucumber, *Australostichopus mollis*, a species with commercial potential, and for which the feeding biology and ecology of juveniles is not well understood (Morgan and Archer, 1999; Stenton-Dozey and Heath, 2009; Maxwell et al., 2009; Slater et al., 2009, 2011a; Slater and Jeffs, 2010; Zamora and Jeffs, 2011).

Australostichopus mollis can be found in shallow waters all along the coast of New Zealand and in the south of Australia in a wide range of habitats from shallow rocky reefs to sandy seafloor and mudflats (Pawson, 1970). As a deposit-feeder, *A. mollis* moves along the seafloor collecting particles (i.e., a mix of minerals, live organisms, decaying material, including faeces of other marine organisms) by extending the tentacles that surround its mouth. However, despite the variety of materials that can be ingested, deposit-feeding sea cucumbers can only digest and use the organic component removed from the sediment (Roberts et al., 2000). The food available for sea cucumbers can be determined by measuring the total organic matter content (TOM) present in the surface sediments of the seafloor, a measure which also includes all microbial residents in the sediment. Naturally occurring levels of TOM where juvenile *A. mollis* can be found are relatively low, around 5% (Slater and Jeffs, 2010). In New Zealand this species can also be found in large numbers under green-lipped mussel (*Perna canaliculus*) farms (Gribben and Bell, 2000, 2001). The TOM levels underneath a mussel farm can be more than twice as high as naturally occurring levels of TOM (Hartstein and Stevens, 2005; Giles et al., 2006).

Sea cucumbers living beneath mussel farms feed on sediments enriched with the biodeposits coming from the mussel farm (Slater and Carton, 2010). Biodeposits are composed of faeces (ingested and digested particles) and pseudofaeces (rejected undigested particles) that have proven to have a high TOM content (typically around 25%) and can induce high growth rates in juvenile *A. mollis* when compared with the natural food available for juveniles living in natural habitats (Slater et al., 2009). Even adult sea cucumbers can grow at high densities when caged

beneath mussel farms (Slater and Carton, 2007). This has drawn the attention of the large mussel aquaculture industry in New Zealand, which has begun to co-culture sea cucumbers to obtain a second crop without much additional effort. Co-culture can also help to reduce the environmental impact of the mussel farm industry which is the largest aquaculture industry in New Zealand producing in excess of 70,000 t a year valued at over US$ 100 million (Alfaro et al., 2010). However, at the early stages of developing this method of aquaculture there is still not enough information about how the growth of juvenile *A. mollis* may be affected by changes in the availability of food under a mussel farm, and in turn, how this might influence the initial stocking biomass. Biodeposit deposition under a mussel farm is not constant or spatially uniform and it depends on several factors such as the size and density of mussels being cultivated and their diet, as well as the dispersal and erosion of biodeposits, which can vary seasonally depending on the hydrodynamic regime to which the farm is exposed (Hartstein, 2003; Giles and Pilditch, 2004; Hartstein and Stevens, 2005; Giles et al., 2006). These changes in deposition result in temporal and spatial changes in the TOM content of sediments beneath mussel farms. For example, the TOM content under a mussel farm in sheltered waters is usually higher than those located in exposed sites, and the TOM content at most farms tends to decrease from spring to winter (Hartstein and Stevens, 2005; Giles et al., 2006).

Sea cucumbers, depending on the species, can react differently to changes in the TOM levels of the food material. They can detect and search for substrates with a higher TOM content (Uthicke and Karez, 1999; Mercier et al., 1999). Depending on the TOM content of the food they can modify their ingestion rates and selectively pick out organic rich particles to suit their nutrient requirements (Massin, 1982; Moriarty, 1982; Hammond, 1983; Rainer and Herndl, 1991; Huiling et al., 2004; Yuan et al., 2006; Paltzat et al., 2008; Liu et al., 2009; Slater et al., 2009; Maxwell et al., 2009; Zamora and Jeffs, 2011). They can also adjust their digestion and absorption of nutrients in relation to the TOM content of the ingested food (Sibuet et al., 1982; Hammond, 1983; Huiling et al., 2004; Maxwell et al., 2009; Liu et al., 2009; Zamora and Jeffs, 2011). All these changes may help the sea cucumbers to cope with the changes in the availability of TOM content of sediments. However, in the case of juvenile *A. mollis* it is not clear how the changes in their foraging behaviour and nutrient acquisition may modify their growth when the mussel biodeposits are the major source of organic material. Therefore, the aim of this study is to evaluate how changes in the TOM content in the feed, using mussel biodeposits as the only

organic material source, affect the feeding behaviour and growth of juvenile *A. mollis* in order to determine their implications for future co-culture efforts.

3.2. Materials and Methods

3.2.1. Experimental Animals

Juvenile sea cucumbers of a similar size were collected from their natural habitat in the Mahurangi Harbour, northern New Zealand by divers. The sample site is characterised by a substratum dominated by silt/mud with large shell fragments (Slater and Jeffs, 2010). The sea cucumbers were transferred to the nearby Leigh Marine Laboratory and held in tanks with flowing 100 µm filtered sea water. The sea cucumbers were left without feed for 48 h to ensure that the gut contents were fully evacuated before being used in the experiments. The sea cucumbers were then weighed to the nearest gram, after the excess water from the respiratory tree was removed by gently squeezing the posterior half of each animal and the external water was blot dried (Sewell, 1990). Only sea cucumbers of similar weight were used in this experiment (20.0 ± 0.4 g, mean ± S.E.) to ensure that the results of the experiment were not biased by differences in the size of the sea cucumbers at the outset of the experiment.

3.2.2. Experimental Feed Treatments

Four feed treatments were used consisting of different proportions of mussel biodeposits. The feeds were prepared using acid washed sand (Ajax Finechem Pty Ltd), with a grain size between 250 to 500 µm, mixed in different proportions with homogeneous biodeposits collected from beneath green-lipped mussel (*P. canaliculus*) cultured in tanks on raw ambient seawater (Slater et al., 2009). To avoid any size class stratification of grains in the feed treatments, the ingredients were mixed thoroughly to a thick paste then spread to form a thin sheet and rapidly frozen at -80 °C. Freezing facilitated storage and delivery of the feed (higher specific gravity and stability when sinking) without affecting its palatability. The proportion of sand to biodeposits for the four feed treatments were 95.8:4.2, 83.3:16.7, 50:50 and 16.7:83.3 resulting in nominal TOM content of 1, 4, 12, and 20% of the dry weight of the feeds respectively.

To determine the actual TOM content of the four feed treatments, three random samples of each prepared feed treatment were analysed. The samples were oven dried at 60 °C for 48 h, weighed and then placed in a furnace for 6 h at 500 °C to ensure complete combustion of organic matter, and then re-weighed. Percentage of TOM was calculated by sample weight lost after combustion (Byers et al., 1978).

3.2.3. Mussel Farm Sampling

The TOM content of the feed treatments was selected in order to represent the uneven distribution of organic content that the sea cucumbers will encounter beneath a typical mussel farm (Fig. 3.1). This was determined by divers taking samples from the surface of benthic sediments beneath a typical mussel farm in the north-eastern New Zealand (90 × 180 m,. W × L; with a water depth ranging from 12 to 16 m). The farm consisted of nine parallel grow out lines each containing 180 droppers of cultured mussels, *P. canaliculus*. On December 2010, sediment samples were taken every ten metres in a grid covering the benthic footprint of the farm which extended 10 m beyond the margin of the farm. The benthic samples were analysed for TOM content as described above after they were sieved (2 mm mesh) to remove large pieces of shell material.

3.2.4. Experiment Design and Rearing Conditions

Sixty gut-evacuated sea cucumbers with an overall mean weight of 20.0 g (± 0.4 S.E.) were randomly selected and allocated to one of twelve tanks. Each tank contained five sea cucumbers with an initial mean stocking biomass of 500.4 g m^{-2} (± 11.2 S.E.). Three out of the twelve tanks were randomly allocated to each feed treatment. The tanks where the sea cucumbers were maintained were square polyethylene tanks with a floor area of 0.20 m^2 (0.55 × 0.35 × 0.21 m, L × W × H with a water depth of 0.18 m). The tanks were supplied with fresh seawater flowing at a rate of 14 l h^{-1}, which was filtered (50 µm mesh) to prevent additional marine organic material entering the experiment. A natural light cycle (12:12 L:D) was maintained during the experiment, although shaded from direct sunlight (L: 0.2-0.3 µE m^{-2} s^{-1}). Seawater temperature was measured every day and was controlled with a seawater chilling unit and maintained at 19.3 °C (± 0.2 S.E.) to avoid possible negative effects of higher temperatures (Slater et al., 2009).

3.2.5. Sample collection procedures

Each sea cucumber allocated to the different feed treatment was photo-identified at the beginning of the experiment so that its growth could be followed through the experiment (Raj, 1998b). The weight of the sea cucumbers was measured as previously explained, both at the outset of the experiment and at the end of the experiment after six weeks. Individual wet weight data were then used to calculate a daily specific growth rate. During the experiment, sea cucumbers were fed weekly with a food ration that was equivalent to the 30% of wet weight of the sea cucumbers per day (Slater et al., 2009). At the end of each week the remaining uneaten feed was completely recovered from each tank, gently rinsed with freshwater to remove salt and oven dried at 60 °C until constant weight in order to determine the ingestion rate. The faeces of the sea cucumbers were easy to separate from the feed and they were removed by siphon every morning. Once a week, the faeces produced between 08:00 and 20:00 (day) and between 20:00 and 08:00 (night) were recovered from each tank, gently rinsed with freshwater to remove salt and oven dried at 60 °C until constant weight in order to determine the faecal production rate.

3.2.6. Data Calculation

The daily specific growth rate (DSGR), ingestion rate (IR); day and night faecal production rate (FPR); apparent absorption efficiency (AAE) and food conversion efficiency (FCE) were calculated as per Yuan et al. (2006):

$DSGR$ (% d^{-1}) = $100*(LN(W_f) - LN(W_i))/t$
IR (g ind^{-1} d^{-1}) = $C/n/t$
FPR (g ind^{-1} d^{-1}) = $(W_{fa}/n)/t$
AAE (%) = $100*(IR - FPR)/IR$
FCE (%) = $100*(W_f - W_i)/(C_t/n)$

Where W_f, is the weight (g) of each sea cucumber at the end of the experiment; W_i, is the initial weight (g) of each sea cucumber at the beginning of the experiment; C, is the dry weight (g) of food consumed in a week in each tank (dry weight food offered - dry weight uneaten food); C_t, is the dry weight (g) of the food consumed during the whole experiment in each tank; W_{fa}, is the

dry weight (g) of the faeces of the sea cucumbers in each tank; *n*, is the number of sea cucumbers in each tank, and *t*, is the time in days.

3.2.7. Statistical Analyses

The mean wet weights of the sea cucumbers in each treatment at the commencement of the experiment were compared using a one-way ANOVA to ensure that the results would not be biased from the outset by any differences in animal sizes in different treatments.

Data for the following experimental variables; IR, FPR, AAE and FCE were compared among treatments using an ANOVA after firstly confirming normality and homogeneity of variances of the data (Quinn and Keough, 2002). Where an overall experimental effect was detected, significant differences were then identified with pairwise comparisons of treatment means using the Tukey's test (Quinn and Keough, 2002).

3.3. Results

3.3.1. Mussel Farm Biodeposit Sampling

The TOM, in the biodeposits sampled beneath the mussel farm ranged from 2.8% to 18.2% TOM which were within the range covered in the laboratory feeding experiments (i.e., 1, 4, 12, and 20% TOM by dry weight). At the margins of the farm footprint the TOM were at their lowest values, typically around 4% TOM. Nearby and beneath the first lines of mussels the TOM levels increased greatly to around 17% TOM (Fig. 3.1). Toward the centre of the farm the TOM levels tended to be more intermediate at around 12% TOM.

3.3.2. Experimental Feed Treatments

The measured TOM content of the four feed treatments (i.e., 1, 4, 12, and 20% TOM by dry weight) was very close to the nominal values and varied little between replicate samples, i.e., 1.0% (± 0.1 S.E.), 3.9% (± 0.1 S.E.), 11.6% (± 0.4 S.E.) and 19.8% (± 0.3 S.E.) respectively.

3.3.3. Sea Cucumber Weight and DSGR

There were no significant differences in the mean sea cucumber wet weight among treatments at the beginning of the experiment (ANOVA, $F_{(11, 48)} = 1.2$, $P = 0.3$) (Fig. 3.2). At the end of the experiment, after six weeks, the wet weight of the sea cucumbers significantly increased in the 4, 12, and 20% TOM feed treatments (ANOVA, $F_{(1, 28)} = 4.4$, $P = 0.045$; ANOVA, $F_{(1, 28)} = 52.3$, $P < 0.001$; ANOVA, $F_{(1, 28)} = 11.5$, $P = 0.002$, for the 4, 12, and 20% TOM feed treatments respectively). However, there were no differences detected among the final wet weights of sea cucumbers from the 4, 12, and 20% TOM feed treatments (Tukey, $P > 0.05$) (Fig. 3.2). The mean DSGR of the sea cucumbers from the 1% feed treatment was negative (-0.1% d^{-1} ± 0.1 S.E.), although no significant differences were detected between the initial and final wet weight of the sea cucumbers of this treatment. The combined mean DSGR for the other three treatments was 0.6% d^{-1} (± 0.1 S.E.).

3.3.4. Ingestion Rate and Faecal Production Rate

The sea cucumbers were observed feeding actively in all the replicate tanks within all treatments within 24 h of the first addition of the food. Throughout the rest of the experiment, evidence of continued feeding was apparent from the production of faeces. Only in the feed treatment with 4% of TOM was the feed fully consumed prior to the weekly food change in all the replicate tanks, from the second week until the end of the experiment. Thus the time until when the feed was fully consumed was used for the calculation of the IR in this treatment. Consumption of faeces, known as “reworking” in other species of sea cucumbers, was not observed on any occasion over the duration of the experiment.

Both the IR and FPR followed a similar trend (Fig. 3.3 and 3.4 respectively) with both decreasing significantly as the TOM availability in the feed increased from 4% to 20% (ANOVA, $F_{(3, 8)} = 29.5$, $P < 0.001$ for the IR and ANOVA, $F_{(3, 8)} = 30.7$, $P < 0.001$ for the FPR). Only the IR and FPR in the 1% TOM feed treatment did not follow this trend and both IR and FPR were smaller than for the 4% TOM feed treatment (Tukey, $P > 0.05$ in both cases). In all the feed treatments the sea cucumbers were feeding more actively during night as FPR were higher at night when compared with the day values ($P < 0.001$ in all cases) (Fig. 3.5).

3.3.5. Apparent Absorption Efficiency

The mean AAE was significantly different among treatments (ANOVA, $F_{(3, 8)} = 35.3$, $P < 0.001$). The AAE increased from the 1% TOM feed treatment to the 20% TOM feed treatment (Tukey, $P < 0.05$) (Fig. 3.5). However, there were no significant differences between the AAE of the 4 and 12% TOM feed treatments (Fig. 3.6).

3.3.6. Food Conversion Efficiency

The mean FCE was significantly different among treatments (ANOVA, $F_{(3, 8)} = 42.47$, $P < 0.001$). The mean FCE was negative in the treatment with 1% of TOM and was smaller than the FCE for the remaining treatments (Tukey, $P < 0.05$ in all cases). The 12 and 20% TOM feed treatments were the only paired comparison of treatment means for FCE that did not show a significant difference (Tukey, $P = 0.9$) (Fig. 3.7).

3.4. Discussion

3.4.1. Food Availability and Growth

The results of this study show that juvenile *A. mollis* are able to maintain similar growth rates when the TOM content in the food decreased from 20% to 4% when the only source of organic food material was *P. canaliculus* biodeposits. The lack of variation in the growth rate of the juvenile sea cucumbers within that TOM range was achieved mainly by changes in the ingestion rate and also through changes in the absorption and food conversion efficiencies. These changes are typical compensatory responses of deposit-feeder animals to changes in the food quality and quantity (Cammen, 1980; Yingst, 1982; Lopez and Levinton, 1987; Jumars and Wheatcroft, 1989; Roberts et.al., 2000). The ingestion rate (dried weight) increased from 16% of their initial wet weight in the 20% TOM feed treatment to 47% of their initial wet weight in the 4% TOM feed treatment. This compensation of the ingestion rate at decreasing TOM levels is independent of the ingredients used to prepare the feed as has been reported previously in *A. japonicus* and *A. mollis* feeding on different formulated diets (Huiling et al., 2004; Yuan et al., 2006; Liu et al., 2009; Slater et al., 2009; Maxwell et al., 2009). Conversely, the absorption and food conversion of the feed became more efficient as the TOM in the food increased and as the sand proportion

decreased and more organic particles were available for digestion and absorption. Another way to increase the nutrient intake by juvenile *A. mollis* when they are limited in the food is to increase the selection efficiency of organic particles as the TOM content in food decreases (Zamora and Jeffs, 2011). Due to these adjustments, the sea cucumbers are able to continue to use low levels of TOM in their food to maintain growth. However, these adjustments appear to be of limited effectiveness at very low TOM in food because no weight gain was detected in the juveniles fed the 1% TOM feed treatment. The lack of growth in the sea cucumbers from the 1% feed treatment is more likely to be due to the low availability of nutrients in the feed and the reduction in the palatability reflected in the reduction of the ingestion rate. Similar results have been reported for *A. japonicus* when the biodeposits are dried during preparation, removing beneficial bacteria, vitamins and fatty acids from them (Yuan et al., 2006). Also, in juvenile *A. mollis* weight loss has been attributed to changes in the palatability of the food and to inadequate feeding rates (Slater et al., 2009). Contrary to the negative effect on growth observed in the 1% TOM feed treatment, in the 4, 12, and 20% TOM feed treatments the organic material available in the feed was sufficient to stimulate feeding and growth, resulting in a combined final mean stocking biomass of 650 g m^{-2} (± 19.8 S.E.) and in a combined mean DSGR of 0.6% d^{-1} (± 0.1 S.E.). This DSGR is similar to the maximum DSGR reported in this species (0.82% d^{-1}) after one month of being fed only with mussel biodeposits (25% TOM) under similar conditions (Slater et al., 2009). Furthermore, despite the wide range of TOM used in this study the mean DSGR of these three feed treatments (i.e., 4, 12, and 20% TOM) was substantially higher than the DSGR reported for juveniles of *A. mollis* caged in their natural habitat (mean 0.30% d^{-1}) after nine months feeding on sediments containing around 5% TOM (Slater and Jeffs, 2010). However, our experiment was only short-term and it is unclear if the growth rates we observed could be maintained over a longer period as bigger sea cucumbers have higher energy requirements (Maxwell et al., 2009). Moreover, when juveniles are being fed with only mussel waste (25% TOM), at a similar feeding rate of this study, their growth rate becomes limited when the stocking biomass reaches 810 g m^{-2} (Slater et al., 2009).

3.4.2. Consequences for Co-culture

The survey of the benthos beneath the mussel farm showed that there is a great deal of small scale spatial variability in the TOM content of the surface sediments which were within the range used for the feeding experiments (i.e., 1, 4, 12, and 20% TOM by dry weight). This wide range

of TOM beneath a mussel farm needs to be considered in future co-culture efforts with sea cucumbers, especially for the selection of suitable mussel farms and for determining the initial stocking biomass of sea cucumbers. Mussel farms with areas of sediment with low TOM levels will support substantially lower sea cucumber biomass because sea cucumbers placed in a low TOM zone (around 4%) will need to process more than three times more material than the sea cucumbers placed in a high TOM zone (around 18%) in order to generate similar growth. It has to be taken into account that in this study the sea cucumbers in the 4% TOM feed treatment were the only ones that consumed the entire weekly food ration before the end of the week; hence their growth rate could have been an underestimation of their full potential. In contrast, in the 12 and 20% feed treatments there was sufficient food remaining to support feeding by additional sea cucumber biomass. Then, for a typical mussel farm, like the one sampled in this study with an average TOM level of 11.1% (± 0.4% S.E.) a conservative initial stocking biomass of juvenile sea cucumbers would be 100 g m^{-2}; i.e., five juveniles of 20 g per m^{-2} (81,000 juveniles in total for the 16,200 m^{-2} of the total farm area). Under ideal conditions (favourable environmental variables, constant food supply, no health issues or loses due to mortality or escapes) this initial biomass should turn into 500 g m^{-2} (i.e., five adults of 100 g per m^{-2}) after nine months growing at a constant growth rate of 0.6% d^{-1}. However, it is likely that external variables that cannot be controlled, such as seawater temperature or the hydrodynamic regime to which the farm is exposed, may be less than ideal and as a consequence growth and survival of the co-cultured sea cucumbers may be affected (Zhou et al., 2006; Slater and Carton, 2007; Paltzat et al., 2008). As co-cultured sea cucumbers are normally caged, food availability will affect their growth as their foraging behaviour will be constrained and they will be unable to search for more organically rich patches of sediment (Da Silva et al., 1986; Mercier et.al., 1999; Uthicke and Karez, 1999; Roberts et al., 2000; Slater, 2010). Therefore, the reduction in the food availability may be one reason why sea cucumbers have been observed to escape from their cages. The negative effect of caging sea cucumbers in low TOM areas has already been observed in juveniles of *A. mollis* caged in their natural habitat (Slater and Jeffs, 2010). Leaving the sea cucumbers free to forage beneath the mussel farm would provide them with additional capacity to manage the natural spatial and temporal variation of food availability and would avoid the expenses associated with cage management. This is not unrealistic because sea cucumbers tend not to move beyond the farm footprint due to the corresponding marked decrease in the TOM content of the sediments at the margins of the farm (Slater and Carton, 2010).

3.5. Conclusions

The results of the current study of juvenile *A. mollis* show the capacity of these sea cucumbers to adjust to changes in the quality of the food, mainly by changes in the food ingestion and absorption which were ultimately expressed in changes in the final wet weight of the sea cucumbers. These changes enabled the growth of the sea cucumbers to be maintained when the TOM content ranged from 4 to 20%. Therefore, the mechanisms used by the juvenile sea cucumbers to adjust to the typical spatial and temporal variability in TOM content of sediments found beneath a mussel farm should be taken into account when selecting a suitable mussel farm and for estimating the initial appropriate sea cucumber stocking biomass. Further research is necessary to determine how other environmental variables such as seawater temperature and the hydrodynamic regime of mussel farms can also modify sea cucumber retention, survival and growth in co-culture beneath mussel farms.

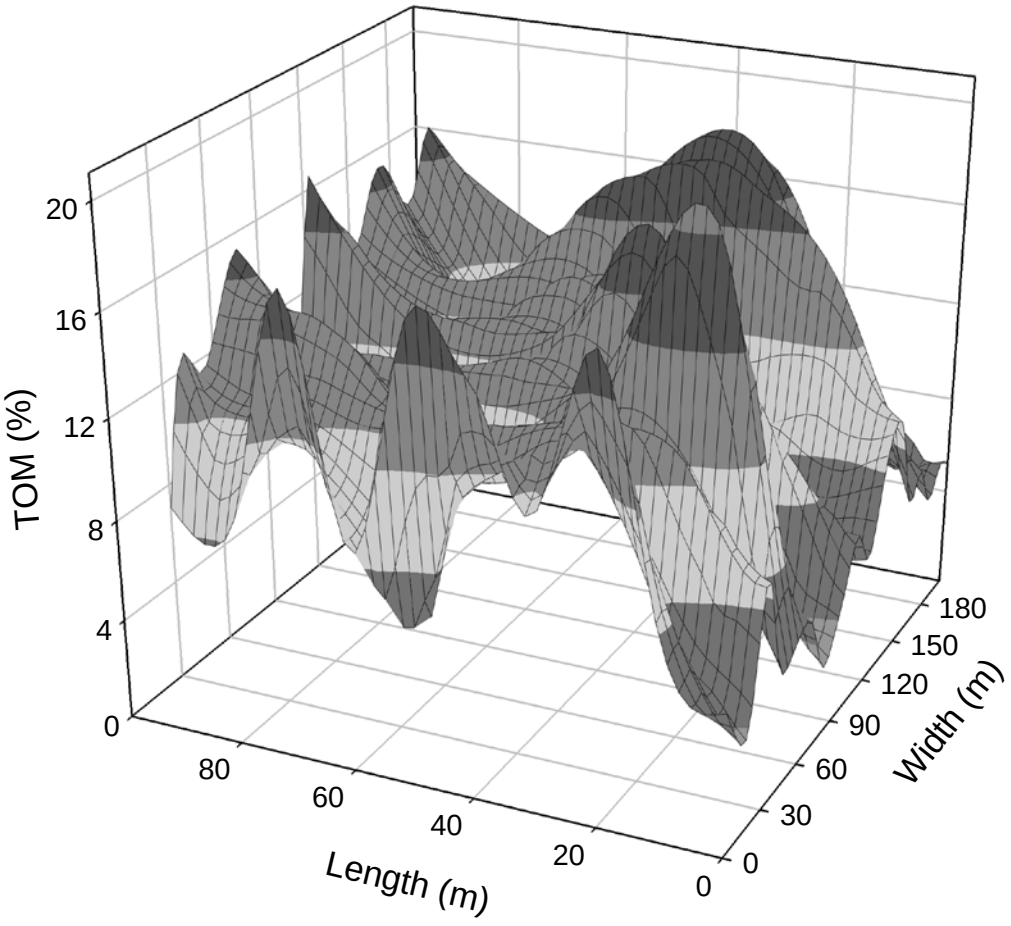

Figure 3.1. Spatial variation in the total organic matter content (TOM) of the sediments beneath a typical *Perna canaliculus* mussel farm (90 m wide, 180 m length) on the north-eastern coast of New Zealand.

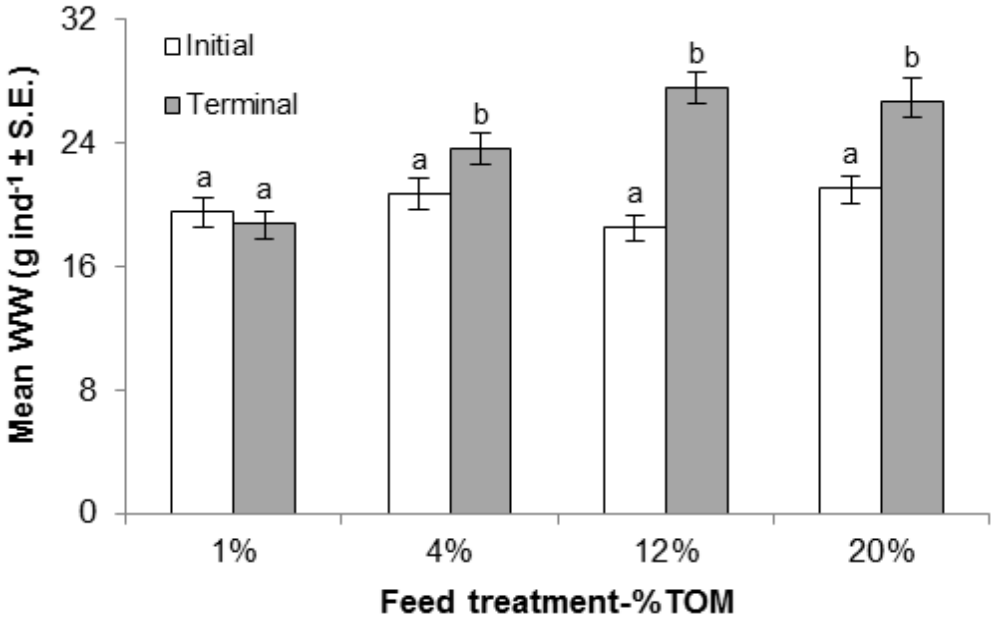

Figure 3.2. Mean initial and terminal wet weight (WW) of *Australostichopus mollis* fed with four different feed treatments containing 1, 4, 12, and 20% TOM derived from *Perna canaliculus* biodeposits over a six week period. Different letters within each treatment represent significant differences ($P < 0.05$).

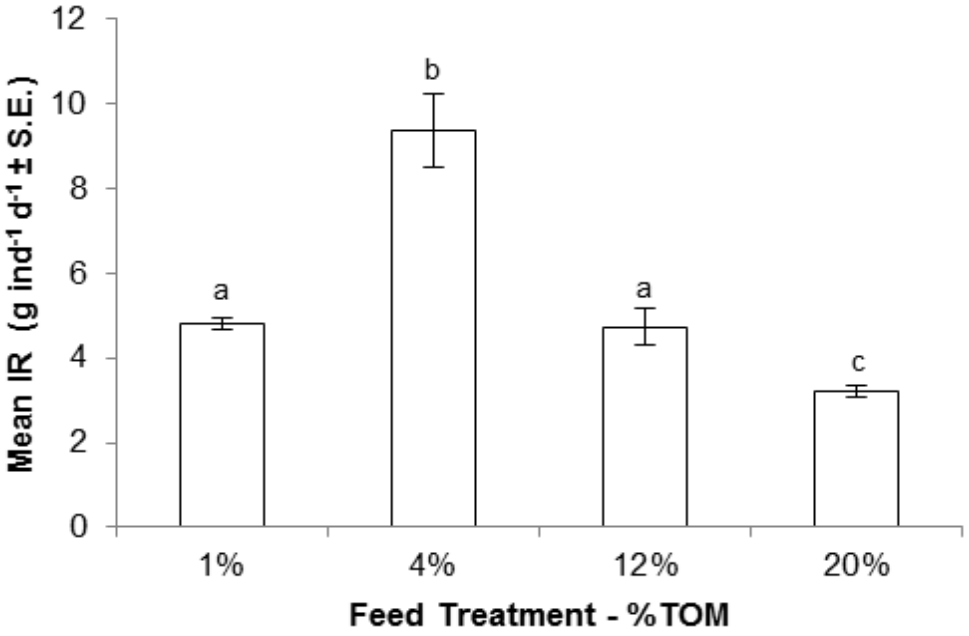

Figure 3.3. Mean ingestion rate (IR) of *Australostichopus mollis* fed with four different feed treatments containing 1, 4, 12, and 20% TOM derived from *Perna canaliculus* biodeposits. Different letters among treatment means represent significant differences ($P < 0.05$).

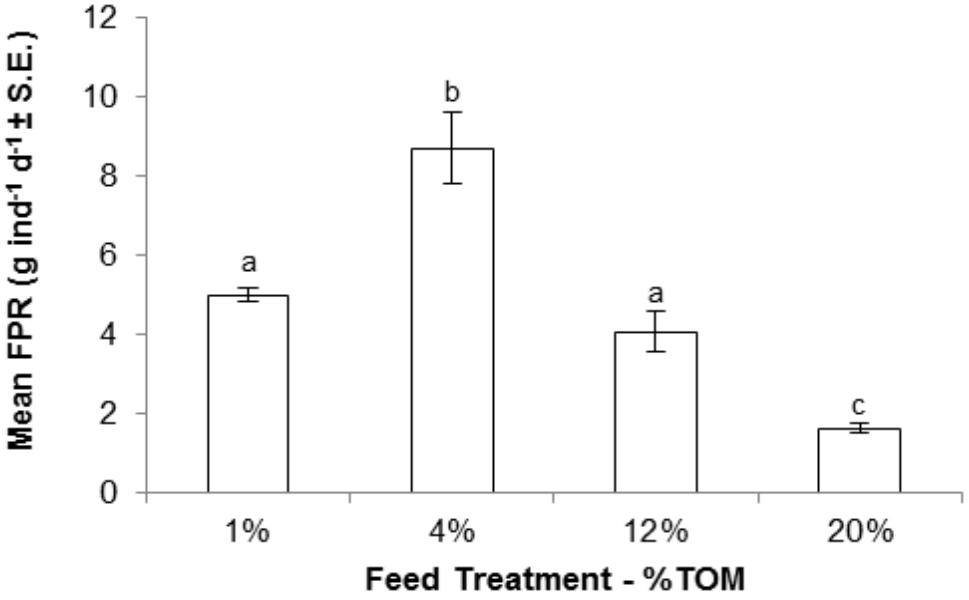

Figure 3.4. Mean faecal production rate (FPR) of *Australostichopus mollis* fed with four different feed treatments containing 1, 4, 12, and 20% TOM derived from *Perna canaliculus* biodeposits. Different letters among treatment means represent significant differences ($P < 0.05$).

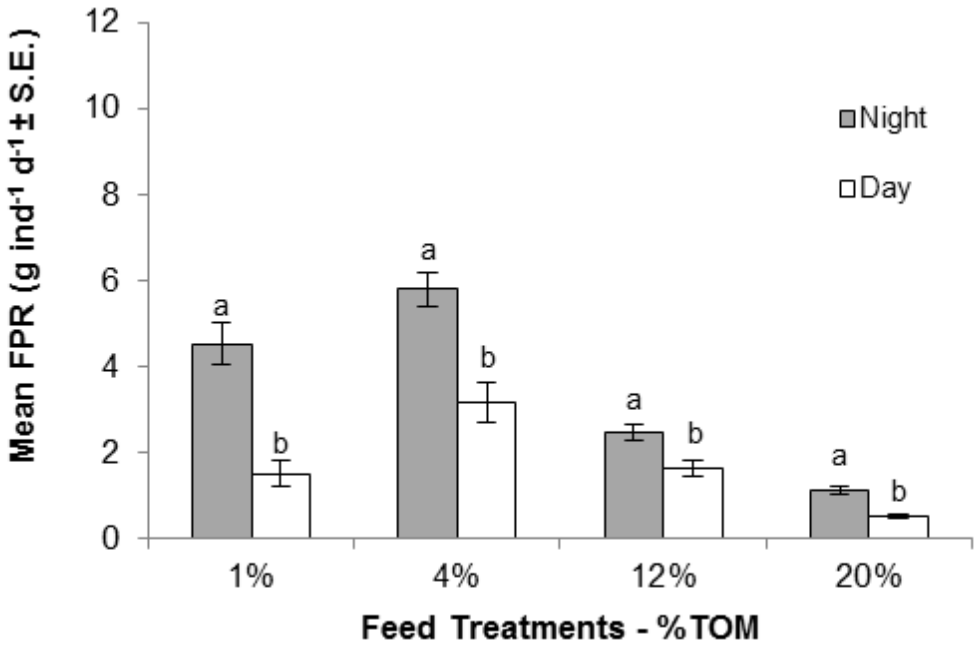

Figure 3.5. Mean night and day faecal production rate (FPR) of *Australostichopus mollis* fed with four different feed treatments containing 1, 4, 12, and 20% TOM derived from *Perna canaliculus* biodeposits. Different letters for night and day means within each treatment represent significant differences ($P < 0.05$).

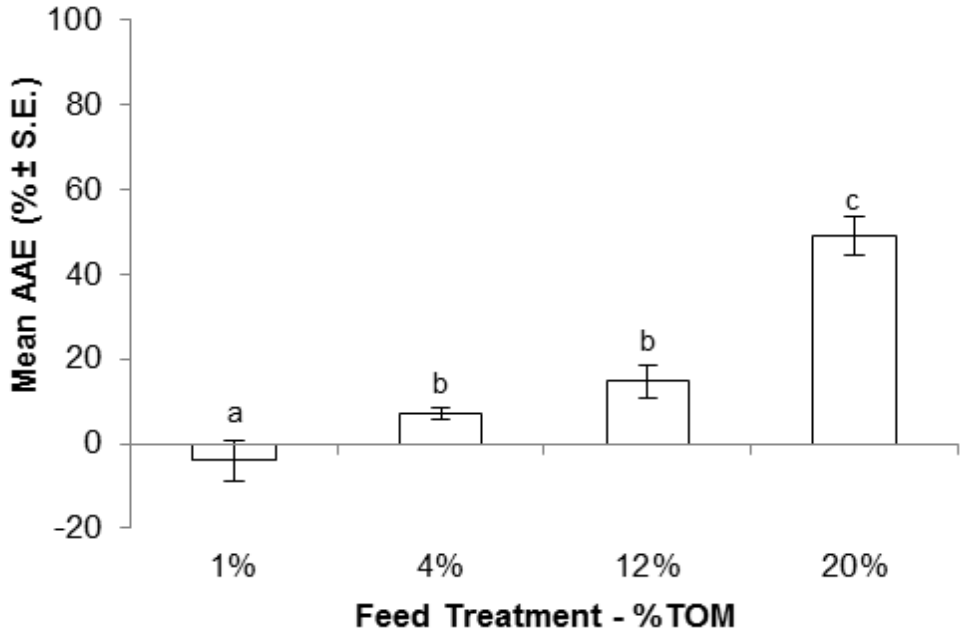

Figure 3.6. Mean apparent absorption efficiency (AAE) of *Australostichopus mollis* fed with four different feed treatments containing 1, 4, 12, and 20% TOM derived from *Perna canaliculus* biodeposits. Different letters among treatment means represent significant differences ($P < 0.05$).

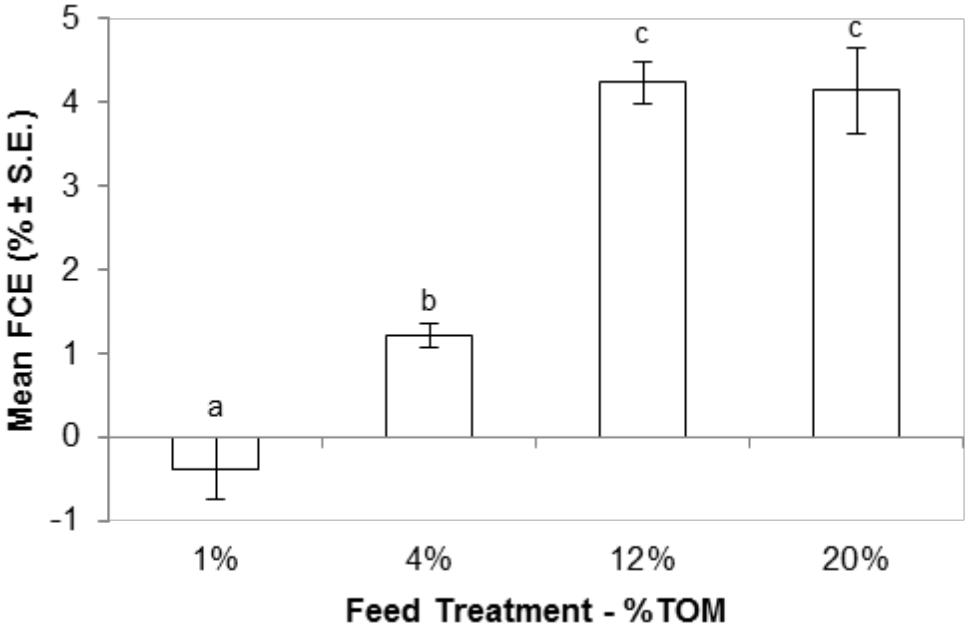

Figure 3.7. Mean food conversion efficiency (FCE) of *Australostichopus mollis* fed with four different feed treatments containing 1, 4, 12, and 20% TOM derived from *Perna canaliculus* biodeposits. Different letters among treatment means represent significant differences ($P < 0.05$).

Chapter Four

Feeding, metabolism and growth in response to temperature in juveniles of the Australasian sea cucumber *Australostichopus mollis*

Abstract

In the present study the importance of seawater temperature for the food intake, food utilization and growth of aquacultured juveniles of the Australasian sea cucumber, *Australostichopus mollis*, was examined. The juveniles (16.5 ± 0.5 g, wet weight) were experimentally exposed to four seawater temperatures (15, 18, 21, and 24 °C) for 105 days, during which they were fed mussel waste, a highly effective natural food source, that is utilized by sea cucumbers under co-culture conditions beneath mussel farms. At each temperature treatment the feeding, metabolism, growth and survival of the juveniles were evaluated. Overall, the sea cucumbers responded negatively to an increase of temperature from 15 to 21 °C with decreased food intake, and growth rates, and elevated metabolism. The survival of juveniles was compromised when held at 24 °C. Most importantly, the food conversion efficiency of juveniles at 15 °C was over seven times greater than for those growing at 21 °C which was reflected in the growth rates (0.71 ± 0.05% d^{-1} versus 0.28 ± 0.05% d^{-1}).The results of this study suggest that consideration of ambient temperature regimes will be a critical factor for the selection of suitable aquaculture locations for *A. mollis* due to the risk of mortality at higher temperatures (≥ 24 °C) and markedly improved growth and food utilization at lower temperatures around 15 °C.

4.1. Introduction

The current high value and demand for sea cucumbers has led to the depletion of many wild populations and this has led to increased interest in developing the aquaculture of several species globally. One of the most important variables to consider for the aquaculture of sea cucumbers is seawater temperature. This is because sea cucumbers are ectothermic so that their body temperature closely follows the ambient seawater temperature which in turn modulates most of their biochemical and physiological processes. Therefore, changes in seawater temperature can alter important parameters for the aquaculture of sea cucumbers, such as their feeding behaviour,

metabolism, growth and even their survival (Mercier et al., 1999; Yang et al., 2005, 2006; Dong et al., 2006, 2008a; An et al., 2007; Ji et al., 2008; Meng et al., 2009). Most sea cucumber species can tolerate a relatively wide ambient temperature range within their natural habitat (Pawson, 1966), although optimal performance is usually restricted to a specific thermal range (Meng et al., 2009). The optimal seawater temperatures are species-specific and have only been determined for a few commercially valuable species (Yanagisawa, 1998). For instance, the optimal seawater temperatures for growth in *Apostichopus japonicus* (temperate species) and in *Holothuria scabra* (tropical species) are around 16 and 30 °C respectively. However, their growth is greatly reduced if the seawater temperature goes beyond or below specific thresholds (Dong et al., 2006; An et al., 2007; Ji et al., 2008; Lavitra et al., 2010).

Information on temperature tolerance is currently lacking in several sea cucumber species recently targeted for commercial aquaculture development, including the Australasian sea cucumber *Australostichopus mollis*. In this species, almost all the previous studies have focused on larval, juvenile and adult feeding biology and ecology, and little attention has been given to the importance of temperature on aquaculture performance (Morgan, 2008a; Slater et al., 2009, 2011a; Maxwell et al., 2009; Slater and Carton, 2010; Slater and Jeffs, 2010; Zamora and Jeffs, 2011, 2012b). A better understanding of how this species responds to changes in seawater temperature will be invaluable for the development of effective commercial aquaculture production for this species.

Australostichopus mollis is a deposit-feeding sea cucumber found over an extensive natural range along the entire coast of New Zealand and southern Australia, covering over 13 ° of latitude (Pawson, 1970). The ambient seawater temperatures within this distribution range vary from below 8 °C in the higher latitude regions and over 20 °C during summer in the lower latitudes of the range (Garner, 1969). This sea cucumber can be found in a wide variety of shallow seawater habitats from rocky reefs to sandy seafloor and mudflats (Pawson, 1970; Mladenov and Campbell, 1998; Morgan, 2011). In these shallow seawater habitats they are also exposed to marked daily and seasonal variations of seawater temperature. Seawater temperature plays an important role in the reproductive cycle of *A. mollis* (Morgan, 2009a). Also in larger sea cucumbers, an increase in seawater temperature (from 14 to 18 °C) affects feeding and produces a significant increase in ammonia excretion and respiration rates (Maxwell et al., 2009). However, for juveniles, there is only an indication that higher seawater temperatures (> 20 °C)

may negatively affect growth and survival (Slater et al., 2009; Slater and Jeffs, 2010). Therefore the main aim of the present study is to determine the response of juvenile *A. mollis* to different temperatures in terms of feeding, ammonia excretion, oxygen consumption and growth. These data will assist in the selection of suitable locations for coastal aquaculture of these sea cucumbers, and begin to define the ideal operating parameters of land-based aquaculture facilities where it may be possible to manipulate seawater temperatures.

4.2. Materials and Methods

4.2.1. Experimental Animals

Juvenile sea cucumbers of a similar size (12 – 25 g) were collected from the Mahurangi Harbour in northern New Zealand by divers during winter (June) of 2010. The depth of the sampling location did not exceed 11 m and the seawater temperature at the time of sampling was 14.8 °C. The sea cucumbers were transferred to the nearby Leigh Marine Laboratory and held in tanks with flowing 100 µm filtered seawater at ambient temperature (15 °C). The sea cucumbers were left without feed for 48 h to ensure that the gut contents were fully evacuated before they were weighed and used in the experiments. The weight of the sea cucumbers was measured to the nearest gram according to the procedure described by Sewell (1990) in which most of the internal and external excess water is gently removed. Individual sea cucumbers were photoidentified so that they could continue to be recognised throughout the experiment from their pattern of body markings (Raj, 1998b).

4.2.2. Experimental Set up

Four seawater temperature treatments were established in order to represent the natural seawater temperatures to which this species may be exposed during the year in the sampling area (Scarsbrook, 2008; Auckland Regional Council, 2008). Three of the experimental temperatures represent the ambient seawater temperatures that can be commonly encountered during winter, spring and summer (15, 18, 21 °C respectively). The last temperature treatment corresponds to an extreme seawater temperature that can be rarely present in the area during summer (24 °C). For each of the four temperature treatments three replicate polyethylene tanks (0.33 x 0.21 x 0.15 m, L x W x H) were used. Four juvenile sea cucumbers of similar weight were randomly selected

and allocated to each of the replicate tanks (16.5 ± 0.5 g; total mean wet weight ± S.E.) (Fig. 4.1). The tanks had a continuous flow (0.2 l min^{-1}) of filtered seawater from a common source for which the temperature was controlled to ± 0.01 °C for each temperature treatment.

4.2.3. Thermal Acclimation, Feeding and Cleaning

The sea cucumbers exposed to the 15 and 18 °C temperature treatments were transferred directly from the ambient seawater temperature to their tanks, because such a temperature change would be possible in the wild with tidal changes in seawater temperature. However, the sea cucumbers for the higher temperature treatments (21 and 24 °C) were allowed to acclimate first to 18 °C for three days prior to being transferred to 21 °C. Sea cucumbers allocated to the 24 °C treatment were acclimated for three days at 21 °C prior to transfer to the 24 °C treatment tanks. Throughout this experiment the sea cucumbers were fed to satiety every two days with mussel waste recovered from beneath tank-cultured green-lipped mussels, *Perna canaliculus* (Slater et al., 2009). Mussel waste was used as a food for the experiment because it has been proven to be an excellent food for juvenile sea cucumbers of this species (Slater et al., 2009; Zamora and Jeffs, 2011, 2012a). The daily feed ration of wet mussel waste was set at 30% of the sea cucumbers' wet weight. The sea cucumbers were fed every two days after uneaten feed had been removed from the tanks. The organic composition of the feed was found to not change significantly over the two day period that it was available for sea cucumbers (*unpublished data*). The sea cucumber faeces were removed twice a day, in the morning and in the evening, by carefully siphoning them out of the tanks.

4.2.4. Weight Measurement

The weight of photographically identified individual sea cucumbers was measured at the outset of the experiment and every 35 days over 105 days. The weight data was then used to measure the growth rate of the sea cucumbers for each temperature treatment as per Ricker (1979):

$$DSGR\ (\%\ d^{-1}) = 100*(LN\ (W_{scf}) - LN\ (W_{sci}))/t$$

Where W_{scf}, is the wet weight of each sea cucumber at the end of the growth experiment; W_{sci}, is the wet weight of each sea cucumber at the beginning of the growth experiment; and t, is the time in days.

4.2.5. Ingestion and Faecal Production Rates

Every week the ingestion rate was measured in each replicate tank by recovering the uneaten feed after a period of two days. During the same period the faeces were recovered in the morning and in the evening and then pooled in order to estimate the faecal production rate. Both the recovered uneaten feed and faeces were carefully rinsed with distilled water to remove salt and then oven dried at 60 °C until a constant weight was achieved. The Ingestion rate (IR) and faecal production rate (FPR) were estimated as per Yuan et al. (2006):

IR (g ind^{-1} d^{-1}) = $C/n_{sc}/t$
FPR (g ind^{-1} d^{-1}) = $(W_{fa}/n_{sc})/t$

Where C, is the dry weight of food consumed in 48 hours in each tank (dry weight food offered - dry weight uneaten food); W_{fa}, is the dry weight of the faeces of the sea cucumbers in each tank pooled together in 48 h; n_{sc}, is the number of sea cucumbers in each tank; and t, is the time in days.

4.2.6. Apparent Absorption and Food Conversion Efficiencies

The apparent absorption efficiency (AAE) and the food conversion efficiency (FCE) were estimated as per Yuan et al. (2006):

AAE (%) = $100*(IR - FPR)/IR$
FCE (%) = $100*(W_f - W_i)/(C_t/n_{sc})$

Where IR, is the ingestion rate in each tank; FPR, is the faecal production rate in each tank; W_f, is the final combined wet weight of the sea cucumbers in each tank; W_i, is the initial combined wet weight in each tank; C, is the dry weight of food consumed in 48 h in each tank (dry weight food offered - dry weight uneaten food); and n_{sc}, is the number of sea cucumbers in each tank.

4.2.7. Ammonia Excretion

Ammonia production by the juvenile sea cucumbers was measured, as it is thought to be the main metabolic waste in echinoderms (Jangoux, 1982). In order to do this, another group of juvenile sea cucumbers were obtained from the collection site in the Mahurangi Harbour during winter (June) of 2010 (26.3 ± 1.2 g wet weight; 1.9 ± 0.1 g dry weight). Twelve randomly selected sea cucumbers were acclimated to each temperature treatment following the methods described above and fed with mussel waste for a week. Then they were starved for two days to let them void their digestive tract. Then each sea cucumber was placed in an underwater sealed plastic chamber (0.75 l) inside a water bath set up to the respective acclimation seawater temperature. The sea cucumbers were left in the chamber for three hours, after which time a seawater sample was taken to measure the ammonia concentration. As a control three additional chambers were sealed underwater without a sea cucumber. Seawater samples from the control chambers and from the chambers with sea cucumbers were analyzed for ammonia concentration.

Ammonia was quantified by the indophenol blue method, avoiding precipitation of calcium and magnesium on the seawater using citrate (Koroleff, 1983). In this method ammonia interacts with phenol in an alkaline solution, using hypochlorite as an oxidizing agent and nitroprusside as a catalyst, then the absorbance of the resulting indophenol is measured at 630 nm. The ammonia excretion rate (AER) was estimated as follows (modified from Maxwell et al., 2009):

$$AER\ (\mu g\ g^{-1}\ h^{-1}) = (A_{sc} - A_c)*F*V/W_{sc}/t$$

Where; A_{sc}, is the absorbance in each seawater sample from a chamber with a sea cucumber; A_c, is the mean absorbance of the control seawater samples from the chambers without a sea cucumber; F, is a factor obtained from a calibration curve using ammonia standards at different concentrations; V, is the volume of the chambers used corrected by the volume of the sea cucumber; W_{sc}, is the dry weight of the sea cucumber; and t, is the time in hours.

4.2.8. Oxygen Consumption

Oxygen consumption in sea cucumbers can be used as a good estimator of their metabolic rate as the use of anaerobic metabolism is thought to be minimal in echinoderms (Lawrence and Lane,

1982). Oxygen consumption was measured in another group of similar sized juvenile sea cucumbers obtained from the collection site during winter (June) of 2010 (19.1 ± 1.4 g wet weight; 1.3 ± 0.1 g dry weight). Twelve randomly selected sea cucumbers were acclimated to each temperature treatment following the methods previously described and fed with mussel waste for a week. Then the sea cucumbers were starved for two days to avoid metabolic responses associated with processing food, i.e., specific dynamic action.

The oxygen consumption was measured through automated, intermittent flow respirometry (Steffensen, 1989). Each acclimated, gut-evacuated sea cucumber was transferred into a 0.3 l acrylic respirometry chamber, contained within a larger 12 l reservoir tank. The seawater used in the respirometry system was filtered (5 µm), UV sterilised, oxygen saturated (with a bubble diffuser) and the temperature was controlled accordingly to each temperature treatment. After the sea cucumber had been sealed in the respirometer chamber a protocol of flush and measurement phases (15 min and 1 h respectively) was established and carried out over 25 h, always starting at 10:00 hrs. As a control for bacterial respiratory activity within the chambers, two additional measurements were made at the beginning and at end of each 25 h measurement run without the sea cucumber present in the respiratory chamber. The oxygen concentration decline during the intermittent measurement phase was quantified using the Hach, IntelliCAL™ LDO101 probe and during this time the oxygen saturation did not go below 80%. The oxygen consumption rate (OCR) in each phase was estimated as follows (modified from Maxwell et al., 2009):

$$OCR\ (\mu g\ g^{-1}\ h^{-1}) = (\Delta DO_{sc} - \Delta DO_{c})*V/W_{sc}/t$$

Where ΔDO_{sc}, is the difference in the oxygen concentration between the beginning and the end of the measurement phase when the sea cucumber was inside the chamber; ΔDO_{c}, is the difference in the oxygen concentration between the beginning and the end of the measurement phase inside the chamber without a sea cucumber; V, is the volume of the chamber corrected by the volume of the sea cucumber; W_{sc}, is the dry weight of the sea cucumber; and t, is the time in hours.

4.2.9. Statistical Analyses

All data were tested for normality and homogeneity of variance using a Shapiro-Wilk and a Levene's test, respectively. When the data were parametric a one-way ANOVA was used to test for significant differences, and when the data was not parametric a Kruskal-Wallis test was used (Quinn and Keough, 2002). In both cases a Tukey post-hoc comparison of means was performed to identify differences among groups (Quinn and Keough, 2002).

4.3. Results

4.3.1. Survival at Different Temperatures

No negative effects were observed in the 15, 18 and 21 °C temperature treatments, in which the juvenile sea cucumbers fed normally throughout the entire experiment. However, in the 24 °C temperature treatment the sea cucumbers stopped feeding and spent most of their time motionless and attached to the walls of the tank with most of them developing skin ulcerations which subsequently compromised their survival.

4.3.2. Growth at Different Temperatures

There was a clear effect of temperature on the mean growth rates of sea cucumbers over 105 days (Fig. 4.2) with overall growth significantly faster at 15 °C than at 18 and 21 °C (ANOVA, $F_{(2, 33)} = 26.9$, $P < 0.001$). At the end of the experiment, the mean wet weight at 15 °C was 22% greater than at 18 °C (ANOVA, $F_{(2, 33)} = 20.3$, $P < 0.001$) and 47% greater than for those growing at 21 °C (ANOVA, $F_{(2, 33)} = 20.3$, $P < 0.001$) (Fig. 4.1). However, these differences in wet weight among treatments became apparent only after seventy days, at which time significant differences between the wet weight of the sea cucumbers growing at 15 and 18 °C, and between the sea cucumbers growing at 15 and 21 °C (ANOVA, $F_{(2, 33)} = 8.4$, $P < 0.001$ in both cases) were detected (Fig. 4.1).

4.3.3. Ingestion Rate, Faecal Production Rate and Apparent Absorption Efficiency at Different Temperatures

There was a clear effect of temperature on feeding, with sea cucumbers feeding more actively at lower temperatures. This resulted in an increase of both the mean IR (Kruskal-Wallis, H = 30.3, d.f. = 2, $P < 0.001$) and the mean FPR (Kruskal-Wallis, H = 29.9, d.f. = 2, $P < 0.001$) as the temperature of the treatments decreased (Table 4.1). The mean IR of the sea cucumbers at 15 °C was 33% greater than those at 18 °C (Tukey, $P < 0.05$) and 106% greater than the mean IR of those at 21 °C (Tukey, $P < 0.05$). The mean FPR of the sea cucumbers at 15 °C was 32% greater than those at 18 °C (Tukey, $P < 0.05$), and 99% greater than the mean FPR of the sea cucumbers at 21 °C (Tukey, $P < 0.05$). However, no significant differences were detected in the mean AAE at different temperatures (ANOVA, $F_{(2, 6)} = 0.5$, $P = 0.60$) because the changes in the mean IR and mean FPR were broadly proportional to one another (Table 4.1).

4.3.4. Food Conversion Efficiency at Different Temperatures

The mean FCE was greatly affected by temperature, increasing as temperature decreased (ANOVA, $F_{(2, 6)} = 240.2$, $P < 0.001$) (Table 4.1). The mean FCE of the sea cucumbers growing at 15 °C was 23% greater than those growing at 18 °C (Tukey, $P < 0.05$) and more than seven times greater than the mean FCE of sea cucumbers growing at 21 °C (Tukey, $P < 0.05$).

4.3.5. Ammonia Excretion and Oxygen Consumption Rates at Different Temperatures

Higher experimental seawater temperatures generated an increase in both the mean AER (ANOVA, $F_{(2, 33)} = 37.2$, $P < 0.001$) and the mean OCR (ANOVA, $F_{(2, 33)} = 103.9$, $P < 0.001$) (Table 4.1). Only the mean AER of the sea cucumbers from the 15 and 18 °C treatments were not significantly different (Tukey, $P > 0.05$). However, the mean AER of the sea cucumbers was almost three times higher at 21 °C compared to 15 °C (Tukey, $P < 0.05$). While the mean OCR of the sea cucumbers increased almost two fold between 15 and 18 °C (Tukey, $P < 0.05$), and was almost two and half times greater in the 21 °C treatment than in the 15 °C treatment (Tukey, $P < 0.05$).

4.3.6. Eenergy Substrates

The atomic ratio of mean oxygen consumed versus mean nitrogen excreted (O/N ratio) was calculated in order to gain a better understanding of the energy substrate utilization at different temperatures (Corner and Cowey, 1968; Ikeda, 1974; Mayzaud and Conover, 1988). This ratio remained relatively low in all temperature treatments being always below 10 (Table 4.1).

4.4. Discussion

4.4.1. Growing at Different Temperatures

Currently there is a need for information on the optimal conditions for hatchery and grow out of most of the commercially valuable sea cucumber species worldwide (Conand, 2004; Ferdouse, 2004; Toral-Granda et al., 2008; Morgan, 2009b). Hatchery culture conditions can be easily controlled as it is usually performed indoors with small volumes of seawater (Xiyin et al., 2004). However, the grow out of juvenile sea cucumbers is usually carried out under less controlled conditions, such as co-culture with bivalves in coastal waters or in shallow ponds on land (Purcell, 2004; Pitt and Duy, 2004; Slater and Carton, 2007; Zhou et al., 2006; Paltzat et al., 2008; Lavitra et al., 2010). Under such conditions, the sea cucumbers are likely to be exposed to a wide range of temperatures, due to seasonal and daily fluctuations, which have the potential to greatly affect production. This is the case for juvenile *A. mollis*, for which the optimum temperature for growth and the thermal tolerances are unknown. The results of our experiment showed that an increase of temperature from 15 to 21 °C markedly reduced feeding and growth in juvenile sea cucumbers and greatly increased metabolism. Previous studies have inferred that the growth of juveniles of this species in the wild declines abruptly at seawater temperatures higher than 20 °C (Slater et al., 2009; Slater and Jeffs, 2010). A negative effect of increasing seawater temperature on feeding, excretion and respiration over a narrower temperature range (14, 16, and 18 °C) than the current study has been reported in much larger *A. mollis* (> 60 g wet weight) fed with diets made from macroalgae and abalone faeces (Maxwell et al., 2009). Regardless of the differences in size, the negative trend in feeding, excretion and respiration rates were more apparent at 18 °C than at 16 °C, which is consistent with the trend identified in the current study. A similar decline in growth response to increasing seawater temperature has been observed in the juveniles of other sea cucumbers species (Yang et al., 2005; Dong et al., 2006;

An et al., 2007; Ji et al., 2008). However, when other sea cucumber species are exposed to stressfully high seawater temperatures, they change their feeding habits or stop feeding, often burrowing into the sediment, or becoming dormant (aestivation) in order to survive. This behaviour often leads to a subsequent loss of body weight and/or metabolic depression (Mercier et al., 1999; Ji et al., 2008). At 24 °C the juvenile *A. mollis* stopped moving and feeding, which could be considered as aestivation, although the subsequent rapid deterioration of the health of juveniles suggests the lack of activity was due to thermal shock. Mortality at the experimental temperature of 24 °C reached 100% after 25 days. Juveniles of this sea cucumber species appear to be living at the upper limits of their thermal tolerance range in northern New Zealand during summer time, where temperatures in shallow waters frequently exceed 23 °C (Scarsbrook, 2008; Auckland Regional Council, 2008). The negative effects for wild populations of sea cucumber may depend on the time of exposure to elevated temperatures, with the possibility that sporadic and short-term exposure to temperatures of 24 °C or greater may be survivable. Furthermore, for juvenile sea cucumbers in the wild, exposure to temperatures around 21 °C during summer could also be sufficient to have a negative effect on growth as has been suggested by previous researchers (Slater et al., 2009; Slater and Jeffs, 2010).

4.4.2. Optimal Temperature for Growth

The lowest experimental temperature treatment of 15 °C produced the fastest growth, without a sign of density dependent limitation during the course of the experiment, going from an initial biomass of 857 g m^{-2} to 1,885 g m^{-2}. This suggests that high levels of cultured biomass (i.e., > 1,800 g m^{-2}) can be sustained without compromising growth rates provided the available food is of sufficiently high quality and that temperature remains within their optimal range. Although it is possible that temperatures lower than 15 °C may further increase the growth of this species. This possibility requires further research to identify the temperature optima and tolerances to extreme cold seawater which will be encountered during winter time in the wild. Assuming 15 °C is near the thermal optima for the juveniles of this species, the most suitable locations for the commercial aquaculture development of this species will be in the most southern regions of New Zealand and Australia. These regions have existing large scale commercial mussel aquaculture, which opens the potential for mussel and sea cucumber co-culture. The use of mussel farms at lower latitudes cannot be disregarded as it will ultimately depend on the thermal regime to which the benthos underneath the farms is exposed. Pond culture of sea cucumbers within these regions

may also be possible; however the careful management of seawater temperatures, especially during summer, will be required to ensure that the aquaculture performance of these sea cucumbers is maintained.

4.4.3. Source of Energy for Metabolism

The food substrates catabolised by the juvenile sea cucumbers for energy can be determined by the relationship between the oxygen consumption and the ammonia-nitrogen excretion rates through the calculation of the atomic O/N ratios (Mayzaud and Conover, 1988). This relationship is thought to be stable regardless of diet because nitrogen excretion is considered to vary little in relation to diet in sea cucumbers (Otero-Villanueva et al., 2004). Low O/N ratio values ($\leq$ 10) indicate that protein is mostly being catabolized, and when the ratios are higher (> 30), lipid is the primary source of energy (Ikeda, 1974). The juvenile sea cucumbers in this study consistently had O/N ratios well below 10 regardless of experimental temperature treatment (Table 4.1), indicating that they are heavily reliant on protein as a source of metabolic energy. This is consistent with previous observations of a high absorption of dietary sources of protein in juveniles of this species (Slater et al., 2011b). Low values of O/N ratios have also been reported for juveniles of *A. japonicus* (around 10) and *Scotoplanes globosa* (3.2) (Yang et al., 2006; Smith, 1983). However, larger size adults of *A. japonicus*, *S. globosa*, *Holothuria atra* and *H. scabra* have higher O/N ratios (> 20) and therefore do not depend much on protein as an energy source, possibly due to changes in their diets as they grow (Smith, 1983; Mukai et al., 1989; Yang et al., 2006). As in this current study, Yang et al. (2006) found that temperature had little or no affect on the O/N ratios of juveniles and adults of *A. japonicus*, suggesting that temperature is not important in determining the selection of substrate for deriving metabolic energy. Therefore, the greatly elevated metabolism in juveniles of *A. mollis* at the higher experimental temperatures in this current study is likely to be responsible for reducing the availability of protein for growth, hence explaining the significant reduction of growth at these temperatures.

4.5. Conclusions

The feeding behaviour, physiology and growth of juveniles of *A. mollis* are greatly affected by seawater temperature in the range of 15 - 24 °C. Overall, the sea cucumbers responded negatively to an increase in seawater temperature in terms of feeding, metabolism and growth.

According to these results, the natural seawater temperature regime should be a major factor in site selection for the out growing of this species in both land-based pond culture and co-culture with coastal shellfish farms, such as mussels. Although this study provides valuable information on the response of juvenile *A. mollis* to seawater temperature, further research is required to determine the optimal temperature for their aquaculture because it may prove to be below the lowest experimental temperature used in the current study, i.e., 15 °C. It may also be necessary to determine if sea cucumbers from different latitudinal regions vary in their feeding, metabolic and growth responses to seawater temperature.

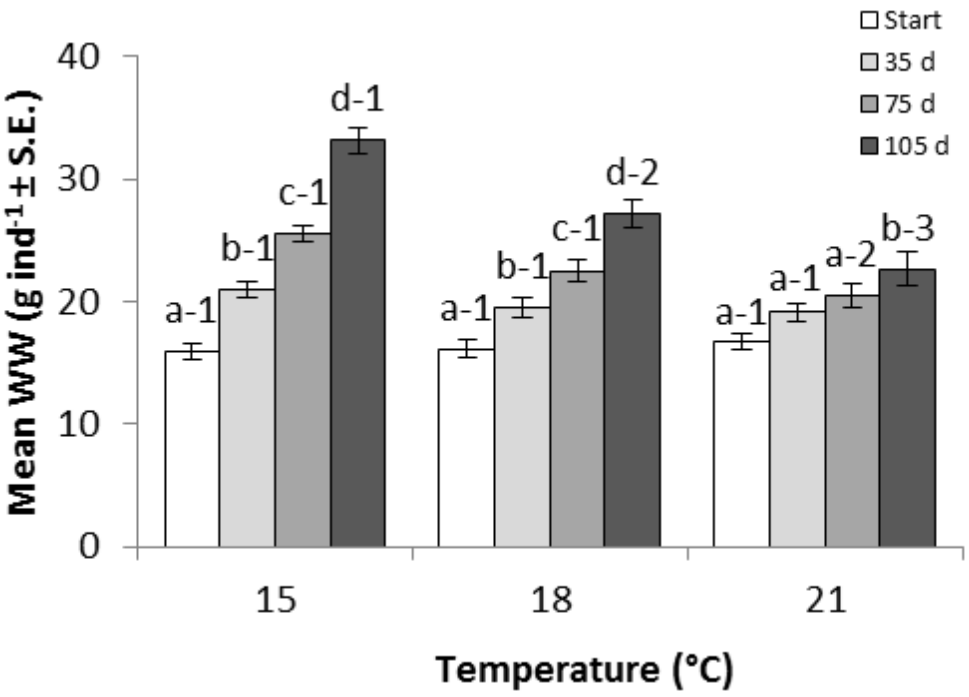

Figure 4.1. Mean juvenile *Australostichopus mollis* wet weight (WW) at the outset of the experiment, after the first 35 days, after 70 days, and after 105 days of growing at different temperatures. Different letters indicate significant differences among the mean wet weights of juvenile sea cucumbers within each temperature treatment, i.e., at different times ($P < 0.05$). Different numbers indicate significant differences in mean wet weights among temperature treatments only for the experimental period represented ($P < 0.05$).

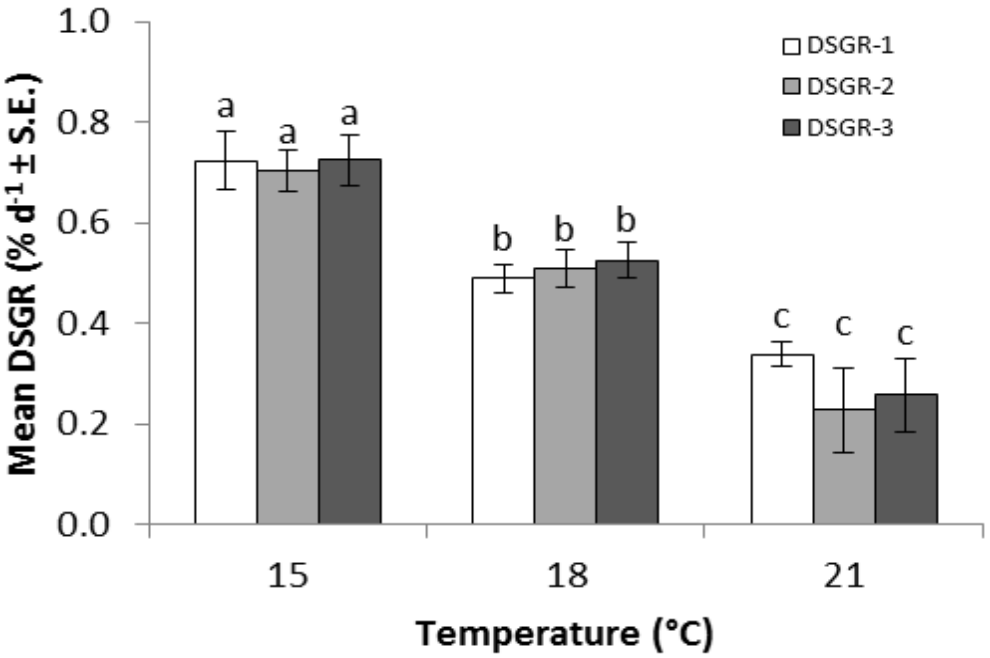

Figure 4.2. Mean daily specific growth rate (DSGR) of the juvenile *Australostichopus mollis* for the first 35 days (DSGR-1), for the second 35 days (DSGR-2), and for the last 35 days (DSGR-3) at different temperatures. Different letters within each temperature treatments and among temperature treatments represent significant differences among means ($P < 0.05$).

Table 4.1. Measured feeding and metabolic responses of the juvenile *Australostichopus mollis* at different experimental temperatures. Ingestion rate (IR), faecal production rate (FPR), apparent absorption efficiency (AAE), oxygen consumption rate (OCR), ammonia excretion rate (AER), oxygen/nitrogen ratio (O/N), food conversion efficiency (FCE). Different letters among temperatures for each parameter represent significant differences ($P < 0.05$). Values in the table are mean ± S.E.

	Temperature		
	15° C	**18° C**	**21° C**
IR (g ind^{-1} d^{-1})	5.18 ± 0.50^{a}	3.88 ± 0.63^{b}	2.52 ± 0.25^{c}
FPR (g ind^{-1} d^{-1})	4.42± 0.26^{a}	3.33 ± 0.12^{b}	2.22 ± 0.18^{c}
AAE (%)	14.72 ± 2.95^{a}	13.91 ± 1.82^{a}	11.80 ± 2.17^{a}
OCR (µg g^{-1} h^{-1})	19.63 ± 1.46^{a}	38.26 ± 4.07^{b}	50.38 ± 2.88^{c}
AER (µg g^{-1} h^{-1})	5.86 ± 0.61^{a}	8.25 ± 1.07^{a}	16.53 ± 0.97^{b}
O/N ratio	2.90	4.07	3.15
FCE (%)	3.97 ± 0.07^{a}	3.23 ± 0.23^{b}	0.55 ± 0.05^{c}

Chapter Five

Macronutrient selection, absorption and energy budget of juveniles of the Australasian sea cucumber *Australostichopus mollis*, feeding on mussel biodeposits at different temperatures

Abstract

The present study was set up to examine the selection and absorption of macronutrients (lipid, protein and carbohydrate) of juveniles of the Australasian sea cucumber *Australostichopus mollis*, feeding on an effective natural feed (mussel waste) at different temperatures. Our results indicate that the juveniles select and absorb lipid more efficiently than carbohydrate and protein at all temperatures. However, the overall magnitude of absorption of carbohydrate and protein make them the main source of nutritional energy for juvenile sea cucumbers. Seawater temperature affects the feeding behaviour of the juveniles, reducing the selection efficiency of macronutrients, while increasing metabolic energy demand, resulting in less energy available for growth. These results show the importance of each macronutrient in the diet of *A. mollis* as a source of energy for growth, which opens up the possibility to replace more expensive nutrient sources, such as protein and lipid, with less costly carbohydrate in order to reduce costs of diet formulation.

5.1. Introduction

A significant bottleneck in the aquaculture of many sea cucumbers species is the availability of highly effective artificial diets to enhance the growth of the early juveniles during the nursery stage or during grow-out when cultured in earthen ponds (Renbo and Yuan, 2004; Pitt and Duy, 2004). The development of artificial diets has been based mostly on traditional methods of trialling different ingredients and observing subsequent growth, rather than an understanding of their intake and absorption of specific macronutrients (Huiling et al., 2004; Yuan et al., 2006; Liu et al., 2009; Seo and Lee, 2011; Seo et al., 2011). Moreover, when measuring food particle selection and absorption in sea cucumbers, most studies have not measured nutrients directly but

used proxy measures such as total organic matter (TOM), organic nitrogen, organic carbon, phytopigments or variations of bacterial levels as an indicator of nutrient selection and absorption, making comparisons among studies complicated (Yingst, 1976; Moriarty, 1982; Hudson et al., 2005; Zamora and Jeffs, 2011; Slater et al., 2011a). This kind of approach has probably slowed the development of effective artificial diets for sea cucumbers. A more systematic approach would be firstly to understand intake, digestion and absorption of macronutrients, which has been so successfully used for many other commercial aquaculture species (Cho and Kaushik, 1990; Guillaume, 2001).

An adequate artificial diet must take into account the specific nutrient requirements of each species as well as their feeding behaviour, and how it is affected by the culture conditions (i.e., salinity, temperature, stocking densities). For example, the feeding biology of sea cucumbers can be modified by changes in seawater temperature, affecting their feed intake as well as their metabolism and therefore, their growth (Yang et al., 2005; Dong et al., 2006; An et al., 2007; Yuan et al., 2009; Zamora and Jeffs, 2012b). However, it is not clear how changes in the seawater temperature affect the specific selection, digestion and absorption of macronutrients from the food in juvenile sea cucumbers. This is because most of the information available on the natural feeding biology of sea cucumbers comes from adults, although some aspects can be generalized for both juvenile and adult sea cucumbers. Deposit-feeding species of sea cucumbers feed predominantly on sedimentary organic detritus of vegetal and animal origin and associated live micro-organisms (Massin, 1982; Roberts et al., 2000). They are known to actively select organic components from among inorganic sediment particles when feeding, most probably by chemical detection using their oral tentacles (Massin, 1982; Uthicke and Karez, 1999; Roberts et al., 2000; Slater et al., 2011a). Once in the alimentary canal, the food is compressed and exposed to the digestive enzymes, which are capable of breaking down carbohydrate (glycosidases), protein (peptide hydrolases) and lipid (ester hydrolases) (Lawrence and Lane, 1982; Roberts et al., 2000). Digestion in sea cucumbers is thought to occur mainly in the first section of the alimentary canal while absorption of the liberated nutrients can occur throughout the alimentary canal (Roberts et al., 2000; Zamora and Jeffs, 2011). These absorbed nutrients would subsequently be utilised for growth and catabolised to meet energy requirements for metabolism. The body of sea cucumbers is composed mainly of protein, mostly as collagen, and carbohydrate, as mucopolysaccharides, with much smaller quantities of lipid (Liu, 2010; Wen et al., 2010; Seo et al., 2011). Although their overall macronutrient composition may be similar, the

ability of different sea cucumber species to digest, absorb and assimilate food varies from species to species (Roberts et al., 2000).

The Australasian sea cucumber, *Australostichopus mollis*, is a commercially important sea cucumber that can be found all along the coast of New Zealand and the southern coasts of Australia (Pawson, 1970). Currently there is only a small fishery in New Zealand with less than 20 t landed annually (Ministry of Fisheries, 2011). Most of the sea cucumbers harvested are exported, mainly to Asia where it fetches over US$ 275 kg^{-1} dry weight (Purcell et al., 2012a). This species has attracted the attention of the aquaculture industry due to the fact that it can be successfully co-cultured with other commercially important species, such as the green-lipped mussel (*Perna canaliculus*), Pacific oysters (*Crassostrea gigas*) and the abalone (*Haliotis iris*) (Slater and Carton, 2007; Maxwell et al., 2009; Zamora and Jeffs, *in review*). However, there is only limited information about the natural feeding biology of *A. mollis* juveniles coming from studies in the wild and in the laboratory, and almost nothing related to their nutrient requirements (Slater et al., 2009, 2011a; Maxwell et al., 2009; Zamora and Jeffs, 2011). Therefore, the aim of this study was to determine the macronutrient selection and absorption in juvenile *A. mollis*, and how these processes are affected by seawater temperature when they are feeding on mussel waste. This information will assist the development of a reliable artificial diet, which is required to significantly reduce production times and costs of nursery culture of this species.

5.2. Materials and Methods

5.2.1. Experimental Animals

Juvenile sea cucumbers of a similar size (14 - 25 g) were collected from the Mahurangi Harbour in northern New Zealand by divers during the winter (June) of 2010. The seawater temperature at the time of sampling was 14.8 °C. The sea cucumbers were transferred to the nearby Leigh Marine Laboratory in seawater and held in tanks with flowing 100-µm filtered seawater at ambient temperature (15 °C). The sea cucumbers were left without feed for 48 h before being weighed and used in the experiments. The sea cucumbers were photo-identified and their weight was measured after the excess water was gently removed (Sewell, 1990; Raj, 1998b).

5.2.2. Experimental Set up

Three seawater temperature treatments were established in order to represent the natural range of seawater temperatures to which this species may be exposed during winter, spring and summer (15, 18, 21 °C respectively) in the sampling area (Scarsbrook, 2008; Auckland Regional Council, 2008). For each temperature treatment, four replicate polyethylene tanks (0.33 × 0.21 × 0.15 m, L × W × H) were used. Five juvenile sea cucumbers of similar weight were randomly selected and allocated to each of the replicate tanks (19.2 ± 0.5 g; total mean wet weight ± S.E.). The tanks had a continuous flow (0.2 L min-1) of 100-µm filtered seawater, for which temperature was controlled to ± 0.01 °C accordingly for each temperature treatment.

5.2.3. Thermal Acclimation, Feeding and Cleaning

The sea cucumbers were acclimated to each of the experimental temperature treatments following the procedures described by Zamora and Jeffs (2012b). Acclimation of the sea cucumbers lasted for two weeks, during which time the sea cucumbers were fed to satiety every two days with green-lipped mussel waste, a highly palatable and effective natural food source for this sea cucumber species (Slater et al., 2009; Zamora and Jeffs, 2012a, 2012b). The proximate composition of the mussel waste was 816 ± 2 g kg^{-1} ash, 90 ± 2 g kg^{-1} carbohydrate, 37 ± 3 g kg^{-1} protein, 6 ± 1 g kg^{-1} lipid in terms of dry weight. The organic composition of the feed was found to not change significantly over the two day period it was available for sea cucumbers (unpublished data). The sea cucumber faeces were removed twice a day, in the morning and in the evening, by carefully siphoning them out of the tanks.

5.2.4. Sea Cucumber Gut Sampling

After two weeks of acclimation the juvenile sea cucumbers were starved for two days to let them void their digestive tracts. Then fresh mussel waste was supplied to the tanks, after which the sea cucumbers started feeding almost immediately. Each sea cucumber was removed from its tank once faeces were observed being produced. The sea cucumber was then immediately placed inside a plastic bag and plunged into iced seawater to arrest their metabolism and digestive enzymes. Each sea cucumber was then dissected and their alimentary canal was extracted and divided in three equal parts by length (Foregut, Midgut, and Hindgut) (Zamora and Jeffs, 2011).

From each part of the gut, the digesta (i.e., mixture of feed and digestive juices) was completely removed and immediately stored at -80 °C for the subsequent biochemical analyses (Zamora and Jeffs, 2011). For comparison, a sample of food was also taken, from each tank one hour after food was delivered to the sea cucumbers, and immediately stored at -80 °C for the subsequent biochemical analyses.

5.2.5. Biochemical Analyses

For all the biochemical analyses, the frozen digesta and food samples were thawed and gently suspended in deionised water. The samples were then centrifuged (9,300 g for 5 min) to remove digestive enzymes with the supernatant. The remaining pellet was freeze-dried and used for the analyses of total organic matter (TOM), lipid, carbohydrate and protein respectively.

Not all of the sampled sea cucumbers had sufficient digesta to allow the biochemical analyses, mostly due to differences in the effect of temperature on the feeding behaviour of this species (Zamora and Jeffs, 2012b). Of the twenty sea cucumbers per treatment, only ten for each of the 18 and 21 °C treatments had sufficient gut contents for analyses, while all the gut contents of the sea cucumbers from the 15 °C treatment were analysed. Moreover, the amount of digesta of each section of the gut coming from a single sea cucumber was insufficient for analyses in all temperature treatments. Therefore, the samples of two randomly selected sea cucumbers within each temperature treatment were pooled, in order to have 30 mg of sample for each biochemical analyses.

5.2.5.1. Total organic matter (TOM)

Percentage of TOM was measured by the loss of weight in the sample after combustion (Byers et al., 1978). The samples were oven dried at 60 °C for 48 h, weighed, and then placed in a furnace for 6 h at 500 °C to ensure complete combustion of organic matter, and then the remaining inorganic material weighed.

5.2.5.2. Lipid

The lipid content was measured gravimetrically after extraction of lipid with a modified Bligh and Dyer (1959) solvent extraction. The freeze-dried samples were sonicated (3 min) in deionised water. Then chloroform and methanol were added, before centrifuging (3,000 g for 10 min). The supernatant was transferred to another centrifuge tube and more chloroform and methanol were added, before centrifuging again (800 g for 5 min). The resulting layer of chloroform was recovered and transferred to a pre-weighed glass tube and placed in a thermal block for solvent evaporation under a stream of nitrogen in order to determine the mass of lipid. The lipid content of the samples was calculated as a percentage of dry weight of the samples.

5.2.5.3. Protein

The protein concentration was measured using a modified Bradford (1976) assay. The freeze-dried samples were sonicated (3 min) in NaOH (1 M), before extraction in a water bath (60 °C for 2 h). After the extraction the samples were centrifuged (3,000 g for 10 min) and diluted. Subsamples were placed into a 96 well plate and mixed with the Bradford reagent before reading their absorbance at 595 nm. The samples were re-extracted two more times with NaOH to recover any protein left to be added to the final count. The protein content of the samples was calculated as a percentage of dry weight of the sample using a bovine serum albumin standard curve as a reference.

5.2.5.4. Carbohydrate

The carbohydrate concentration was measured using the phenol sulphuric acid method (Dubois et al., 1956). The freeze-dried samples were sonicated (3 min) in deionised water. Then, diluted subsamples were mixed with the sulphuric acid and with the phenol solutions and were left to cool for 15 min. After centrifugation (3,000 g for 10 min) the absorbance (at 490 nm) of the samples was measured in a 96 well plate reader. The carbohydrate content of the sample was calculated as a percentage of dry weight of the sample using a D-glucose standard curve as a reference.

5.2.6. Macronutrient Selection Efficiency

The selection efficiency of the juvenile sea cucumbers for each macronutrient was determined as the mean difference between each macronutrient available in the supplied food and in the foregut of the sea cucumbers. The selection efficiency then is expressed as a percentage of the macronutrient available in the supplied food (Zamora and Jeffs, 2011).

5.2.7. Macronutrient Absorption Efficiency

Absorption efficiency, defined as the mean percentage of ingested organic material moving across the gut wall, was calculated separately for each macronutrient as the observed mean percentage of each macronutrient loss between the foregut and the faeces (Zamora and Jeffs, 2011).

5.2.8. Energy Budget

In order to assess the energy budget of the juveniles feeding on mussel waste at different temperatures the following equation was used (Ricker, 1968):

$$C = P + F + U + R$$

Where *C*, is the energy ingested through the food; *P*, is the energy available for growth; *F*, is the energy lost in the faeces; *U*, is the energy lost through excretion (in this case ammonia); and *R*, is the energy lost due to respiration. The data used to calculate *C*, *F*, *U* and *R* respectively were taken from a previous study with juvenile *A. mollis* under identical holding conditions and of similar size (Table 5.1) (Zamora and Jeffs, 2012b).

The energy available for growth, *P*, was calculated as follows: $P = C - F - R - U$. While *C* and *F* were calculated by estimating the amount of energy of each macronutrient ingested (in the foregut) and eliminated as faeces daily using the following conversion factors for lipid (39.57 J mg^{-1}), protein (23.66 J mg^{-1}) and carbohydrate (17.17 J mg^{-1}) (Otero-Villanueva et al., 2004). While *U* and *R* were calculated by transforming the amount of oxygen consumed and ammonia

excreted daily into energy, using the following conversion factors for oxygen (19.79 J mg^{-1}) and ammonia (24.85 J mg^{-1}) (Elliot and Davison, 1975).

5.2.9. Statistical Analyses

Significant differences in the mean value of each macronutrient within each gut section in each temperature treatment, and among temperature treatments were assessed using one-way ANOVAs. One-way ANOVAs were also used to detect differences in the mean selection and absorption efficiencies among temperature treatments. Prior to the analyses, percentage values were transformed using the arcsine function and the data were then tested for normality and homoscedasticity (Quinn and Keough, 2002). The post hoc Holm-Sidak test was used to identify the source of differences among individual pairs of means when significant overall experiment-wise differences among means were detected (Quinn and Keough, 2002).

5.3. Results

5.3.1. Changes in TOM along the Gut, Selection and Absorption

There were significant differences among the mean TOM content measured in the food, foregut, midgut, hindgut and faeces at 15 °C (ANOVA, $F_{(4, 35)} = 22.25$, $P < 0.001$), 18 °C (ANOVA, $F_{(4, 20)} = 24.17$, $P < 0.001$) and 21 °C (ANOVA, $F_{(4, 20)} = 6.11$, $P = 0.005$). The TOM content was significantly higher (around 3%) in the foregut than in the food at 15 °C (Holm-Sidak, $P < 0.001$) and at 18 °C (Holm-Sidak, $P < 0.001$) (Fig. 5.1). However, at 21 °C there was no significant difference between the mean TOM content of the food and foregut (Holm-Sidak, $P = 0.073$) (Fig. 5.1). The mean TOM content of the digesta of the sea cucumbers decreased significantly by 3 - 4% in all of the temperature treatments, as it moved from the foregut along the alimentary canal until it was eliminated as faeces (Holm-Sidak, $P < 0.001$ in all temperature treatments) (Fig. 5.1). When comparing the mean TOM content of each gut section at different temperatures, there were only significant differences between the 15 and 21 °C treatments, with the TOM content being higher at 15 °C for both the foregut and midgut (Holm-Sidak, $P < 0.001$ in both cases) (Fig. 5.1).

There were significant differences in the mean TOM selection efficiency at different temperatures (ANOVA, $F_{(2, 17)} = 14.18$, $P < 0.001$). The TOM selection efficiency was lower at 21 °C than at 15 °C (Holm-Sidak, $P < 0.001$) and at 18 °C (Holm-Sidak, $P < 0.001$) (Fig. 5.5). However, temperature did not affect the overall TOM absorption efficiency, being similar in all temperature treatments (ANOVA, $F_{(2, 17)} = 0.66$, $P = 0.53$) (Fig. 5.6).

5.3.2. Changes in Lipid along the Gut, Selection and Absorption

There were significant differences among the mean lipid content of the food, foregut, midgut, hindgut and faeces at 15 °C (ANOVA, $F_{(4,35)} = 25.59$, $P < 0.001$), 18 °C (ANOVA, $F_{(4, 20)} = 22.03$, $P < 0.001$) and 21 °C (ANOVA, $F_{(4, 20)} = 4.52$, $P = 0.017$). The lipid content was significantly higher in the foregut than in the food, being three times higher at 15 °C, and twice as high at 18 and 21 °C (Holm-Sidak, $P < 0.001$ for all temperature treatments) (Fig. 5.2). In all temperature treatments the lipid content of the digesta significantly decreased as it moved along the alimentary canal from the foregut until it was eliminated as faeces. The lipid content of the faeces decreased to almost a third of that measured in the food at 15 °C, one sixth at 18 °C, and half at 21 °C (Holm-Sidak, $P < 0.001$ for all temperature treatments) (Fig. 5.2). When comparing the mean lipid content of the different gut sections at different temperatures, there were only significant differences between the lipid content of the foregut of the 15 and 21 °C treatments, with higher mean lipid content observed at 15 °C (0.5% higher) (Holm-Sidak, $P < 0.001$) (Fig. 5.2).

There were significant differences in the lipid selection efficiency at different temperatures (ANOVA, $F_{(2, 17)} = 13.32$, $P < 0.001$). The lipid selection efficiency was significantly higher (almost twice as high) at 15 °C than at 18 °C (Holm-Sidak, $P < 0.001$) and 21 °C (Holm-Sidak, $P = 0.002$) (Fig. 5.5). Temperature also affected the mean lipid absorption efficiency (ANOVA, $F_{(2, 17)} = 5.00$, $P = 0.02$), with significant differences being only found between the mean lipid absorption efficiency at 18 and 21 °C (Holm-Sidak, $P < 0.001$) (Fig. 5.6).

5.3.3. Changes in Protein along the Gut, Selection and Absorption

There were significant differences among the mean protein content measured in the food, foregut, midgut, hindgut and faeces at 15 °C (ANOVA, $F_{(4, 35)} = 4.31$, $P = 0.007$), 18 °C

(ANOVA, $F_{(4, 20)} = 4.05$, $P = 0.024$) and 21 °C (ANOVA, $F_{(4, 20)} = 3.81$, $P = 0.029$). Significant differences were found between the protein content of the food and the foregut only in the 18 °C temperature treatment (Holm-Sidak, $P < 0.05$) (Fig. 5.3). However, in all temperature treatments the protein content of the digesta decreased significantly by 1 - 1.5% between the foregut and faeces. (Holm-Sidak, $P < 0.001$ in all temperature treatments) (Fig. 5.3). There were no differences among the temperature treatments in the protein content for each of the three gut sections (foregut, midgut and hindgut) analysed separately (Holm-Sidak, $P > 0.05$ in all comparisons) (Fig. 5.3).

Neither the mean protein selection and absorption efficiencies were significantly different among the temperature treatments (ANOVA, $F_{(2, 17)} = 0.83$, $P = 0.46$; and ANOVA, $F_{(2, 17)} = 0.42$, $P = 0.66$ respectively) (Fig. 5.5 and 5.6).

5.3.4. Changes in Carbohydrate along the Gut, Selection and Absorption

There were significant differences among the mean carbohydrate content measured in the food, foregut, midgut, hindgut and faeces at 15 °C (ANOVA, $F_{(4, 35)} = 9.71$, $P < 0.001$), 18 °C (ANOVA, $F_{(4, 20)} = 5.57$, $P = 0.008$) and 21 °C (ANOVA, $F_{(4, 20)} = 9.23$, $P < 0.001$). The carbohydrate content of the foregut was 1 - 2 % significantly higher than the food in each of the temperature treatments (Holm-Sidak, $P < 0.001$ for each of the three temperature treatments) (Fig. 5.4). In all three temperature treatments the carbohydrate content of the digesta significantly decreased by 2 - 3% as it moved along the alimentary canal from the foregut until it was eliminated as faeces (Holm-Sidak, $P < 0.001$ in all temperature treatments) (Fig. 5.4). For each gut section there was no significant difference in the carbohydrate content among the temperature treatments (Holm-Sidak, $P > 0.05$ in all comparisons) (Fig. 5.4).

There were significant differences in the mean selection efficiency of carbohydrate at different temperatures (ANOVA, $F_{(2, 17)} = 7.77$, $P = 0.005$). The carbohydrate selection efficiency decreased with increasing temperature from 15 °C to 21 °C, being almost three times higher at 15 °C (Holm-Sidak, $P < 0.001$) (Fig. 5.5). Conversely, the seawater temperature did not affect the carbohydrate absorption (ANOVA, $F_{(2, 17)} = 0.90$, $P = 0.429$) (Fig. 5.6).

5.3.5. Macronutrient Selection and Absorption Preference

There were significant differences among the mean selection efficiencies of the macronutrients at 15 °C (ANOVA, $F_{(2, 27)} = 89.21$, $P < 0.001$), 18 °C (ANOVA, $F_{(2, 13)} = 22.09$, $P < 0.001$) and 21 °C (ANOVA, $F_{(2, 13)} = 30.35$, $P < 0.001$) (Fig. 5.5). The proportion of lipid in the foregut of juvenile sea cucumbers compared to the food was significantly greater than for protein (Holm-Sidak, $P < 0.001$ in all temperature treatments) and carbohydrate (Holm-Sidak, $P < 0.001$ in all temperature treatments) (Fig. 5.5).

Likewise, there were significant differences among the mean absorption efficiencies of the macronutrients at 15 °C (ANOVA, $F_{(2, 27)} = 39.43$, $P < 0.001$), 18 °C (ANOVA, $F_{(2, 13)} = 40.90$, $P < 0.001$) and 21 °C (ANOVA, $F_{(2, 13)} = 7.55$, $P = 0.012$). Overall, lipid was absorbed more efficiently than protein and carbohydrate, but these differences were less pronounced as the seawater temperature increased. At 15 °C the juvenile sea cucumbers were more efficient absorbing lipid than protein (Holm-Sidak, $P < 0.001$) and more efficient absorbing protein than carbohydrate (Holm-Sidak, $P = 0.005$) (Fig. 5.6). At 18 °C lipid was absorbed more efficiently than protein (Holm-Sidak, $P < 0.001$) and carbohydrate (Holm-Sidak, $P < 0.001$) (Fig. 5.6). At 21 °C lipid was absorbed more efficiently than carbohydrate (Holm-Sidak, $P = 0.004$) (Fig. 5.6).

5.3.6. Energy Source and Energy Budget

Most of the energy ingested by the juvenile sea cucumbers in all temperature treatments was in the form of carbohydrate, then protein and finally lipid (Table 5.1). The amount of each nutrient ingested decreased with increasing seawater temperature, with lipid ingestion decreasing almost three times while the ingestion of carbohydrate and protein were reduced almost by half as temperature went from 15 to 21 °C (Table 5.1). These changes in the amount of nutrient ingestion resulted in juveniles ingesting more than two times more energy at 15 °C than at 21 °C (Table 5.2). Most of the energy ingested by sea cucumbers was lost through the faeces, especially carbohydrate and protein. While more than half of the ingested lipid was absorbed in all temperature treatments (Table 5.1). The energy lost through respiration and ammonia excretion was doubled and tripled respectively as the temperature increased from 15 °C to 21 °C, although the energy requirements used for these functions was relatively low overall (Table 5.2). Thus the juveniles had almost three times more energy available for growth at 15 °C than at 21

°C, mostly due to a combination of greater energy intake and lower metabolic demand. Whereas, differences in absorption at the lower experimental temperature were not an important contributor to this overall difference in energy availability (Table 5.2).

5.4. Discussion

This study provides the first report of the major nutrient requirements for the juveniles of this sea cucumber species, providing further insight into the selection and absorption of organic particles for this species and how these processes are influenced by seawater temperature. While *A. mollis* is capable of digesting and absorbing lipid, protein and carbohydrate present in the mussel waste over the range of temperatures from 15 to 21 °C, there were marked differences in their abilities to uptake and absorb different macronutrients over this temperature range.

5.4.1. Macronutrient selection and absorption at different temperatures

Selective feeding behaviour appears to be a critical consideration for the development of effective artificial diets for juvenile sea cucumbers, as the feed needs to be in a form and amount that can be recognised and preferentially ingested by the juveniles (Slater et al., 2009, 2011a, 2011b; Zamora and Jeffs, 2011). In this study selective feeding was observed for lipid, carbohydrate and protein. However, the selection of lipid was proportionally much more pronounced than carbohydrate and protein, which could be a response to the relatively low lipid availability in the mussel waste (0.6%) (Seo and Lee, 2011). The consistency in the relativity of the intake results, for the three macronutrients over the three temperature treatments, would suggest that the sea cucumbers are capable of selecting organic particles for ingestion, rather than selectively consuming particles on the basis of their composition of individual macronutrients. It is unclear what factors make the mussel waste attractive and stimulate feeding in A. mollis (Lawrence et al., 2007). However, it appears that the mechanisms involved in the detection and ingestion of organic particles can be affected not only by food availability, but also by seawater temperature, whith increases from 15 to 21 °C reducing the overall selection efficiencies of the macronutrients (Zamora and Jeffs, 2011).

In terms of absorption efficiency, most of the lipid ingested were absorbed (i.e., over 80% at 18 °C), while most of the carbohydrate and protein ingested were eliminated in the faeces (i.e.,

around 80 and 70% for carbohydrate and protein respectively in all temperature treatments) following a similar trend as in another sea cucumber, *Apostichopus japonicus* (Seo and Lee, 2011; Seo et al., 2011). The absorption of lipid was most affected by temperature compared to protein and carbohydrate, which may be due to differences in the temperature dependent activity of specific digestive enzymes or due to an effect on the structure and functioning of the digestive tract cells, affecting nutrient absorption (Gao et al., 2008, 2009).

5.4.2. Energy budget of juveniles at different temperatures

Information on energy budgets and how they are affected by seawater temperature and diet has been important for underpinning development of artificial diets in *A. japonicus* (Yuan et al., 2006, 2009; An et al., 2007; Liu et al., 2009; Xia et al., 2013). Mussel waste has proven to be a highly effective feed for juvenile *A. mollis* and the results of this study reveal how the energy provided by the mussel waste is utilised at a range of seawater temperatures. Independently of temperature, the juveniles of this species of sea cucumber seem to rely on all macronutrients as a source of energy for growth. However, an increase in seawater temperature negatively affected the amount of energy ingested through food, but also the energy absorbed and available for growth (Zamora and Jeffs, 2012b). This kind of pattern was not the same in a previous study, in which the energy available for growth increased or decreased with temperature depending on the diet fed to largerjuveniles (Maxwell et al., 2009). Whereas a similar negative effect on energy intake has been observed in juvenile *A. japonicus* when temperature increases near the threshold for aestivation (between 26 - 30 °C) (Yang et al., 2005; Yuan et al., 2009). In juvenile *A. japonicus* an increase of temperature from 5 to 25 °C also increases the metabolic costs, mostly from supporting increased respiration, which can require over 30% of energy ingested at temperatures around 20 °C, and thereby also reducing the energy available for growth (Yuan et al., 2009; An et al., 2007). Comparatively, the combined energy lost through excretion and respiration in *A. mollis* (less than 1% of ingested energy) is similar to those observed in sea urchins, which can also have low excretion and respiration depending on temperature and diet (Otero-Villanueva et al., 2004). Overall, the results of the current study indicate the importance of controlling seawater temperature in order to maximize the energy ingestion and absorption and therefore growth of the juvenile *A. mollis* in an aquaculture situation.

5.4.3. Formulation of artificial diets

The results of this study are useful for assisting in the development of the first effective artificial diet for the aquaculture of juvenile *A. mollis*. Artificial diets have been extensively studied in another temperate sea cucumber, *A. japonicus* (Huiling et al., 2004; Yuan et al., 2006; Liu et al., 2009; Seo and Lee, 2011; Xia et al., 2013). Artificial diets have proven to not only accelerate growth, but could also improve the tolerance of sea cucumbers to stressful culture conditions (Wang et al., 2009; Zhang et al., 2010). These diets typically contain a high proportion of inorganic matter (190 – 730 g kg^{-1}), with carbohydrate (100 – 660 g kg^{-1}), protein (150 – 400 g kg^{-1}) and lipid (20 – 100 g kg^{-1}) in descending contribution. Mussel waste has a lower nutrient profile (i.e., 820 g kg^{-1} ash, 90 g kg^{-1} carbohydrate, 40 g kg^{-1} protein, 6 g kg^{-1} lipid in terms of dry weight), which due to selective feeding reduces the ash content and increases the organic composition in the foregut (i.e., 770 g kg^{-1} ash, 110 g kg^{-1} carbohydrate, 50 g kg^{-1} protein, 15 g kg^{-1} lipid in terms of dry weight) effectively meeting the nutritional requirements of juvenile A. mollis. In this study we demonstrated that *A. mollis* relies on all macronutrients as a source of energy, indicating that this species needs a proportionally balanced diet with more carbohydrate than protein and lipid. Knowing the indicative macronutrient dietary requirement helps the formulation of an artificial diet for *A. mollis*. However, also essential is identifying reliable, palatable and cost-effective sources of these macronutrients (Slater et al., 2011b). There is also the possibility to replace expensive lipid and protein sources with cheaper carbohydrate sources, but this option needs further evaluation in order to identify adequate replacement levels that satisfy the nutrient and energy requirements of this species (Cho and Kaushik, 1990; Guillaume, 2001; Kennedy et al., 2007; Seo and Lee, 2011).

5.5. Conclusions

Lower seawater temperature (15 °C versus 18 and 21 °C) greatly increased the energy available for growth of juvenile *Australostichopus mollis* through a marked increase in overall energy intake and reduced metabolic demand when fed on mussel waste. By contrast the absorption efficiency of macronutrients (lipid, protein and carbohydrate) changed little over this same temperature range. Independently of the seawater temperature, the juveniles are able to use carbohydrate, protein and lipid as a source of energy, which opens up the possibility to replace more expensive nutrient sources, such as protein and lipid, with less costly carbohydrates in order to reduce costs of diet formulation.

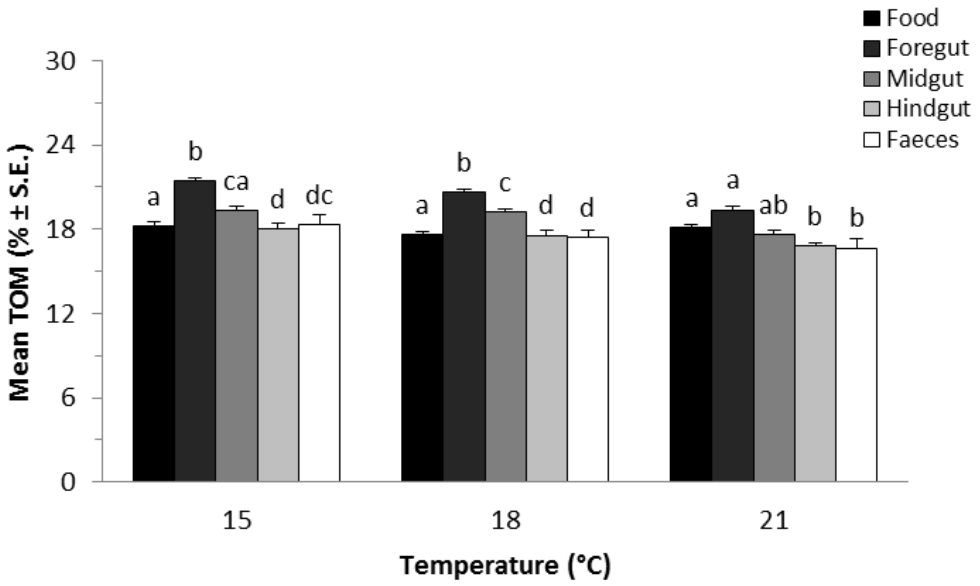

Figure 5.1. Mean total organic matter (TOM) content of the food, in the digesta from different gut sections (foregut, midgut and hindgut) and in the faeces coming from juvenile *Australostichopus mollis* feeding on mussel waste at three different temperatures. Different letters within each temperature treatment represent significant differences between means ($P < 0.05$).

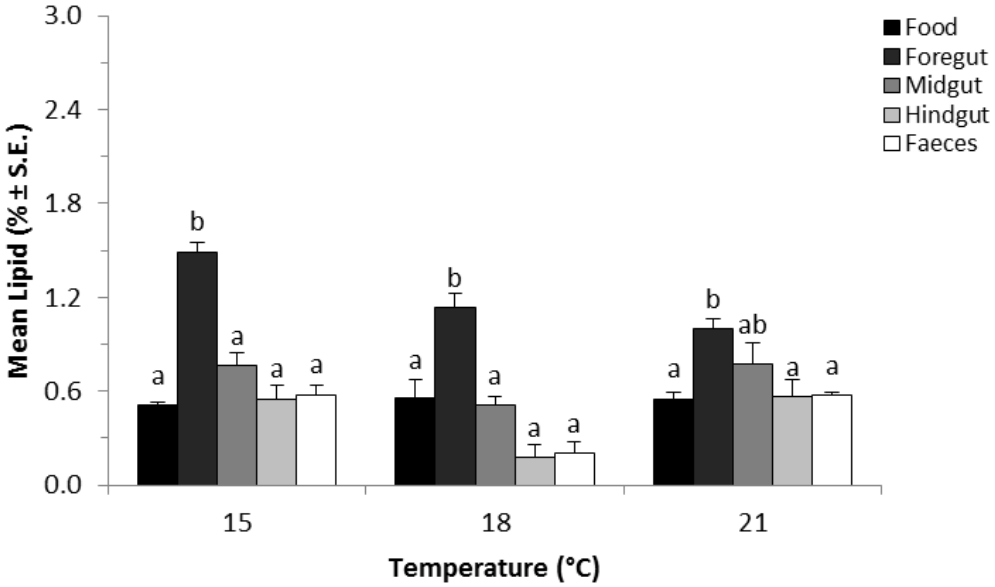

Figure 5.2. Mean lipid content of the food, in the digesta from different gut sections (foregut, midgut and hindgut) and in the faeces coming from juvenile *Australostichopus mollis* feeding on mussel waste at different temperatures. Different letters within each temperature treatment represent significant differences between means ($P < 0.05$).

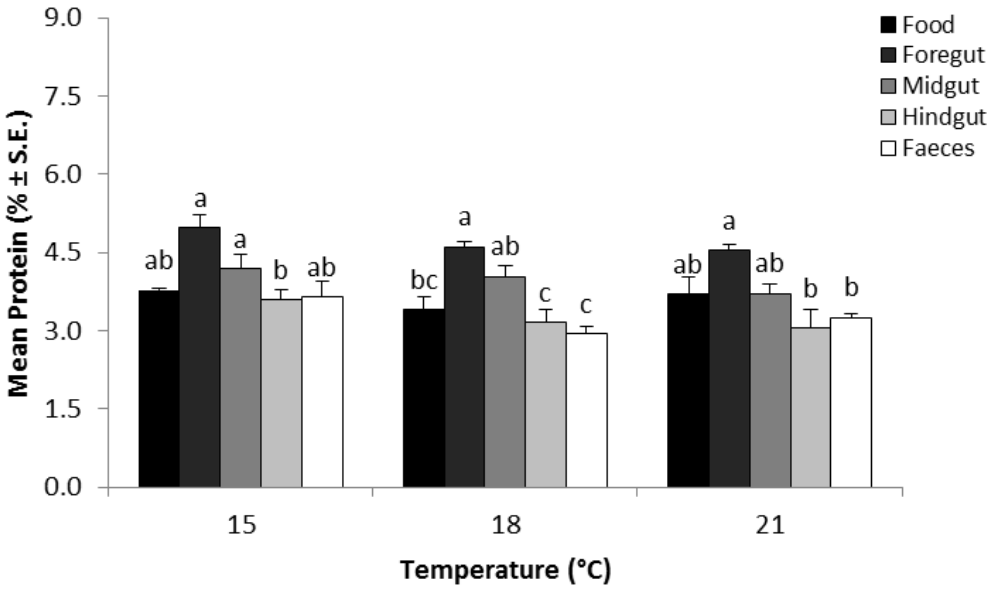

Figure 5.3. Mean protein content of the food, in the digesta from different gut sections (foregut, midgut and hindgut) and in the faeces coming from juvenile *Australostichopus mollis* feeding on mussel waste at different temperatures. Different letters within each temperature treatment represent significant differences between the means ($P < 0.05$).

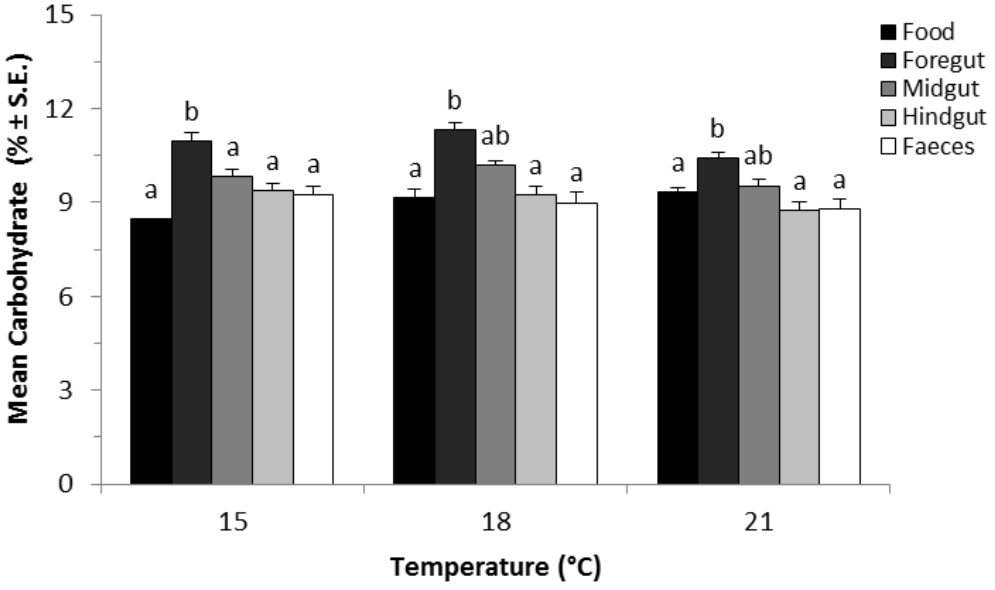

Figure 5.4. Mean carbohydrate content of the food, in the digesta from different gut sections (foregut, midgut and hindgut) and in the faeces coming from juvenile *Australostichopus mollis* feeding on mussel waste at different temperatures. Different letters within each temperature treatment represent significant differences between means ($P < 0.05$).

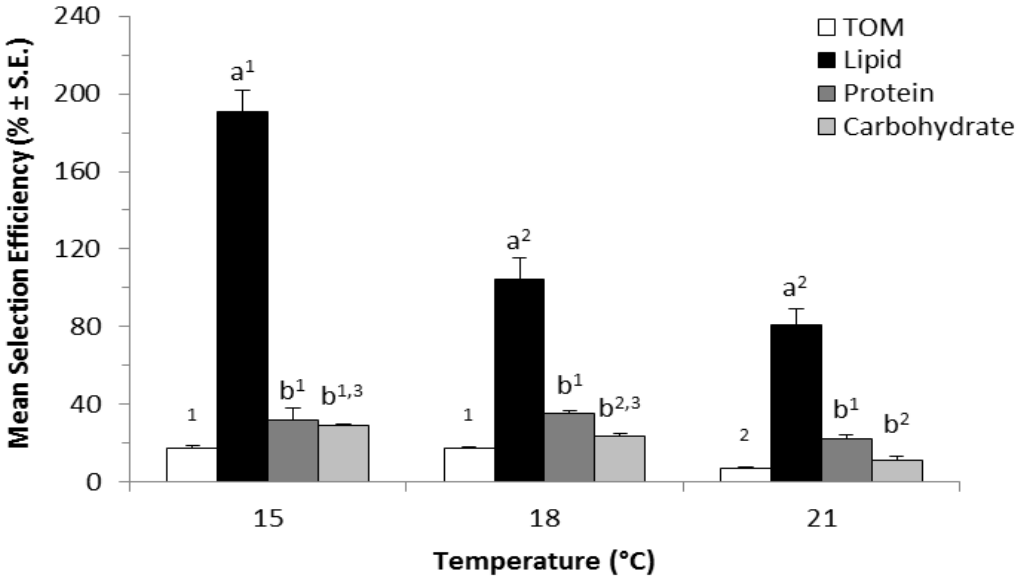

Figure 5.5. Mean selection efficiency of nutrients of juvenile *Australostichopus mollis* feeding on mussel waste at three experimental temperatures. Different letters within each temperature treatment represent significant differences between means ($P < 0.05$). Different numbers among temperature treatments for each nutrient represent significant differences between means ($P < 0.05$).

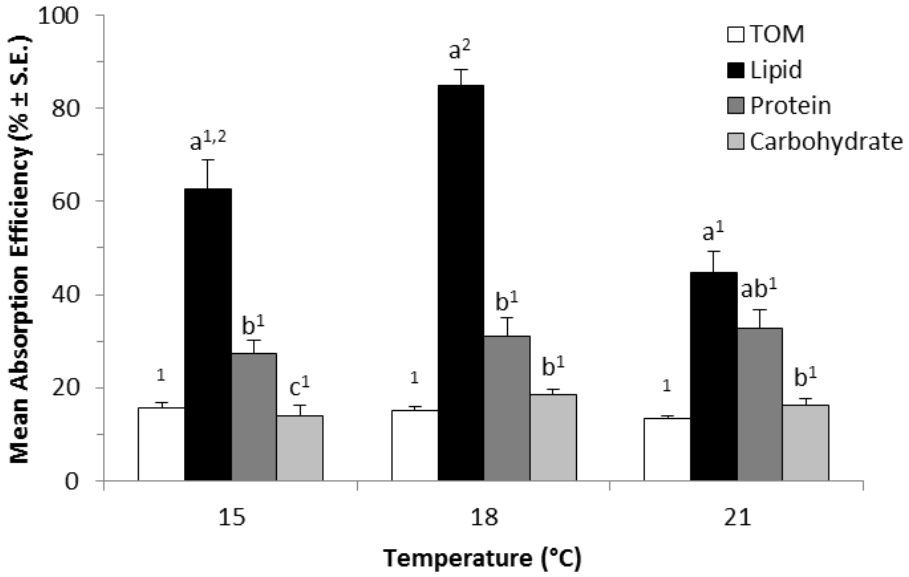

Figure 5.6. Mean absorption efficiency of macronutrients of juvenile *Australostichopus mollis* feeding on mussel waste at different temperatures. Different letters within each temperature treatment represent significant differences between means ($P < 0.05$). Different numbers among temperatures for each nutrient represent significant differences between means ($P < 0.05$).

Table 5.1. Juvenile *Australostichopus mollis* ingestion (IR), faecal production (FPR), respiration (OCR) and excretion (AER) rates modified (expressed as mg per individual per day) from Zamora and Jeffs (2012b) when feeding on mussel waste at different temperatures. Values of each macronutrient IR and FPR were calculated using the information obtained in this study of their relative content in the foregut and faeces respectively. Apparent absorption values (AA) of each macronutrient were calculated as AA = IR - FPR. Numbers in brackets correspond to energy equivalent in Joules (J) of each nutrient.

	15 °C	**18 °C**	**21 °C**
IR (mg ind^{-1} d^{-1})	5,180	3,880	2,520
Lipid IR (mg ind^{-1} d^{-1})	77.28 (3,058 J)	43.91 (1,738 J)	25.15 (995 J)
Carbohydrate IR (mg ind^{-1} d^{-1})	567.23 (9,739 J)	439.06 (7,539 J)	262.74 (4,511 J)
Protein IR (mg ind^{-1} d^{-1})	258.20 (6,109 J)	178.79 (4,230 J)	114.25 (2,703 J)
FPR (mg ind^{-1} d^{-1})	4,420	3,330	2,220
Lipid FPR (mg ind^{-1} d^{-1})	25.60 (1,013 J)	6.85 (271 J)	12.77 (505 J)
Carbohydrate FPR (mg ind^{-1} d^{-1})	409.58 (7,033 J)	299.23 (5,138 J)	195.01 (3,348 J)
Protein FPR (mg ind^{-1} d^{-1})	161.58 (3,823 J)	98.17 (2,322 J)	72.24 (1,709 J)
AA (mg ind^{-1} d^{-1})	760	550	300
Lipid AA (mg ind^{-1} d^{-1})	51.67 (2,045 J)	37.06 (1,467 J)	12.38 (489 J)
Carbohydrate AA (mg ind^{-1} d^{-1})	157.65 (2,707 J)	139.84 (2,404 J)	67.73 (1,162 J)
Protein AA (mg ind^{-1} d^{-1})	96.62 (2,228 J)	80.63 (1,908 J)	42.01 (994 J)
OCR (mg ind^{-1} d^{-1})	0.61	1.08	1.63
AER (mg ind^{-1} d^{-1})	0.32	0.38	0.62

Table 5.2. Parameters of the energy budgets and allocation of energy consumed (%/*C*) of juvenile *Australostichopus mollis* feeding on mussel waste at different temperatures as estimated from integration of macronutrient selection and absorption data with ingestion, faecal production, respiration and excretion rates data from Zamora and Jeffs (2012b). *C* = Energy consumed trough food, *U* = Energy lost through ammonia excretion, *R* = Energy lost through respiration, F = Energy lost through faeces, and *P* = Energy available for growth.

	15 °C		18 °C		21 °C	
	E (J day^{-1})	%/*C*	E (J day^{-1})	%/*C*	E (J day^{-1})	%/*C*
C	18906.24	100.00	13506.66	100.00	8209.56	100.00
U	7.92	0.04	9.49	0.07	15.43	0.19
R	12.15	0.06	21.41	0.16	32.44	0.40
F	11868.56	62.78	7731.40	57.24	5562.81	67.76
P	7017.60	37.12	5744.36	42.53	2598.88	31.66

Chapter Six

Evaluation of transportation methods of juveniles of the Australasian sea cucumber *Australostichopus mollis*

Abstract

The transport of juvenile sea cucumbers to grow-out sites is problematic as they are prone to damage and mortality. The response of juvenile *Australostichopus mollis* to simulated transport conditions with and without seawater for different periods of time (2, 4, 8, 12, and 24 h) at different temperatures was evaluated in this study. Sea cucumbers were placed in plastic bags with seawater or wrapped in a seawater-soaked cloth. Monitoring of evisceration, skin lesions, feeding activity and survival were used to evaluate the success of transportation. The juveniles of this species can withstand being without seawater for up to 8 h with no consequences to their survival and feeding activity. Complete immersion of the juveniles in seawater was found to be the only option for transportation over longer periods, but preventive measures should be taken to avoid detrimental levels of temperature, oxygen and pH. Overall, the existing constraints to moving juvenile sea cucumbers to grow-out sites can be overcome through preventing desiccation during short-term transport without seawater, and by the control of temperature for long-term transportation with seawater. The results suggest that in juvenile sea cucumbers, feeding activity and the presence of skin lesions are more sensitive indicators of the success of transport than evisceration and survival.

6.1. Introduction

The large demand for sea cucumber products in Asian markets, together with their high market value, has generated a great deal of pressure on the wild populations of many species of sea cucumber around the world, particularly in tropical regions (Ferdouse, 2004; Kinch et al., 2008; Choo, 2008). This has led to the development of hatchery and nursery technology as a source of juveniles for restocking of wild populations and aquaculture production (Southward et al., 2005). Juvenile production has been economically feasible only for the most commercially important species, such as the temperate sea cucumber, *Apostichopus japonicus*, the tropical sandfish,

Holothuria scabra, and the white teatfish, *Holothuria fuscogilva* (Renbo and Yuan, 2004; Mills et al., 2012; Jimmy et al., 2012). Whether the hatchery-produced juveniles are destined for restocking or aquaculture, they need to be transported from the hatchery site to the grow-out location. The transport of juvenile sea cucumbers exposes the animals to a series of potential stressors, such as capture from the culture system, handling, transportation, and subsequent release. Depending on the method used for transport, different levels of physiological stress and mechanical abrasion can be generated, leading to adverse impacts on the behaviour, physiology, and immune system of the sea cucumbers and often resulting in infection with disease, reduction in growth rates and mortalities (Iversen et al., 1998; Van der Meeren, 1991; Purcell et al., 2006a; Singh et al., 2004; King, 2009).

Transportation methods for both juvenile and adult sea cucumbers have only been briefly described for two species (*A. japonicus* and *H. scabra*) (e.g., Sui et al., 1991; Pitt, 2001; Xiyin et al., 2004; Song and Liu, 2005; Purcell et al., 2006a; Sun et al., 2006; Robinson and Pascal, 2012). According to the available literature, the ideal transport conditions for juvenile sea cucumbers appear to be highly species-specific and are hard to comply with fully when resources are limited. For example, the temperate sea cucumber, *A. japonicus*, can be transported without seawater for extended periods of time without major problems, whereas the same technique does not work for juveniles of *H. scabra* (Purcell et al., 2006a; Sun et al., 2006). Therefore, reliable and cost-effective transport methods need to be determined for juveniles of each sea cucumber species.

In Australasia, the hatchery technology for the production of juveniles of *Australostichopus mollis* is being developed in order to take advantage of the increasing global demand for sea cucumbers (Zamora and Jeffs 2013). This species is an aspidochirotid sea cucumber that is similar in appearance to *A. japonicus*, with a high market value in Asia of more than US$ 275 kg^{-1} dried (Purcell et al., 2012a). Individuals of this species have been transported to, and released for co-culture, underneath mussel and oyster farms, or transferred into land-based tank holding facilities (Slater and Carton, 2007; Maxwell et al., 2009; Slater et al., 2009; Zamora and Jeffs 2012a). However, the transport of juvenile *A. mollis* to grow-out locations has proven to be problematic. Transport outcomes for juveniles have been best for short trips of less than an hour where the sea cucumbers were transported inside tanks with large quantities of seawater with the animals attached to seaweed to avoid abrasion and evisceration (*Personal Observation*).

Although effective, this method is not practical, or economically viable, especially for transporting large numbers of juveniles over longer distances. Therefore, the main objective of this study was to determine more effective transport conditions for juvenile *A. mollis* by experimentally comparing different transportation methods. Success of the transportation method being tested was evaluated by monitoring the occurrence of evisceration, skin lesions, as well as the subsequent feeding activity, and survival of the juvenile sea cucumbers.

6.2. Materials and Methods

6.2.1. Source of Juveniles

Juvenile sea cucumbers were collected from the Mahurangi Harbour in northern New Zealand by divers in April 2012. The sea cucumbers were transferred immediately, in a 100-l plastic tank half-filled with fresh seawater from the collection site, to the nearby Leigh Marine Laboratory. Once in the laboratory the juveniles were held in tanks with flowing 100-µm filtered seawater at ambient temperature. Juveniles were weighed to ± 1 g according to the procedure described by Sewell (1990) and only those juveniles weighing between 12 and 24 g, a size typical for transfer to grow-out, were retained for subsequent experimentation. Individual sea cucumbers were photo-identified so that they could continue to be recognized and monitored throughout the experiment from their pattern of body markings (Raj, 1998b).

The selected juveniles were allowed to fully recover from handling by holding them in rectangular tanks with a continuous flow of 50-µm filtered fresh seawater at 17 ± 0.2 °C, similar to the ambient temperature at their site of collection. The juveniles were allowed to acclimate to the new conditions for two weeks during which time they were fed with mussel biodeposits collected from underneath tank-cultured green-lipped mussels, *Perna canaliculus* (Zamora and Jeffs, 2011).

6.2.2. Experimental Set up

Two potential transportation systems for juvenile sea cucumbers were simulated experimentally. The first one was an open system in which the sea cucumbers were exposed to the air. The second system was a closed one in which the juveniles were placed in plastic bags and exposed

to different temperatures, following a similar protocol presented by Purcell et al. (2006a) for juveniles of the tropical sea cucumber *H. scabra*. For both open and closed transportation systems, the effect of the duration of the exposure to the holding conditions was evaluated. All the individual sea cucumbers used for experimentation were starved 48 h prior being exposed to the experimental conditions.

6.2.3. Open System Transport Simulation

To determine the length of time that the juvenile sea cucumbers could be maintained in the absence of seawater without deleterious effects, a total of 20 starved juveniles were individually placed on top of plastic trays uncovered at ambient air temperature of 22.2 °C (± 0.5 S.D.) for the experimental duration. Five randomly selected juveniles from the trays were returned to the holding tanks every hour on the hour, over a period of four hours. The wet weight of the sea cucumbers was measured before returning them to the holding tanks to measure their change in weight due to water loss. After returning the sea cucumbers to the holding tanks, their condition was regularly evaluated in terms of the presence of skin lesions, feeding activity and survival. These were evaluated every 30 min during the first 6 h and then every 12 h for the following seven days. The same procedure was carried out simultaneously in another group of 20 starved juveniles, but with the sea cucumbers individually wrapped with a seawater-soaked cotton cloth (SSCC) to prevent desiccation when they were placed on their respective plastic trays.

6.2.4. Closed System Transport Simulation

For closed system transport simulation the sea cucumbers were housed in insulated polystyrene boxes (50 × 30 × 30 cm, L × W × H) as are typically used for transporting a wide range of live seafood species. The sea cucumbers were either held with small volumes of seawater in individual plastic bags or wrapped with a SSCC, and then placed inside polystyrene boxes which were subject to three different types of temperature control. The ambient temperature treatment (AT) was maintained in the shade at ambient temperature, the ice pack treatment (IP) included colling ice-packs inside the boxes to lower the temperature and were maintained in the shade, the refrigeration treatment (RT) involved placing the boxes inside a refrigeration unit set to 4 °C. Temperature inside each box was measured throughout the experiment, in order to have an idea of the variation of temperature inside each shipping box. The mean temperatures throughout the

whole experimental time at the AT, IP and RT transport conditions were 20.4 °C (± 0.7 S.D.) 10.2 °C (± 0.4 S.D.) and 6.1 °C (± 0.2 S.D.) respectively. A total of 120 randomly selected juvenile sea cucumbers were used in this experiment, with 60 of these sea cucumbers individually wrapped with a SSCC and placed in a separate plastic bag (14.5 cm × 20.5 cm, W × H). Each plastic bag was filled with 500 mL of air and sealed to avoid any leakage. The other 60 juveniles were individually placed in plastic bags without the SSCC, and filled with 250 mL of seawater at 17 °C and 250 mL of air before sealing the plastic bag. The sealed bags containing the sea cucumbers were then randomly assigned and immediately taken to one of three transport conditions (i.e., 20 bagged juveniles in SSCC and 20 bagged juveniles in seawater in the AT, IP and RT transport condition treatments respectively).

For each of the three transport treatments (i.e., AT, IP, RT), four randomly selected juveniles from both the SSCC and seawater treatments were taken out at 2, 4, 8, 12, and 24 h. The juveniles were then weighed and returned to the holding tanks with seawater at 17 °C and their condition was regularly evaluated in terms of the presence of skin lesions (i.e., skin ulcerations), feeding activity (i.e. presence of faeces) and survival. These were evaluated every 30 min during the first 6 h and then every 12 h for the following seven days.

To better understand how the holding experimental conditions changed for the juvenile sea cucumbers depending on the transport conditions, the temperature, pH and dissolved oxygen of the seawater associated with each sea cucumber was measured immediately after their removal from the transport conditions at 2, 4, 8, 12, and 24 h. After removing the juveniles from the plastic bags, samples of seawater were taken from the bags or squeezed from the SSCC inside the bag, for measuring seawater parameters. Seawater parameters were also measured from controls which consisted of plastic bags without a sea cucumber but containing either a SSCC or 250 mL of seawater that were removed from the treatments at every sampling time for each temperature treatment. The seawater parameters were measured using calibrated instruments; waterproof pH spear probes (Eutech), oxygen probes (Hach, IntelliCAL™ LDO101) and handheld digital thermometers.

6.2.5. Statistical Analyses

For both transport simulation experiments, the effect of time of exposure on the weight loss of the juveniles within and among treatments was tested with ANOVA. Data were tested for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests respectively prior to analyses. When overall significant differences were detected with the ANOVA, the Holm-Sidak method was used for subsequent pairwise comparisons of treatment means (Quinn and Keough, 2002). In situations where the data were found to be non-parametric, comparisons among treatments were made with Kruskal-Wallis tests (Quinn and Keough, 2002). Eviscerated sea cucumbers were excluded from statistical comparisons of weight loss in sea cucumbers.

Differences in the number of sea cucumbers surviving, feeding, developing skin lesions and eviscerating at different times of exposure were determined using 2 × 2 contingency tables, with time and the response as variables. For these analyses, frequency data for each response variable were pooled into two groups; ≤ 8 h (i.e., 2, 4 and 8 h, n = 12 sea cucumbers), and > 8 h (i.e., 12 and 24 h, n = 8 sea cucumbers). The Fisher's Exact test was used for determining significance because more than 20% of the cells of the contingency tables had less than five observations (Quinn and Keough, 2002).

For the closed system transport simulation, the changes in temperature, pH and oxygen of the seawater samples taken at different times were compared within and among transport conditions treatments separately for the sea cucumbers held in a SSCC and in seawater. All data were tested for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests respectively prior to any statistical analysis. The temperature data were compared using a non-parametric Kruskal-Wallis test and a Tukey's test was used a posteriori to identify the source of the differences. The pH and oxygen data were compared using two-way ANOVAs with treatment and time as factors and pH and oxygen as dependent variables respectively. The Holm-Sidak method was used for pairwise comparisons of means when overall experiment-wide differences were detected (Quinn and Keough, 2002). Data from eviscerated sea cucumbers were excluded from the pH and oxygen analyses.

6.3. Results

6.3.1. Open System Transport Simulation (Exposed to the Air)

6.3.1.1. Weight loss of juveniles

There were no significant differences in the initial weight of the sea cucumbers that were exposed to the air for different times (ANOVA, $F_{(7, 39)} = 2.11$, $P = 0.07$). The sea cucumbers that were exposed to the air lost weight as time passed regardless of whether they were in a SSCC or not (ANOVA, $F_{(3, 15)} = 5.28$ - uncovered, $P = 0.01$; ANOVA, $F_{(3, 16)} = 28.93$, $P < 0.001$ - SSCC) (Table 6.1), however, there was no difference in the mean weight loss for sea cucumbers in a SSCC versus those that were not over the evaluated times (ANOVA, $F_{(1, 32)} = 0.14$; $P = 0.71$) (Table 6.1).

6.3.1.2. Evisceration and survival of juveniles

None of the sea cucumbers eviscerated during the simulated transport conditions. Seven days after being returned to the holding tanks there were no differences in the number of sea cucumbers that had survived after simulated transport either uncovered or in a SSCC (Fisher Exact, $P = 1.00$) (Table 6.1). Only two of the 20 sea cucumbers that were left uncovered died within the 7 days of observation. The first one died 24 h after being exposed for 4 h uncovered, and the second one died 7 days after being exposed for 3 h uncovered (Table 6.1).

6.3.1.3. Feeding and skin lesions in juveniles

Seven days after being returned to the holding tanks all the experimental sea cucumbers that were in a SSCC during simulated transport conditions were feeding. Whereas, only 40% of the juveniles that were uncovered during the transport experiment were feeding (Fisher Exact, $P < 0.001$). Furthermore, the juveniles that were in a SSCC did not have any skin lesions, whereas 90% of the sea cucumbers that were uncovered developed skin injuries (Fisher Exact, $P = <0.001$) (Table 6.1).

6.3.2. Closed System Transport Simulation With or Without Seawater

6.3.2.1. Weight changes of juveniles

There were no significant differences among the initial weight of the sea cucumbers that were held in plastic bags with or without seawater for different times (ANOVA, $F_{(29, 119)} = 0.96$, P =

0.53) (Table 6.2 and 6.3). Only the sea cucumbers that were in a SSCC significantly lost weight progressively as time passed in all transport treatments (ANOVA, $F_{(2, 8)} = 35.48$, $P < 0.001$ for the AT treatment; ANOVA, $F_{(4, 11)} = 46.81$, $P < 0.001$ for the IP treatment; ANOVA, $F_{(4, 11)} = 68.29$, $P < 0.001$ for the RT treatment) (Table 6.2 and 6.3). However, weight loss differences in the juvenile sea cucumbers among the different transport treatments only became apparent after 8 h (ANOVA, $F_{(2, 6)} = 66.00$, $P < 0.001$). At this time the weight loss of the sea cucumbers was lower in the RT treatment than in the AT and IP treatments (Holm-Sidak, $P < 0.001$ in both cases) (Table 6.2).

6.3.2.2. Evisceration of juveniles

Evisceration events occurred in the sea cucumbers only during the experimental simulated transport conditions before they were returned to the holding tanks. Evisceration was more common in the juveniles exposed for longer times (> 8 h) in the experimental conditions (Table 6.2 and 6.3). However, a significantly higher number of juvenile sea cucumbers eviscerating was only detected for those held in the AT treatment in a SSCC, with 88% of the sea cucumbers eviscerating after 24 h (Fisher Exact, $P = <0.001$) (Table 6.2).

6.3.2.3. Survival of juveniles

The overall survival of juveniles after exposure to simulated transport conditions was relatively high (> 80%) regardless of the transport condition treatment and time of exposure (Table 6.2 and 6.3). However, survival was 60% among sea cucumbers in the RT treatment in a SSCC. Survival after transportation appeared to depend on the time of exposure and the holding conditions, with more juveniles surviving when exposed for shorter periods of time (≤ 8 h) than those exposed for longer periods of time (between 12 and 24 h) (Fisher Exact, $P = <0.001$ for the juveniles in a SSCC in the RT treatment, and Fisher Exact, $P = <0.01$ for the juveniles held in seawater in the IP treatment) (Table 6.2 and 6.3).

6.3.2.4. Feeding of juveniles

The number of juveniles feeding was clearly affected by the amount of time that they had previously spent in the experimental transport conditions. For juveniles in a SSCC, all juveniles

held in experimental conditions for ≤ 8 h were feeding after 7 days, whereas none of the sea cucumbers held in experimental conditions for longer periods (12 and 24 h) were feeding after 7 days (Fisher Exact, P = <0.001 for all three transport condition treatments) (Table 6.2). For juveniles held with seawater in experimental conditions, the number of sea cucumbers feeding after 7 days did not significantly change with time of exposure in the AT treatment (Fisher Exact, P = 0.16) but tended to decrease over time (Table 6.3). Whereas, all the juveniles held in seawater in the IP and RT treatments stopped feeding ≥ 4 h of exposure to experimental conditions, and therefore no significant differences were detected over time (Fisher Exact, P = 0.12 for both temperature treatments) (Table 6.3).

6.3.2.5. Skin lesions in juveniles

The appearance of skin lesions after exposure to experimental transport conditions was more pronounced in the sea cucumbers held in a SSCC than in those held in seawater. Under both conditions, the number of sea cucumbers with skin lesions tended to increase with time of exposure (Table 6.2 and 6.3). However, a significant increase in the presence of skin lesions with time was detected only for the sea cucumbers held with seawater in the RT treatment (Fisher Exact, P = 0.02) (Table 6.3).

6.3.2.6. Variations of temperature, pH and oxygen

The parameters that were measured from the seawater taken from the plastic bags (i.e., temperature, pH and oxygen concentration) varied depending on the transport conditions (i.e., AT, IP and RT treatments) and on whether the juveniles were held in seawater or in a SSCC (Fig. 6.1 and 6.2). The temperature to which the sea cucumbers were exposed was more stable in the AT treatment than in the IP and RT treatments in which the temperature gradually decreased for both the juveniles held in seawater and in a SSCC (Kruskal-Wallis, H = 20.53, d.f. = 2, P = <0.001 – juveniles in seawater; Kruskal-Wallis, H = 40.60, d.f. = 2, P = <0.001 – juveniles in a SSCC) (Fig. 6.1 and 6.2). However, for both juveniles held with seawater and in a SSCC, there were no significant differences in the temperature measured between the IP and RT treatments (Tukey, P > 0.05, for both juveniles held in seawater and in a SSCC) (Fig. 6.1 and 6.2). When comparing the temperatures at which the juveniles held in seawater were exposed in all transport conditions with those of the juveniles in a SSCC, there were significant differences (Kruskal-

Wallis, H = 75.70, d.f. = 5, P = <0.001) (Fig. 6.1 and 6.2). Overall lower temperatures of exposure were detected for the juveniles held in seawater, reaching lower temperatures around 5 °C after 8 h of exposure in the IP and RT treatments, while higher temperatures around 10 °C where reached only after 12 h of exposure for the juveniles in a SSCC in the RT treatment (Tukey, $P < 0.05$, for both treatments) (Fig. 6.1 and 6.2). Whereas in the case of pH and oxygen concentration, significant differences were only detected for the sea cucumbers held in seawater (Two way ANOVA, source of variation: Transport condition ($F_{(2, 38)} = 51.31$, $P < 0.001$), Time ($F_{(4, 38)} = 33.45$, $P < 0.001$), Interaction ($F_{(8, 38)} = 8.28$, $P < 0.001$) for pH; and Two way ANOVA, source of variation: Transport condition ($F_{(2, 38)} = 81.02$, $P < 0.001$), Time ($F_{(4, 38)} = 1.37$, $P = 0.26$), Interaction ($F_{(8, 38)} = 11.05$, P = <0.001) for oxygen) (Fig. 6.1 and 6.2). The pH decreased with time in the AT treatment more than in the IP and RT treatments (Holm-Sidak, $P < 0.001$) (Fig. 6.2). Whereas, in the case of oxygen, its concentration drastically decreased with time in the AT treatment (Holm-Sidak, $P < 0.001$), but increased in the IP and RT treatments (Holm-Sidak, $P < 0.001$ for both IP and RT treatments) (Fig. 6.2). When compared with the control values of pH and oxygen, again major differences were found only for the sea cucumbers held with seawater, in which the levels of the controls were always higher for each transport condition treatment at all times (Fig. 6.1 and 6.2).

6.4. Discussion

Adverse physiological and behavioural consequences due to handling and transportation have been documented for several commercially important aquatic taxa (Iversen et al., 1998; Singh et al., 2004; Purcell et al., 2006a; King, 2009). According to our results, when juvenile *A. mollis* are exposed to inappropriate transport conditions they will develop skin lesions, eviscerate, stop feeding and die. The magnitude of these negative consequences was highly dependent on the duration of exposure to transport conditions, especially the temperature and whether or not the juvenile sea cucumbers were held in seawater. Efficient transportation of hatchery-produced juveniles will be useful for the development of future culture-based stocking programs in Australasia (Zamora and Jeffs, 2013). As showed in this study juvenile *A. mollis* could be successfully transported if the adequate measures are taken as discussed below.

6.4.1. Short-Term Transportation of Juveniles

Ideally juvenile sea cucumbers would be transported without seawater as has been reported for lobsters (Van der Meeren, 1991), abalone (Heasman et al., 2004), gastropods (e.g., *Trochus* sp.) (Dobson, 2001) and scallops (Mincher, 2008). However, this study shows that juveniles of *A. mollis* can be transported without seawater only for a limited amount of time (< 1 h when uncovered and ≤ 8 h in a SSCC). The use of a SSCC to wrap the juvenile sea cucumbers substantially reduced the negative effects of being without seawater, allowing the viable transport times to be extended. This mitigation is effective, provided temperature does not go below 10 °C during transport. Compared to juveniles of other sea cucumber species, *A. mollis* is similar to *A. japonicus* which can withstand being transported without seawater in a moist environment for up to 10 h (Song and Liu, 2005), whereas juveniles of *H. scabra* fared poorly when held without seawater in damp sponges (Purcell et al., 2006a). Therefore, juvenile *A. mollis* could be transported fairly long distances without seawater, especially if the hatchery and final destination of the juveniles are close to an airport, although this is often not the case.

6.4.2. Long-Term Transportation of Juveniles

If the transportation of juveniles of *A. mollis* lasts more than 8 h, the use of seawater appears to be the only effective option. When transporting the juveniles with seawater at ambient temperature they were able to survive for 24 h without any skin lesions, and with only a slight effect on their subsequent feeding behaviour if the time of transport exceeded 12 h. This negative effect on the feeding behaviour could be due to the significant decrease in pH and oxygen concentration of the seawater observed during transportation. These changes in the transport seawater are most likely due to an increase in the typically low metabolism of *A. mollis* juveniles triggered by stress, and resulting in elevated oxygen demand and increased release of respiratory carbon dioxide into solution (Zamora and Jeffs, 2012b). Therefore, when transporting juvenile sea cucumbers for long periods of time in seawater preventive measures should be taken, in order to avoid harmful temperature, pH and oxygen levels. Starvation immediately prior to transport and reduction of the seawater temperature during transport should help to reduce sea cucumber metabolism and minimize excretion by the animals. Another common preventive measure is the injection of oxygen into the seawater during transport, or the use of a sealed transport container filled with oxygen which can then dissolve into the enclosed seawater (Song and Liu, 2005; Purcell et al. 2006a). However, if preventive measures cannot be applied, the seawater used for transport should be changed at regular intervals (Sui et al., 1991; Robinson and Pascal, 2012).

The control of pH has not been used in the transport of sea cucumbers, however, depending on the species of sea cucumber, the pH levels during transport might be important as it has been found for some species of fish (Singh et al., 2004; King, 2009).

6.4.3. Control of Temperature during Transportation of Juveniles

Temperature is an important factor for transportation of sea cucumbers. For juvenile *A. mollis* an increase in seawater temperature can increase their metabolism, decrease their feeding activity, reduce their subsequent growth rates, and can be lethal (Maxwell et al., 2009; Zamora and Jeffs, 2012b). In order to avoid the negative effects of an increase in seawater temperature during transport in other sea cucumber species (i.e., *H. scabra* and *A. japonicus*) it has been recommended that transport should be done during the cooler hours of the night and early morning (Song and Liu, 2005; Purcell et al., 2006a; Robinson and Pascal, 2012). However, this study has shown that not only an increase in seawater temperature during transport, but also an excessive decrease in temperature (below 10 °C) can be harmful to juvenile sea cucumber and therefore, should also be avoided when transporting juvenile *A. mollis*.

6.4.4. Success Indicators of Juveniles Transportation

In this study, feeding activity was one of the best indicators of transport stress. For example, 7 days after experimental transport, some juveniles that did not eviscerate or have skin injuries had still not recommenced feeding. This suggests that feeding behaviour is a more sensitive indicator of transport induced stress than evisceration or skin lesions. In other marine invertebrates transportation stress has been observed to cause subsequent behavioural changes. For example, the daily burrowing cycle in the sea cucumber *H. scabra* is altered after transportation, as well as their initial feeding activity which makes them more vulnerable to predators when released in the wild for enhancement purposes (Dance et al., 2003; Purcell et al., 2006a). In the case of *A. mollis*, the factors that appeared to most affect feeding after transportation were the duration and the temperature during the transport period. Nevertheless, feeding behaviour was not the only important indicator of transport success in juvenile *A. mollis*, as some juveniles that were feeding after the transport experiment also had developed skin injuries that were capable of progressing to cause subsequent mortality. Skin lesions in cultured sea cucumbers can be caused by different biotic or abiotic factors, and depending on their nature can be highly contagious and lethal

(Purcell and Eeckhaut, 2005). In this study skin injuries were more common in those juveniles that were exposed directly to the air, suggesting that desiccation was the most likely explanation. However, skin lesions were also present among the juveniles held with seawater at cooler temperatures for a long time (RT treatment), suggesting that skin lesions may be a stress response that is not exclusive to desiccation. Therefore, both feeding activity and the presence of skin injuries need to be taken into account, and carefully monitored after transportation of juvenile sea cucumbers.

6.5. Conclusions

Transportation of juveniles of the sea cucumber *A. mollis* can be done without seawater for short periods of time only (i.e., up to 8 h) when desiccation is avoided, making the process more cost effective and easier to handle. However, for transportation times of more than 8 h the only effective option is to transport the juveniles in seawater, and requires careful consideration of the potential negative effects of changes in seawater temperature, pH and oxygen during transport. For the purpose of controlling experimental variables our research was undertaken with idealized transport conditions, however, in practice temperature and pressure fluctuations, vibration and agitation may reduce estimated transport times due to additional stress on the animals. Regardless, this study indicates that feeding behavior, as well as the presence of skin lesions, after transportation are more sensitive indicators of the transportation success in juvenile sea cucumbers.

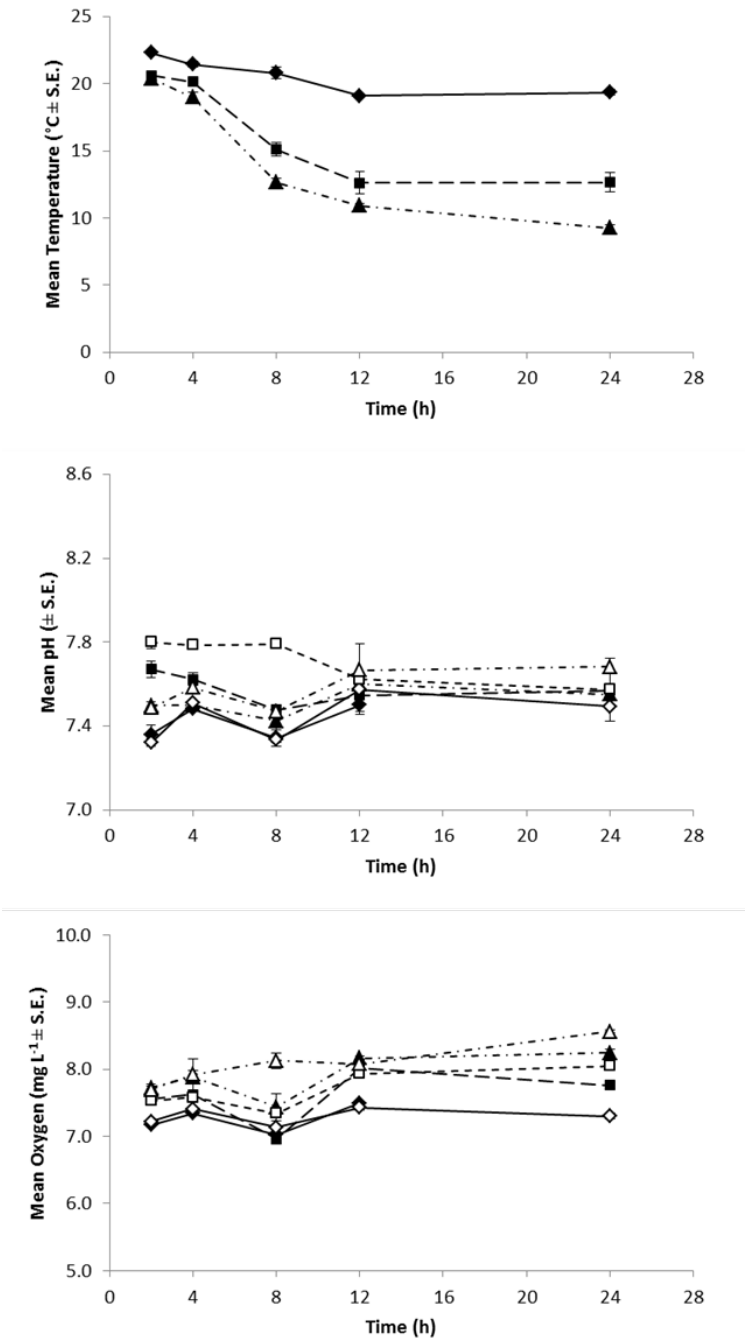

Figure 6.1. Mean temperature, pH and oxygen levels from water samples extracted from the seawater-soaked cotton cloth (SSCC) wrapping juveniles of *Australostichopus mollis* at ambient temperature (AT = ♦), ambient temperature and ice-packs (IP = ■) and inside a refrigeration unit (RT = ▲) over the 24 hour transport simulation period inside a shipping box. Values coming from eviscerated sea cucumbers were not used to estimate the levels of oxygen and pH. For both pH and oxygen the mean values of controls without sea cucumbers are also presented for each transport condition treatment (i.e., AT = ◊, IP = □, and RT = Δ).

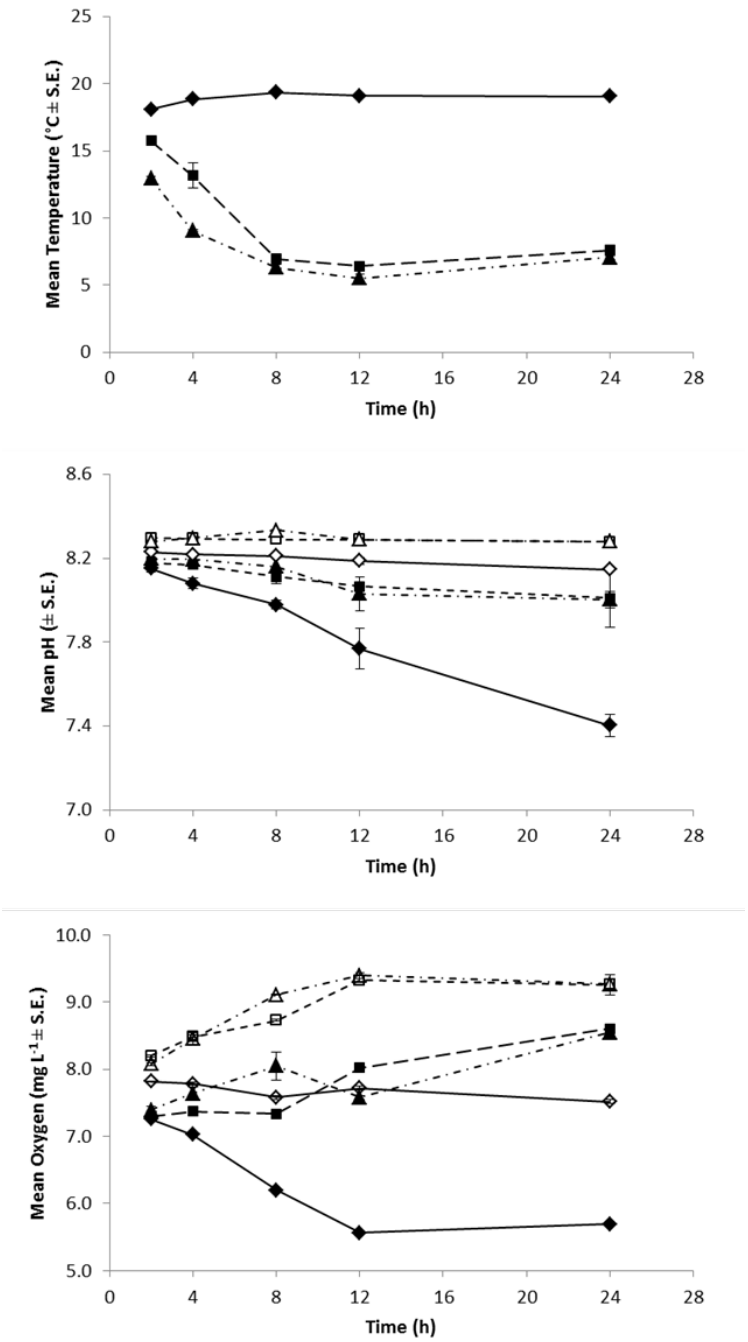

Figure 6.2. Mean temperature, pH and oxygen levels from seawater samples extracted from the bags with juvenile *Australostichopus mollis* held with seawater at ambient temperature (AT = ♦), ambient temperature and ice-packs (IP = ■) and inside a refrigeration unit (RT = ▲) over the 24 h transport simulation period inside a shipping box. Values coming from eviscerated sea cucumbers were not used to estimate the levels of oxygen and pH. For both pH and oxygen the mean values of controls without sea cucumbers are also presented for each temperature treatment (i.e., AT = ◊, IP = □, and RT = Δ).

Table 6.1. Weight (W) change, and percentage (%) of juvenile *Australostichopus mollis* that survived, developed skin lesions, and were feeding 7 days after experimental simulation of transportation, with and without cover (seawater-soaked cotton cloth, SSCC) to aerial exposure (AE) during different transportation times (1, 2, 3 and 4 h). Different letters within each temperature for the percentage of weight lost indicate significant differences ($P < 0.05$).

	Time (h)	Initial W. (g) (Mean ± S.E)	Final W. (g) (Mean ± S.E)	W. Lost (%) (Mean ± S.E)	S. Lesions (%)	Survival (%)	Feeding (%)
AE (without SSCC)	1	16.46±1.93	16.21±1.94	1.59±0.33[a]	80	100	60
	2	20.82±1.29	19.85±1.05	4.46±1.14[b]	80	100	60
	3	17.80±1.40	16.80±1.25	5.51±0.16[b]	100	80	20
	4	25.01±2.03	23.40±2.03	6.58±1.00[b]	100	80	20
AE (with SSCC)	1	21.23±1.32	20.87±1.23	1.62±0.33[a]	0	100	100
	2	19.93±2.34	19.26±2.18	3.15±0.56[a]	0	100	100
	3	20.96±1.50	19.76±1.44	5.74±0.37[c]	0	100	100
	4	19.49±1.92	18.13±1.74	6.94±0.49[c]	0	100	100

Table 6.2. Weight (W) change, and percentage (%) of juvenile *Australostichopus mollis* that survived, developed skin lesions, and were feeding 7 days after experimental simulation of transport conditions of being wrapped with a seawater-soaked cotton cloth (SSCC) and held in a plastic bag at ambient temperature (AT), ambient temperature and ice-packs (IP) and inside a refrigeration unit (RT) during different transportation times inside a shipping box (2, 4, 8, 12, and 24 h). Eviscerated sea cucumbers were excluded from calculations of mean weight lost. Different letters within each temperature for the percentage of weight lost indicate significant differences ($P < 0.05$).

	Time (h)	Initial W. (g) (Mean ± S.E)	Final W. (g) (Mean ± S.E)	W. Lost (%) (Mean ± S.E)	S. Lesions (%)	Evisceration (%)	Survival (%)	Feeding (%)
AT	2	17.97±2.99	17.40±2.84	3.08±0.74[a]	0	0	100	100
	4	23.53±4.54	21.96±4.28	6.74±1.03[b]	0	0	100	100
	8	29.80±3.16	28.47±2.59	11.29±0.47[c]	50	25	100	75
	12	26.41±3.07	20.33	15.20	75	75	100	0
	24	31.10±5.75	----	----	50	100	25	0
IP	2	25.05±4.38	24.27±4.34	3.33±0.66[a]	0	0	100	100
	4	26.72±2.69	25.24±2.75	5.82±1.18[a]	0	0	100	100
	8	28.99±3.59	21.24±1.94	17.79±0.38[b]	50	25	100	75
	12	33.25±4.38	22.35±1.48	23.10±2.24[c]	50	25	100	0
	24	32.69±4.65	24.68±2.09	12.39±0.80[d]	25	50	50	0
RT	2	21.39±3.99	20.73±3.82	2.92±0.70[a]	0	0	100	100
	4	25.00±0.96	23.83±0.83	4.65±0.56[a]	0	0	100	100
	8	23.05±3.25	21.88±4.64	4.65±1.26[a]	25	25	100	75
	12	29.36±2.77	22.50±2.77	19.64±1.04[b]	25	25	0	0
	24	34.29±7.16	22.78±10.38	13.49±0.44[c]	25	50	0	0

Table 6.3. Weight (W) change, and percentage (%) of juveniles *Australostichopus mollis* that survived, developed skin lesions, and were feeding 7 days after experimental simulation of transport conditions of being held in a plastic bag with 500 mL of seawater at ambient temperature (AT), ambient temperature and ice-packs (IP) and inside a refrigeration unit (RT) during different transportation times inside a shipping box (2, 4, 8, 12, and 24 h). Eviscerated sea cucumbers were not used in mean weight lost estimations. Different letters within each temperature for the percentage of weight lost indicate significant differences ($P < 0.05$).

	Time (h)	Initial W. (g) (Mean ± S.E)	Final W. (g) (Mean ± S.E)	W. Lost (%) (Mean ± S.E)	S. Lesions (%)	Evisceration (%)	Survival (%)	Feeding (%)
AT	2	27.91±6.99	27.55±6.55	-0.29±2.49[a]	0	0	100	100
	4	22.83±2.24	22.56±2.11	1.06±1.12[a]	0	0	100	75
	8	29.03±3.05	29.25±3.28	-0.71±2.81[a]	0	0	100	75
	12	29.96±5.10	31.41±5.58	-4.33±4.00[a]	0	0	100	50
	24	26.49±3.03	24.62±3.55	-0.47±2.24[a]	0	25	75	50
IP	2	32.05±3.71	32.32±3.72	-0.91±0.77[a]	0	0	100	100
	4	27.96±4.32	27.97±4.78	0.74±1.86[a]	0	0	100	0
	8	31.89±5.83	32.49±5.91	-2.02±2.01[a]	0	0	100	0
	12	27.47±3.85	27.42±3.25	-0.57±2.24[a]	25	0	100	0
	24	31.53±5.66	28.38±12.55	-5.73±1.08[a]	0	50	0	0
RT	2	26.35±3.81	26.36±3.61	-0.34±0.73[a]	0	0	100	100
	4	20.09±1.20	21.03±1.44	-4.51±1.05[a]	0	0	100	0
	8	31.20±5.99	28.73±7.70	-0.63±0.60[a]	25	25	100	0
	12	27.44±0.73	27.54±1.45	1.77±1.63[a]	75	25	100	0
	24	23.73±1.20	26.31±1.83	-4.67±4.03[a]	50	25	25	0

Chapter Seven

General Discussion

7.1. Introduction

A review of the research on *A. mollis* in relation to the aquaculture development for this species (Chapter One) identified a number of areas where information was lacking and was in need of further research. These findings were used to guide the specific aims for the remainder of the research presented in this thesis. Consequently, in the following chapters new information regarding the physiology, ecology, feeding behaviour, nutrient absorption and utilization, as well as growth and effective transport of juvenile *A. mollis* is presented and discussed in some detail within the conclusion of each research chapter. In summary, the research presented in the thesis provides evidence of the capability of juvenile *A. mollis* to cope with low food availability in sediments and their ability to maintain growth rates when feeding on sediments containing markedly different levels of organic content (Chapters Two and Three). This new information increases the understanding of the feeding behaviour of this species, especially the importance of food availability as a means for controlling feeding behaviour. Then the response of the juveniles to different seawater temperatures in terms of feeding behaviour, metabolism, nutrient utilization and growth was evaluated (Chapters Four and Five). These two chapters increased our understanding on how the sea cucumbers will perform under different seawater temperatures as well as the juveniles nutrient requirements. Finally, this research provides technical information for the transportation of juvenile sea cucumbers from the hatchery or nursery to the grow-out locations (Chapter Six).

7.2. Contribution of the Research to the Development of Artificial Diets

Th e results presented in this thesis showed that sea cucumbers do not necessarily need a high organic content in their food in order to generate acceptable growth (0.6% d^{-1}) (Zamora and Jeffs, 2012b). Using palatable feeds with low organic content could be useful in terms of reducing the organic waste produced (faeces and uneaten food) since most of the food would be consumed, and the organic content of the faeces would be low (Zamora and Jeffs, 2012b).

However, if the organic content of the feed is low, the sea cucumbers would need to process more food than when feeding on diets with high organic content to satisfy their requirements for nutrients and growth. Therefore, in order to supply an adequate quantity of food, the amount of organic content in the feed (high or low TOM content), together with the stocking density and other variables that also affect feeding behaviour (e.g., seawater temperature) have to be considered. An artificial diet needs to contain an appropriate overall organic content, and it must also have an adequate balance of macronutrients, that match the requirements of the sea cucumbers. This study provided some of the first results for beginning to determine the specific macronutrient requirements of juvenile sea cucumbers of any species, providing an initial guidance for the formulation of artificial diets. The results showed that the juvenile *A. mollis* are able to selectively consume, digest, and absorb lipid, protein and carbohydrate to varying extents, emphasizing their respective importance as a source of energy for growth and metabolism. Since the amount of carbohydrate and protein ingested and absorbed were comparatively higher than lipid for a natural feed (mussel waste), it is suggested that their inclusion in any artificial diet should be higher. Accordingly, the results suggest that any artificial diet prepared for *A. mollis* should have at least 3% lipid, 6% protein and 12% carbohydrate in terms of dry weight. This is similar to the proportions of macronutrients found currently in artificial diets for *A. japonicus* (Yuan et al., 2006; Liu et al., 2009; Seo and Lee, 2011).

Future development of an artificial feed for juvenile *A. mollis* will rely on identifying digestible and cost effective artificial sources for each macronutrient that also provide sufficient subunits (e.g., amino and fatty acids). This is important, since it is not just a matter of simulating the proportions of macronutrient in a highly palatable natural food in order to generate good growth (Slater et al., 2011b). In addition, the palatability of feed ingredients will also be an important consideration because it is still not clear which components of the food is responsible for attracting sea cucumbers towards the food and stimulating ingestion (Lawrence et al., 2007). The addition of specific food attractants and ingestion stimulants, such as glycine and betaine, are commonly used in artificial feeds in aquaculture to facilitate the use of feed ingredients that otherwise would not be ingested but can also be beneficial for the sea cucumbers growth (Coman et al., 1996; Felix and Sudharsan, 2004). The way in which artificial food is presented to juvenile sea cucumbers also needs to be evaluated in terms of particle size and texture (e.g., in the form of dry or wet pellets, or as a slurry or paste). Ultimately, the development of artificial diets is

necessary to develop a more reliable source of nutrition and to reduce the hatchery and nursery production time of juveniles. In addition, artificial diets can also play a role in improving other aquaculture practices, such as assisting with inducing the gonad maturation of the broodstock (Morgan, 2004b).

7.3. Contribution of the Research to the Development of Transport Methods

The transportation of live juvenile sea cucumbers from hatchery and nursery culture systems to grow-out locations has been found to be challenging for a number of sea cucumber species, and could be very expensive, especially if the use of seawater is required (Sun et al., 2006). The research presented in this thesis demonstrated the capability of the juveniles of *A. mollis* to withstand long periods of time (up to 8 h) without seawater, which would be a typical transport duration to the grow-out locations. The feeding activity and survival of juvenile sea cucumbers were not affected during this time, which would allow the transportation of juveniles in a more cost effective way than moving while immersed in seawater. The ability to transport juvenile sea cucumbers for 8 hours without seawater allows for hatchery and nursery facilities to more easily supply juveniles over a much wider area, by eliminating the necessity for the hatchery to be nearby to the grow-out locations. For example, a hatchery located near a major city airport in New Zealand would have the potential to supply juvenile sea cucumbers throughout the country via air shipping without seawater. However, it is still not clear whether or not it would be necessary to precondition the juveniles of this species to the grow-out conditions prior to their release following transportation to the grow-out location (Dance et al., 2003; Purcell et al., 2006a). The behaviour of hatchery produced juveniles could be different under artificial holding conditions, and therefore needs to be evaluated after transportation and release into the grow- out sites.

7.4. Contribution of the Research for the Selection of Culture Sites and Stocking Densities

A better understanding of how *A. mollis* responds to extrinsic variables, such as seawater temperature, is essential to the development of efficient commercial aquaculture of this sea cucumber species. A knowledge of the optimal extrinsic parameters for culturing this species would not only allow ideal conditions to be maintained under controlled aquaculture conditions in a hatchery or nursery situation, but would also be useful for selecting more suitable grow-out

locations where animals are exposed to ambient environmental conditions (Renbo and Yuan, 2004; Agudo, 2006; Purcell, 2012). The results of the research presented in this thesis show that food availability and seawater temperature can greatly influence the feeding activity of juveniles of *A. mollis*, and therefore the growth of this species (Zamora and Jeffs, 2011; Zamora and Jeffs, 2012a, 2012b).

Sufficient food needs to be available for juvenile *A. mollis* in order to guarantee fast growth rates. When there is a low organic content in sediments (e.g., bellow 4%) and a high stocking biomass of sea cucumbers (e.g., around 500 g m^{-2}) it is highly likely that the available food will be rapidly depleted. Under such conditions the sea cucumbers will not grow and can start losing weight, due to an inability to gather sufficient nutrition despite processing more sediment than when feeding on high organic content sediments (Zamora and Jeff, 2011). Under mussel farms, the organic material does not distribute evenly horizontally across the seafloor sediments, which is why the sea cucumbers would have to actively forage in order to seek out areas of sediment with high organic matter (Zamora and Jeffs, 2012a). For this reason stocking densities of sea cucumbers need to be adjusted according to the food availability in the grow-out location, with low organic content locations being able to support proportionately less sea cucumber biomass compared with locations with sediments that are rich in organic matter. Ideally, locations with a high organic matter input to the sediment throughout the year should be chosen for the effective grow-out of *A. mollis*, such as areas beneath mussel farms. The sediments impacted by a typical mussel farm have an average organic content of 11% (TOM) which, according to the results of this research, would be more than capable of sustaining stocking densities of *A. mollis* of around 500 g m^{-2}, provided the food under mussel farms is continually renewed. Under different culture conditions, such as in earthen ponds or tanks, when food availability has to be carefully managed, artificial diets may be added to greatly increase the organic content of the sediments in order to achieve higher stocking densities than are achievable under natural grow-out conditions. When there are high levels of organic matter in the sediments, the ingestion rate of juvenile *A. mollis* has been shown to decrease, which would potentially allow higher stocking densities of sea cucumbers (Qin et al., 2009). It is likely that there will be a limit for the enrichment of sediments with artificial diets since there is a possibility of decomposition of organic rich uneaten feed and faeces which will require more frequent removal and higher water flows in order to maintain optimum water quality. Therefore, maintaining high availability of organic

food for sea cucumbers will lead to correspondingly increased operational costs associated with cleaning and water pumping.

Seawater temperature also proved to be an important factor for influencing the growth of juvenile *A. mollis* when food availability is not an issue (Zamora and Jeffs, 2012b). This species responded negatively to an increase in seawater temperature from 15 to 24 °C in terms of feeding, metabolism, growth, and survival. Seawater temperatures around 24 °C were lethal to juvenile sea cucumbers held under aquaculture conditions. Therefore, seawater temperature, especially during summer, should be given paramount consideration when selecting commercial grow-out locations. Seawater temperature maintained at around 15 °C would be the ideal conditions for siting sea cucumber grow-out operations. Sea cucumbers reduced their feeding activity and growth at seawater temperatures of 21 °C (Zamora and Jeffs, 2012b). In addition, it has been shown that the capability of juvenile sea cucumbers to select and absorb organic particles from the sediment is also affected at this temperature, suggesting that their ability to feed on and reduce the organic content of the sediments during summer could be compromised (Slater and Carton, 2009, MacTavish et al., 2012). Therefore, in grow-out situations where seawater temperature cannot be controlled, it will be important to adjust the supply of artificial feed in relation to increased seawater temperature, with the amount of food being reduced to avoid the accumulation of organic matter from the food in sediment. Seawater temperature could also be an important factor influencing the timing of transfer of juvenile *A. mollis* from the hatchery or nursery controlled conditions to the grow-out location (Purcell, 2004; Renbo and Yuan, 2004; Hair, 2012). According to the results obtained in this research, it is strongly recommended to wait to transfer the sea cucumbers at the end of summer time, in order to allow the juveniles to grow as much as possible before entering to the summer season again.

Given the wide latitudinal range in the distribution of this species, it could be expected that individuals from different locations would respond differently to the natural variation of seawater temperature in terms of growth and feeding behaviour (Purcell et al., 2012b). The results of this study are more representative of populations from northern New Zealand which can be exposed to naturally higher summer seawater temperatures (over 20 °C) than populations in southern parts of the country, e.g., below 10 °C. Concomitantly, it is possible that *A. mollis* from colder waters in southern New Zealand may show a negative response to elevated seawater temperatures at a lower point (around 18 °C or below) than observed for individuals from

populations in northern parts of the country (Maxwell et al., 2009). Variation in the performance of *A. mollis* from source populations that experience a range of different natural thermal regimes may provide some additional flexibility for the selection of potential grow-out locations for this sea cucumber. This possibility warrants further investigation through future research.

In the current research, one or more extrinsic variables were controlled individually for experimental purposes, which is not the normal situation for a commercial aquaculture operation. Under commercial grow-out conditions (in earthen ponds, sea ranching or as part of integrated multi-trophic aquaculture operation) the sea cucumbers will be exposed to the variation of several environmental parameters simultaneously, and the synergistic effect of these upon the sea cucumbers will determine the success of the culture (Renbo and Yuan, 2004; Agudo, 2012). Some of these varaiables that were not examined in the course of this current study, such as salinity, oxygen levels, predators, presence of shelter, and extreme weather conditions, will also need to be taken into account when evaluating the success of the commercial aquaculture of *A. mollis*.

7.5. General conclusions

Overall, the body of research presented in this thesis will provide useful information for the development of commercial aquaculture of *A. mollis*, by increasing the understanding of the physiology, ecology, feeding behaviour, growth, handling, as well as nutrient absorption and utilization. However, further research is required for further advancing the development of artificial diets, optimising juvenile transport conditions, and improving our knowledge of the response of *A. mollis* to a wider range of extrinsic parameters likely to be encountered during aquaculture. Such research would help to further improve nursery production and transport of juveniles, and facilitate the selection and management of suitable locations for grow-out of this sea cucumber species, independently of whether it is for pond culture, sea ranching or integrated multi-trophic aquaculture.

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