Developing hatchery culture of the tropical sea cucumber, *Holothuria scabra,* using micro-algae concentrates

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Statement on the Contribution of Others

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Abstract

Major bottlenecks to further development and expansion of hatchery-based production of tropical sea cucumbers include our lack of knowledge of their nutritional requirements and appropriate foods for larval stages. Live micro-algae have been used as the main source of food for hatchery culture. However, mass culture of adequate volumes of high quality cultured micro-algae is both labour and resource demanding and is often inappropriate for small-scale hatcheries in developing countries that generally lack the required resources and technical capacity. Alternative potential food sources for larvae include highly-concentrated marine micro-algae that is now available commercially. This study assessed the potential of commercially available micro-algae concentrates as a replacement for live cultured micro-algae during hatchery culture of sandfish, *Holothuria scabra*. It examined ingestion and digestion of micro-algae concentrates by larval sandfish (Chapter 3), the nutritional value of micro-algae concentrates for larval sandfish during hatchery culture (Chapter 4), the relationships between hyaline spheres formation, larval food composition and subsequent settlement success (Chapter 5), and the ingestion, digestion and nutritional value of live micro-algae and micro-algae concentrates for newly settled sandfish juveniles (Chapter 6).

Ingestion and digestion of two live (TISO and *Chaetoceros muelleri*) and six concentrated micro-algae products (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) by sandfish auricularia larvae of different ages were assessed using epifluorescence microscopy. The commercial micro-algae products were purchased from an Australian distributor. They were: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova* sp. (Haptophyceae) (Pavlova 1800®); (3) mono-cultured *Tetraselmis* sp. (Chlorophycophyceae) (Tetraselmis 3600®); (4) mono–cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®); (5) mono-cultured *Thalassiosira pseudonana* (Bacillariophyceae) (3H 1800®); and (6) a mix of four concentrated single micro-algae species: *Isochrysis* sp., *Pavlova* sp. *Thalassiosira pseudonana* and *Tetraselmis* sp. (Shellfish Diet 1800®). This is the first study to report the use of epifluorescence microscopy with larval echinoderms and experiments were conducted using 2, 6 and 10 day old auricularia larvae. Seven of the eight micro-algae tested were ingested and digested by larvae with digestion occurring more rapidly in older larvae. *C. muelleri* was rapidly digested by 6-day and 10-day

old larvae but results indicate that *C. muelleri* is unsuitable as a food for 2-day old sandfish larvae. TISO was well ingested by sandfish larvae in both live and concentrated forms, and live TISO was the most suitable of the micro-algae tested in terms of ingestion and digestibility. All commercially available micro-algae concentrates tested were readily ingested and digested by *H. scabra* larvae with the exception of *Thalassiosira pseudonana* (3H 1800®) which was not ingested by larvae of any of the three ages tested. Results show potential for using micro-algae concentrates as alternatives to live micro-algae in hatchery culture of sandfish. The three most digestible of the ingested Instant Algae® products were used in a subsequent experiment to assess the relative nutritional values of digestible micro-algae, as a basis for optimising a diet for hatchery culture of sandfish, and to provide information on the nutritional requirements of sandfish larvae.

Three Instant Algae® (Reed Mariculture Inc., Campbell, CA, USA, 95008) products: (1) mono-cultured *Isochrysis sp.* (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova sp.* (Haptophyceae) (Pavlova 1800®); and (3) mono-cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®) were used to feed sandfish larvae both singly and in ternary combination to assess their nutritional efficacy. Two-day auriculariae were held at a starting density of 0.3 mL^{-1} and were initially fed a daily ration equivalent to the dry weight of 10,000 cells mL^{-1} of Isochrysis 1800®. This ration was increased by the dry weight equivalent of 1000 cells mL^{-1} of Isochrysis 1800® per day as larval development proceeded. Post-settled larvae fed TW 1200® were significantly larger than those fed the ternary diet, Isochrysis 1800® or Pavlova 1800®. There were significant differences in the mean (±SE) survival of auriculariae and post-settled larvae between treatments and survival to settlement was significantly higher ($P < 0.05$) for larvae fed TW 1200® (13.7 ± 0.7%) alone. Laval development, competency and survival were significantly correlated with dietary levels of total protein, lipid and nitrogen-free extract (NFE, equivalent to carbohydrate), and with total polyunsaturated fatty acid (PUFA) content of the diets, and the levels of some specific fatty acids (FA). The proportion of late auriculariae with hyaline spheres (day 13), numbers of competent doliolariae (day 15) and the total length of post-settled larvae (day 21) were all positively correlated with dietary NFE and palmitic acid (16:0) contents, as well as dietary EPA:DHA ratio. This study is the first comprehensive assessment of the nutritional value of micro-algae concentrates for sandfish larvae based on their nutrient compositions. Results confirm the feasibility of using commercially available micro-algae concentrates as a sole food source for hatchery culture of sandfish, and are the first to report successful hatchery culture of sandfish larvae without using

live micro-algae. All micro-algae concentrates used in this study proved nutritious for sandfish larvae and supported normal growth and development and relatively high survival, through settlement.

The following experiment investigated the influence of diet composition on hyaline sphere (HS) development in auricularia larvae of sandfish, and the subsequent relationships between the presence and size of HS and competency through settlement and early juvenile performance. Two-day old larvae were fed one of three commercially available micro-algae diets that varied in their nutrient compositions: (1) *Isochrysis sp.* (Haptophyceae); (2) *Pavlova sp.* (Haptophyceae); and (3) *Thalassiosira weissflogii* (Bacillariophyceae) or a ternary combination of the three. There were positive significant correlations between HS development in late auriculariae on days 10, 11, 12 and 13 post-fertilisation, and the proportion of competent doliolariae on day 15, post-settlement size (day 21) and post-settlement survival (day 25). The dietary components that most strongly influenced these relationships were carbohydrates (as NFE) and the polyunsaturated fatty acids arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The results confirm a strong relationship between HS formation in late auriculariae of sandfish and subsequent larval competency through settlement. As such, the presence and size of HS is a reliable indicator of subsequent performance for sandfish. Given that HS development was influenced by the nutrients available to sandfish auriculariae, there is clear opportunity for development of more appropriate diets for hatchery culture of this species that will improve HS formation and larval performance supporting improved hatchery production.

Information on the nutritional requirements and preferred diets of sea cucumber juveniles is extremely limited and this has hindered development of hatchery culture methods. The final experiment in this study assessed ingestion, cell wall digestion and relative nutritional values of two live micro-algae (*Isochrysis aff. galbana* (TISO) and *Chaetoceros muelleri*) and six concentrated micro-algae products (Instant Algae®, Reed Mariculture Inc.) for early juveniles of sandfish, *Holothuria scabra*. The six Instant Algae® products were: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova* sp. (Haptophyceae) (Pavlova 1800®); (3) mono-cultured *Tetraselmis* sp. (Chlorophycophyceae) (Tetraselmis 3600®); (4) mono–cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®); (5) mono-cultured *Thalassiosira pseudonana* (Bacillariophyceae) (3H 1800®); and (6) a mixture of four micro-algae species: *Isochrysis* sp., *Pavlova* sp. *Thalassiosira pseudonana*

and *Tetraselmis* sp. (Shellfish Diet 1800®). Seven of the eight micro-algae tested were ingested by juveniles with the exception of live TISO. Faeces excretion times varied between ingested diets that passed through the juvenile gut in less than 1 h. The cell walls of five of the eight micro-algae tested were partially or mostly digested (*Chaetoceros muelleri*, TW1200®, Palova 1800®, Isochrysis 1800® and Shellfish 1800®), while the cell walls of Tetraselmis 3600® and 3H 1800® remained intact. Juvenile growth rates were significantly different between diet treatments over the duration of a 14-day growth trial. Mean (±SE) length of early juveniles at the end of the growth trial was highest for those fed live *C. muelleri* (4.10 ± 0.03mm) followed by TW 1200® (3.49 ± 0.05mm). Juvenile survival did not differ significantly between diet treatments and was highest for those fed *C. muelleri* (79.33 ± 6.11%) followed by TW1200® (78.33 ± 1.20%). Pearson's correlation tests were used to identify key correlations between the levels of specific nutrients and juvenile performance (growth and survival). A significant positive correlation between growth and dietary protein content ($P < 0.05$), and a highly significant positive correlation between growth and dietary EPA: DHA ratio ($P < 0.01$) provide new information to inform diet selection for juvenile sandfish that will help improve juvenile performance and support improved hatchery production.

Hatchery culture of sandfish larvae and early juveniles using micro-algae concentrates, without the use of live micro-algae, is a significant output from this study, and results support development of cheaper, simpler hatchery rearing protocols for this species. This study also determined detailed nutritional compositional data for the commercial micro-algae products used, and on this basis, it was possible to deduce new information relating to key nutrients for sea cucumber larvae. The results provide a basis for fine-tuning diets used in hatchery culture of sandfish, and other sea cucumbers, to better address their nutritional requirements. Such a development is likely to result in improved quality of hatchery produced juveniles as well as improved hatchery production.

Publications

Peer-reviewed journal publications resulting from this thesis

1. Duy, N.D.Q., Pirozzi, I., Southgate, P.C., 2015. Ingestion and digestion of live micro-algae and micro-algae concentrates by sandfish, *Holothuria scabra*, larvae. *Aquaculture* 448, 256-261.
2. Duy, N.D.Q., Francis, D.S., Pirozzi, I., Southgate, P.C., 2016 Use of micro-algae concentrates for hatchery culture of sandfish, *Holothuria scabra*. *Aquaculture* 464, 145-152.
3. Duy, N.D.Q., Francis, D.S., Southgate, P.C., 2016. Development of hyaline spheres in late auricullariae of sandfish, *Holothuria scabra*: is it a reliable indicator of subsequent performance? *Aquaculture* 465, 144-151.
4. Duy, N.D.Q., Francis, D.S., Southgate, P.C., 2016. The nutritional value of live and concentrated micro-algae for early juveniles of sandfish, *Holothuria scabra*. *Aquaculture* 473, 97-104.

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CHAPTER 1

GENERAL INTRODUCTION[1]

1.1. Introduction

Sea cucumbers (Echinodermata: Holothuroidea) have been exploited for human consumption and used for their perceived medicinal properties for centuries (Hamel et al., 2001), and over 50 species are now traded within the global dried sea cucumber, 'trepang' or 'bêche-de-mer' market (Purcell et al., 2012). Although some sea cucumber fisheries have existed for centuries, they generally follow 'boom-and-bust' patterns of exploitation (Anderson et al., 2011; Conand, 2004; Hair et al., 2016) and increasing global demand for dried sea cucumbers has resulted in major declines in natural sea cucumber stocks in many countries in the Asia-Pacific region (Jimmy et al., 2012, Mills et al., 2012). This has stimulated growing interest in development of methods for hatchery propagation, mariculture, and stock enhancement of sea cucumbers in a number of countries (Purcell et al., 2012). By 2014, China had produced an estimated 200,000 metric tonnes (wet weight) of the temperate sea cucumber, *Apostichopus japonicus,* from commercial aquaculture which is roughly equal to double the total global wild-harvest (Han et al., 2016). However, aquaculture of tropical sea cucumber species greatly lags that of temperate species and grow-out systems are yet to be adequately developed for the tropical sea cucumbers (Raison, 2008) with limited production achieved from pond culture (Duy, 2012). Research interest has been particularly focused on sandfish, *Holothuria scabra*, that is high value species among tropical sea cucumbers (Robinson, 2013).

Sandfish are thought to be a good candidate for aquaculture in the tropics (Battaglene et al., 1999; Bell and Gervis, 1999; Jimmy et al., 2012) because they are amendable to hatchery production (Duy, 2012; Purcell et al., 2012), and juveniles and adults feed on organic matter and detritus in sediments and require no additional food input during culture (Mills et al., 2012; Pitt and Duy, 2004). However, after nearly three decades of research, little is known about the

[1] The contents of this Chapter together with data from Chapters 3, 4, 5 and 6, have been submitted for publication as: Duy, N.D.Q., Southgate, P.C. Hatchery culture and nutrition of larval and juvenile sea cucumbers with an emphasis on sandfish, *Holothuria scabra. Reviews in Aquaculture* (submitted)

nutritional requirements, feeding mechanics or food assimilation in sandfish larvae or juveniles and this has hindered development of hatchery production of sandfish, which is characterised by poor survival to the juvenile stage of this species (Purcell et al., 2012).

1.2. Hatchery culture methods of sea cucumbers

1.2.1. Reproductive cycle and spawning induction

Sea cucumber sexes are separate but, without apparent sexual dimorphism, examination of the gonad is required to determine the sex of an individual. The sex ratio of sandfish in the natural population is close to 1: 1 (Hamel et al., 2001) and the minimum size at sexual maturity is around 180 g (Agudo, 2006). Annual spawning periods of sandfish in the wild differ in different geographical areas as a result of differences in temperature and salinity (see Hamel et al., 2001). For example, in Moreton Bay Australia, the peak of the spawning period is from September to November (Morgan, 2000) while in New Caledonia, peak spawning periods fall in December to February and August to September (Hamel et al., 2001). Sandfish broodstock in captivity can be induced to spawn all year round by conditioning in ponds or tanks (Duy, 2010; Pitt and Duy 2004); however, the period of holding broodstock in tanks might relate inversely to fecundity and to the hatching rate of fertilised eggs in sandfish (Morgan, 2000).

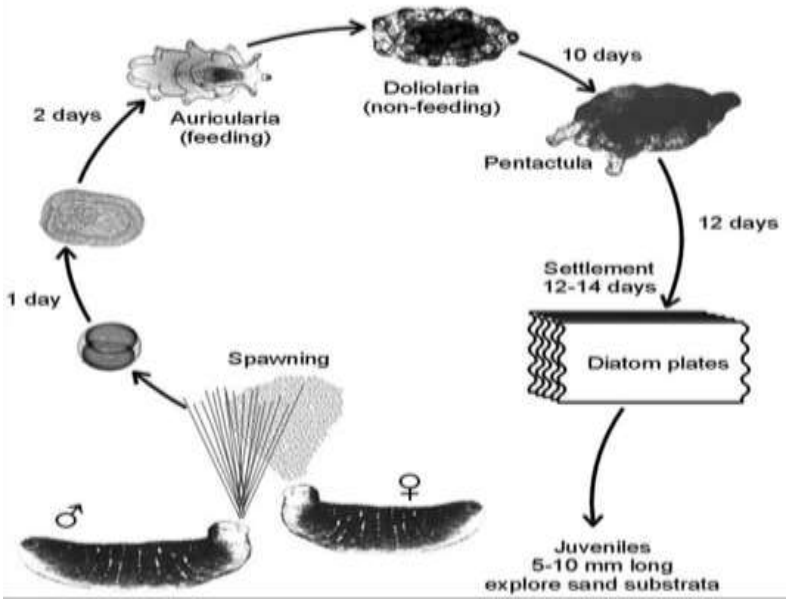

Fig.1.1. The lifecycle of cultured sandfish *Holothuria scabra* described by Battaglene (1999).

Induction of spawning is generally conducted in tanks with various stressors including thermal stimulation, gonad (male only) extraction method, water pressure, dry treatment, food stimulants (addition of dried *Spirulina*) or combination of two or three above methods (Agudo, 2006). However, the most common spawning induction method used in current sandfish hatcheries is to use thermal shock by heating the water in spawning tank to 3-5 °C above that of ambient temperature, then transferring broodstock into the tank (Duy, 2010) with or without the addition of dried *Spirulina* (Duy, 2012). Males normally spawn first and this generally triggers females to release eggs (Duy, 2010). Alternatively, an in-vitro fertilisation (IVF) technique can be simply used by stripping gametes from mature sea cucumber adults (Eeckhaut et al., 2012). However, this method is not commonly used in sandfish hatcheries.

1.2.2. Established larval culture methods

1.2.2.1. Larval culture methods

There are several stages within the hatchery production of sandfish which have different husbandry and food requirements (Fig.1.1). The first auricularia stage is planktonic and larvae are generally fed live cultured micro-algae (Agudo, 2006; Duy, 2010; Hamel et al, 2001; Knauer, 2011). The recommended larval rearing density for sandfish is 0.3-0.5 larvae mL^{-1} (Battaglene, 1999; Duy, 2012) and up to 1.0 larva mL^{-1} (Asha and Diwakar, 2012). High survival, good growth rates and fastest development of auriculariae were obtained at salinity between 33 and 35 ppt (Asha et al., 2011).

1.2.2.2. Food and feeding

Generally, the larval diet of sea cucumbers is composed of a combination of two or three live micro-algae (Table 1.1). Although a mixture of two or three live micro-algal species are preferred for sandfish auriculariae (Battaglene, 1999; Agudo, 2006; Duy, 2010), single-species diets composed of diatoms *Chaetoceros muelleri* or *C. calcitrans,* have been strongly indicated as the most suitable and desirable diet during the larval rearing period (Knauer, 2011; Duy, 2012; Gamboa et al., 2012). However, the reasons that *C. muelleri* or *C. calcitrans* are the most suitable and effective micro-algae remain unclear because of lack of knowledge of the nutritional requirements of holothurian larvae.

Table 1.1. Micro-algae commonly used as larval diets for the hatchery culture of sea cucumbers.

Species	Micro-algae recommended for hatchery culture	Author(s)
Apostichopus japonicus	*Dunaliella salina, Phaeodactylum tricornutum*, *Chaetoceros* and marine yeasts	Chen (2003)
A. japonicus	*Chaetoceros muelleri* at 15,000 cells mL^{-1} day^{-1}	Sun and Li (2013)
Australostichopus mollis	*Chaetoceros muelleri* at 3,000 cells mL^{-1} day^{-1}	Morgan (2008)
Isostichopus fuscus	A mixture dominated by *Rhodomonas* sp. and *Dunaliella* sp. or *Chatoceros* sp. and *Dunaliella* sp.	Mercier et al. (2012)
Stichopus horrens	A mixture of *Chaetoceros muelleri*, *Dunaliella* sp. and *Chlorella pyrenoidosa* (7 : 2 : 1)	Hu et al. (2013)
Stichopus sp.	A mixture of marine yeast (*Rhodotorula*) and micro-algae *Rhodomonas* sp. and *Dunaliella* sp.	Hu et al. (2010)
Bohadschia marmorata	*Chaetoceros calcitrans* and *Isochrysis galbana*	Laxminarayana (2005)
Holothuria atra	*Isochrysis galbana* and *Chaetoceros calcitrans*	Laxminarayana (2005)
Holothuria scabra	*Rhodomonas salina*, *Chaetoceros muelleri* and *Chaetoceros calcitrans*	Battaglene (1999)
H. scabra	*Chaetoceros muelleri* and *Rhodomonas salina*, mixed in equal parts, or *Isochrysis aff. galbana* (first days) and then mixed with *Chaetoceros* sp. (4-5 days later)	Agudo (2006) Duy (2010) Knauer (2011)
H. scabra	*Rhodomonas salina, Chaetoceros calcitrans, Chaetoceros mulleri, Tetraselmis chuii, Isochrysis galbana* and *Pavlova lutheri*	Giraspy and Ivy (2005), Ivy and Giraspy (2006)
H. scabra	*Isochrysis galbana*, 10,000 -20,000 cells mL^{-1} day^{-1}	James et al. (1994), Morgan (2001)
Holothuria spinifera	*Chaetoceros calcitrans* alone or in combination with *Isochrysis galbana*	Asha and Muthiah (2006)
Holothuria vegabunda	*Isochrysis* sp. (day 3), then fed a mixture *C. muelleri, C. calcitrans* and *Tetraselmis* sp.	Dabbagh et al. (2011)

Food ration is a factor that influences larval performance of sandfish. However, little is known about the importance of micro-algae ration on sea cucumbers. James et al. (1994) and Morgan (2001) reported that larval sandfish fed *Isochrysis galbana* can develop into competent

doliolariae, but Battaglene (1999) and Knauer (2011) reported the opposite. These conflicting results may be explained by the different feeding rates of *I. galbana* in these trials. Morgan (2001) revealed that larval survival was reduced and growth and development was inhibited at an algae cell density of 40,000 cells mL^{-1}, whereas, Knauer (2011) fed auriculariae with slightly higher amounts based on equal dried weight of *C. muelleri* at the late auricularia stage. Further studies are necessary to better validate optimal densities of specific micro-algae at each larval stage for sandfish.

1.2.3. Metamorphosis and settlement induction

The method used for inducing settlement of *Apostichopus japonicus* involves the use of polycarbonate and polyethylene sheets preconditioned in seawater to develop a natural biofilm (Xiyin et al., 2004). This method was later successfully adapted for *H. scabra* (Agudo, 2006) and *Australostichopus mollis* (Zamora and Zeffs, 2013). In the temperate species, *Stichopus japonicus*, Ito and Kitamura (1997) indicated that the density of benthic diatom should be more than at 200,000 cells cm^{-2} for effective metamorphosis induction. "Algamac 2000" (Aquafauna Biomarine, Hawthorne, California, USA) can be used as a settlement cue for sandfish (Battaglene, 1999), and soluble extracts of the sea grass *Thalassia hemprichii* can induce metamorphosis and settlement on clean plastic surfaces (Mercier et al., 2000). Sandfish larvae settled preferentially on the leaves of the sea grass *T. hemprichii* in the presence or absence of natural biofilm (4.8-10.5%) compared with sand or crushed coral substrate (<1.5%), and settlement can be delayed up to four days in the absence of substrates, but results in very low survival (Mercier et al., 2000). Similarly, in the sea cucumber *Apostichopus japonicus*, the use of *Ulvella* film treated with antibiotics can induce metamorphosis in up to 80 % of larvae after 87 h (Matsuura et al., 2009). James et al. (1994) suggested using extract of *Sargassum* sp. for coating settlement plates for sandfish. Recently, settlement plates coated with a paste of *Spirulina* sp. that can induce settlement and provide an initial food source for newly settled individuals were introduced (Pitt and Duy, 2004; Duy, 2010); they reduce operational costs and potential contamination of cultures by harmful animals, such as copepods (Mills et al., 2012). Although the efficacy of dried *Spirulina* in settlement induction of metamorphosed sandfish larvae has been reported, further studies are required to optimise its use both alone, or combined with other potential food sources, as an effective settlement cue.

1.2.4. Newly settled juvenile culture

1.2.4.1. Culture methods

Newly settled juvenile culture is a critical stage in sea cucumber hatchery production (Ramofafia et al., 2003; Purcell et al., 2012). Methods for growing and feeding newly-settled sea cucumbers have generally been adopted from those used for gastropods like abalone (Battaglene, 1999). Generally, benthic diatoms (e.g. *Nitzschia* spp., *Navicula* spp.) are used to condition settlement plates and they provide both settlement induction cues and a supplemental food source for post-settlement larvae (Agudo, 2006). Newly settled juveniles are raised using this method until their length reaches about 5 mm, when they can be transferred to nursery tanks (Battaglene et al., 1999) or hapa nets in ponds (Duy, 2010; Pitt and Duy, 2004).

1.2.4.2. Foods and feeding

Although benthic diatoms and shrimp starter feed are commonly used as a food source for juveniles in sandfish hatcheries, the relative efficiencies of these feeds are not well understood (Pitt & Duy, 2004; Watanabe et al., 2012). Micro-algae, such as *Chaetoceros muelleri* and *Skeletonema* spp. have been used as a food source for culturing early juveniles of sandfish (Duy, 2012; Juinio-Meñez et al, 2012) but little research has examined the effect of individual micro-algae species on growth and survival of early post-settled juveniles. Also, little is known about the key dietary components for newly settled juvenile sea cucumbers. Diatom-conditioned plates might take up to 5 months to produce (Ito and Kitamura, 1997), whereas diatoms, such as *Chaetoceros* and *Skeletonema* are relative easy to growth in tropical conditions within a week (Battaglene, 1999). However, micro-algae including micro-algae concentrates, have not been investigated as a potential food source for early juvenile culture.

1.3. The move away from live micro-algae in the hatchery culture of sea cucumbers

One of the major impediments to routine mass production of sea cucumber hatcheries is that current established hatchery practices are heavily based on live micro-algae as an indispensable food source for larval culture. Mass culture of adequate volumes of high quality cultured micro-algae is both labour and resource demanding (Coutteau and Sorgeloos, 1992), and is often inappropriate for small-scale hatcheries in developing and small-island nations that often lack the technical resources and skilled personnel required for successful hatchery operation. Production of appropriate quantities of high quality live micro-algae, as a larval food source, is a common bottleneck (Southgate et al., 2016). Moreover, micro-algae culture can be a source

of bacterial contamination (Mercier et al., 2004) and predators (e.g. copepods and ciliates) in larval rearing tanks (Duy, 2010; Mills et al., 2012). These disadvantages of live feed culture underscore the importance of identifying alternative diets as replacements for live micro-algae.

Broad use of commercially available micro-algae concentrates as food for sea cucumber larvae would, for example, support development of simplified larval rearing protocols that are more appropriate for developing nations. Moreover, nutrient compositions of micro-algae concentrates are generally consistent, while those of live micro-algae can vary according to culture conditions such as growth phase, culture medium composition and temperature (Brown et al., 1997; Martínez-Fernández et al., 2006; Pacheco-Vega and Sanchez-Saavedra, 2009; Pernet et al., 2003). As such, the use of 'off-the-shelf' foods potentially supports development of simpler and more reliable culture protocols for sea cucumber hatcheries.

Several other criteria must be considered relating to the potential use of artificial diets, such as spray-dried micro-algae, concentrated micro-algae and formulated feeds as replacements for live micro-algae. Overfeeding can be a problem for sea cucumber larvae (Ahsa, 2004; Morgan, 2001, 2008, 2009) resulting in deteriorating water quality, which is often the case with the breakdown of uneaten formulated food particles (Holme et al., 2009). Non-living and 'artificial' feed particles are generally negatively buoyant and settle more rapidly than live micro-algae, potentially resulting in rapid fouling of the culture medium (Langdon, 2003). Thus, optimisation of rations to reduce particle settlement and excessive food wastage and water fouling may enhance the effectiveness of these foods during hatchery culture.

The negatively buoyacy of non-living food particles does, however, have an advantageous aspect for post-settlement larvae culture of sea cucumbers. This characteristic is well suited to the feeding behaviour of post-settlement sea cucumber larvae because, at settlement, sandfish larvae become grazers and forage detrital organic matter and are capable of digesting carbon sources from microbial and fungal degradation (Lopez, 1987; James et al., 1994). On this basis, non-living foods including concentrated algae could be used as food sources for settlement stages of sandfish.

1.4. Use of other commercial products as alternative diets for larval sea cucumbers

In general, the potential of using artificial or formulated foods and non-living micro-algae preparations for larval culture of marine species has been well examined. Microencapsulated feeds and spray-dried algae have been reported to be widely used for hatchery culture of crustaceans (Robinson et al., 2005; Spolaore et al., 2006; Holme et al., 2009), fish (Langdon, 2003), bivalves (Knauer and Southgate, 1999; Teitelbaum and Fale, 2008; Marshall et al., 2010) and echinods (Azad et al., 2010). For instance, a commercial microencapsulated feed ("E-Z Larva", Zeigler Bros.) was successful in supporting larval culture of the sea urchin *Lytechinus variegatus* (George et al., 2004). Similarly, microencapsulated foods "Frippak CD#2" and "Lansy-Shrimp" (INVE Aquaculture), supported normal growth of sea urchin, *Psammechinus miliaris* and *Paracentrotus lividus*, larvae and better growth of 10-day post-settlement sized juveniles than a live micro-algae diet (Liu et al., 2007a, 2007b). Clearly, these products may have potential as a food source for larval sandfish.

Given the dependence of larval sandfish culture on live micro-algae as a larval food source, experimental studies on alternative diets have been conducted to replace live feeds. For example, Pitt and Duy (2004) reported that a mixture (50:50) of spray-dried *Schizochytrium* sp. "Algamac 2000" (Aquafauna Biomarine, Hawthorne, California, USA), and dried *Spirulina* and "Frippak #2CD" (INVE Aquaculture, Belgium), can be used as foods to culture sandfish larvae to settlement, with relatively high survival rates of 12.7% and 3.8%, respectively, compared to commonly reported rates of survival in many sandfish hatcheries of around 1% (Raison, 2008; Purcell et al., 2012).

In the recent years, some successes in larval sea cucumber culture have been reported using micro-algae concentrates. For instance, Shellfish Diet 1800® (Instant Algae®, Reed Mariculture Inc.) was used as food throughout the culture period of sandfish larvae (Hair et al., 2011) although some live cultured micro-algae were used initially. Shellfish Diet 1800® is composed of *Isochrysis* sp. (30%), *Tetraselmis* sp. (20%), *Pavlova* sp. (20%) and *Thalassiosira weissflogii* (30%). Larvae showed 30% survival at day 9, transformed to the doliolaria stage by day 11 and settled by day 15. In another trial on the sea cucumber *Isostichopus badionotus,* Zacarias-Soto et al. (2013) fed larvae twice a day with a mixture of commercial concentrates of *Tetraselmis* sp. and *Isochrysis* sp. (Instant Algae, Reed Mariculture, Inc., Campbell, CA, USA) following the feeding protocol recommended by Agudo (2006) of 20,000, 20,000–30,000, and 30,000–40,000 cells/mL for early, mid, and late auriculariae, respectively. Once larvae reached the late auricularia stage, they were co-fed with live cultured *Chaetoceros*

muelleri and/or concentrates of *Thalassiosira weissflogii* (Instant Algae, Reed Mariculture, Inc.) to induce metamorphosis to doliolariae and the settlement of pentactulae larvae. This protocol was maintained until the juveniles reached 3 cm in length, after which they were fed with powdered *Macrocystis* sp. and *Sargassum* sp. sieved to <55 μm particle size and enriched with "Algamac-2000" (Bio-Marine, Inc., Hawthorne, CA, USA). Despite low larval survival (2–5%), the result of this trial showed the potential of using micro-algae concentrates as a sole food source for hatchery culture of this species.

1.5. The nutritional requirements of *H. scabra* larvae

1.5.1. Protein

Most studies on diets for sea cucumbers have focused on the juvenile stage and little is known about the protein requirements of larvae. In addition, little research has examined protein requirements of newly settled juvenile sea cucumbers but have focused mainly on older/larger juveniles. Huiling et al. (2004) reported that the optimal dietary protein content for juveniles of *Apostichopus japonicus* is 21.5%. Likewise, Seo and Lee (2011) indicated that a diet containing 200 g kg^{-1} protein (170 g kg^{-1} digestible protein) with 20 g kg^{-1} lipid (13 g kg^{-1} digestible lipid) may be sufficient for optimum growth of juveniles of this species. Slater et al. (2009) stated that artificial protein sources used to feed juveniles of the sea cucumber *Australostichopus mollis*, consistently showed high apparent digestibility, ranging from 75.1% for fish meal to 98.1% for casein. They also found that low-cost protein sources, such as meat meal, showed promise for future diet formulation, but high protein content may reduce ingestion rates and thereby lower overall digestive efficiency in juveniles.

Giraspy and Ivy (2008) reported that the commercially available preparation "Algamac Protein Plus" (42.9% crude protein) supported good growth rates of golden sandfish (*H. lesson*) juveniles (1.7 mm in length) as an individual feed, and a mixed feed composed of a 1:1 mixture of 'Algamac 2000' (39.0% protein) and 'Algamac Protein Plus' produced greater growth rates and survival. In addition, shrimp starter feeds generally comprising of 35-40 % protein content, have been shown to strongly improve growth rates and survival of *H. scabra* juveniles (Pitt and Duy, 2004). Oroco et al. (2014) reported that the growth rate and digestion efficiency of cultured juvenile *H. scabra*, increase with the protein content.

However, protein utilisation might vary according to the feed ingredients and sources used. For example, spray-dried *Spirulina* contains about 60% crude protein and contains all essential

amino acids, though with reduced amounts of methionine, cysteine and lysine when compared to the proteins of meat, eggs and milk; but it did not support strong growth of juvenile *H. lessoni* (Giraspy and Ivy, 2008). In contrast, green-lipped mussel (*Perna canaliculus*) bio-deposits or wastes with a crude protein content of 5.1% proved to be highly palatable and supported good growth rates of juvenile *A. mollis* (Slater et al., 2009; Zamora and Jeffs, 2011). Fu et al. (2005) reported the presence of proteases in the digestive tract of *A. japonicus*. Whereas, Yokoyama (2013) recently reported that this species has limited capacity to digest animal protein. Moreover, a study on *Apostichopus japonicus* (Tang et al., 2007) reported that from auricularia to the doliolaria stage, protease activity decreases then increases again during the later stages of development (pentactulae) resulting in higher protease activity in juveniles than in larvae.

There is only one report on the essential amino acids (EAA) requirements of juveniles of the temperate sea cucumber *A. stichopus* (Sun et al., 2004). Based on a growth experiment over a 40-day period to test five feed formulations, the results indicated that the weight gain rate was maximal for *A. stichopus* when the diet was rich in threonine, valine, leucine, phenylalanine, lysine, histidine and alganine.

1.5.2. *Lipids*

Lipids are important dietary components for marine invertebrate larvae providing energy, maintaining the integrity of structural membrane and functioning as precursors for crucial steroid of marine larvae (Holme et al., 2009). During larval development of sea cucumbers, the hyaline spheres (HS) seem to be associated with the storage of nutrients, presumably lipids, which are subsequently utilised for metamorphosis (Chen et al., 1991; Dautov and Kashenko, 1995), and the presence of HS in late auricularia larvae preceding metamorphosis is thought to be an important indicator of larval competence (Dautov and Kashenko, 1995; Chen et al., 1991; Ramofafia et al., 2003; Morgan, 2008; 2009; Sun and Li, 2013). However, the relationship between larval performance of sandfish and lipid content of larval diet is still unclear.

Lipid appears to play a limited role in energy storage and energy provision in growing and estivating juvenile sea cucumbers (Yang et al., 2006). Significant lipid accumulation is limited to the gonads in mature individuals, and thus, protein appears to form the primary energy source and tissue energy store in growing juveniles (Dong et al., 2006; Yang et al., 2006). Most

invertebrate species, including sea cucumbers, possess active elongase and desaturase enzymes that enable them to significantly modify dietary fatty acids (Kelly and Scheibling, 2012). Dietary polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are important for normal development of many invertebrate larvae such as bivalves (Knauer and Southgate, 1999), crustaceans (Holme et al., 2009) and echinoids (Liu et al., 2007a; Liu et al., 2007b; Carboni et al., 2012), but little is known about their roles in holothurians. Yu et al. (2015) reported that the fatty acid compositions of sea cucumber (*A. japonicus*) tissues can be significantly influenced by diets with different inclusion levels of corn meal and soybean meal, and that sea cucumbers might have the ability to synthesise some degree of 20:1n-9, 22:1n-9, 20:3n-3, 20:4n-6 and 20:5n-3 when they were less available in the diet. Therefore, further study is needed to provide greater understanding of the relationships between dietary lipids and the resulting growth and survival of sea cucumbers, particularly in larvae and early juveniles for which there is very limited information.

1.5.3. Carbohydrates

Most deposit-feeding holothurians have little cellulase activity in their gut and they do not appear to assimilate macro-algae before it is decomposed by bacteria and fungi (Yingst, 1976; Lopez, 1987). Slater et al. (2011) developed a new method for determining apparent digestibility of carbohydrates by sea cucumber (*A. mollis*) juveniles, and found that apparent digestibility was moderate but did not exceed 50% for wheat starch, tapioca starch, carrageenans, and pre-gelatinised maize starch. They also suggested that artificial carbohydrate sources may require pre-fermentation to improve digestibility by sea cucumbers.

High levels of dietary carbohydrate consistently induced rapid growth in juvenile *Stichopus (Apostichopus) japonicus* and *A. mollis* (Slater et al., 2009; Zhou et al., 2006) and *H. scabra* (Orozco et al., 2014). Diatoms generally contain relatively high levels of easily digestible carbohydrate (Orozco et al., 2014) and species of diatoms, such as *Sketonema* sp. and *C. muelleri* have been reported to supported good performance of sandfish larvae (Battaglene, 1999; Duy, 2012; Knauer, 2011). However, little is known about carbohydrate requirements of sea cucumber larvae and early juveniles. A greater understanding of the relationships between the dietary carbohydrates and the resulting growth and survival of larvae and early juvenile sandfish is therefore critically important for future studies in this field.

1.6. Conclusion

The major bottlenecks to further development and expansion of hatchery-based production of tropical sea cucumbers include our lack of knowledge of their nutritional requirements and appropriate foods for larval and early juvenile stages. Despite nearly three decades of research, both nutritional and biological aspects of sea cucumber culture remain understudied, particularly for sandfish. Live micro-algae are still used as the main source of food for hatchery culture of sandfish. However, mass culture of adequate volumes of high quality cultured micro-algae is both labour and resource demanding and is often inappropriate for small-scale hatcheries in developing countries that generally lack the required resources and technical capacity. Alternative food sources for larvae, such as highly-concentrated marine micro-algae, that is now available commercially, have potential for use in hatchery culture of invertebrates (Reed and Henry, 2014) and preliminary results suggest some success in their use as a substitute for live micro-algae when rearing sandfish larvae (Hair et al., 2011).

Although there has been a recent increase in research to develop improved hatchery techniques for sandfish, further research should be conducted experimentally to improve larval diets and simplify hatchery procedures. The nutritional values of micro-algae determined experimentally in growth trials not only reflect the nutrient compositions of micro-algae but also the ability of larvae to digest them and the efficiency with which their nutrients are assimilated because not all micro-algae can be ingested and digested efficiently by invertebrate larvae. On this basis, determination of whether a given micro-alga is ingested and digested by larvae of the target species is an important first step in assessing its suitability as a larval food source and important in developing effective diets for hatchery production.

In addition to appropriate characteristics for ingestion and subsequent digestion, suitable foods for sea cucumber larvae must have appropriate nutritional compositions. Determining relationships between dietary nutrient compositions and larval growth, development and survival is essential for optimising larval feeding protocols and deducing the nutritional requirements of larvae which, in the case of sandfish and holothurians more generally, are poorly known. Therefore, a greater understanding of the relationships between the nutritional composition of larval diets and larval performance is critically important for future studies in this field to identify key dietary components supporting larval growth and survival of sandfish.

1.7. Overall objective, aims and statement of organisation

The overall objective of this study is to investigate the possibility of replacing live micro-algae with commercially available micro-algae concentrates for sandfish larvae, and early juveniles, with a view to simplification of hatchery culture methods for this species. It includes deduction of new information on the nutritional requirements of sandfish larvae and early juveniles, through correlation of diet compositions and larval/early juvenile performance. The major aims of this study were to:

1. Assess the ingestion and digestion of live micro-algae and micro-algae concentrates for sandfish auriculariae (Chapter 3)
2. Assess the nutritional value of digestible micro-algae concentrates for sandfish and the relationships between larval development and nutrient compositions (Chapter 4).
3. Assess the relationship between the hyaline sphere development and nutrient composition of larval diets (Chapter 5).
4. Assess nutritional value of live micro-algae and micro-algae concentrates for sandfish early juveniles in terms of ingestion, digestion and early juvenile performance (Chapter 6).

This thesis is presented in a Thesis-by-Publication format. Each research chapter represents a succinct study that has either been published or submitted. The status of each chapter at the time of thesis submission is indicated using footnotes associated with chapter titles.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Study sites

This study was conducted within the Australian Centre for International Agricultural Research (ACIAR) projects FIS/2010/054 "Mariculture development in New Ireland, Papua New Guinea" and FIS/2010/042 "Expansion and Diversification of Production and Management Systems for Sea Cucumbers in the Philippines, Vietnam and northern Australia" led by Professor Paul Southgate at the University of the Sunshine Coast (USC). Most experiments of this study (reported in Chapters 3, 4, 5 and 6) were conducted at the National Fisheries Authority (NFA), Nago Island Mariculture Research Facility (NIMRF) in Kavieng, Papua New Guinea (PNG) (2°34'57.50"S, 150°46'51.86"E).

2.2. General hatchery culture methods for experimental conduction

The broodstock used for experiments in PNG were collected near Limanak Island (2°34′S 150°48′E) in the authorised closed fishing area. They were held for a week in raceways at the NIMRF prior to spawning induction.

Broodstock were induced to spawn using thermal shock followed by the addition of dried *Spirulina* sp. to the culture water (Agudo, 2006; Duy, 2010). Eggs were hatched in tanks filled with 1 µm filtered seawater (FSW) at a density of 1 egg mL^{-1}. After 2 days, auriculariae larvae in the main larval culture tanks were counted and used for the larval trials. Early juveniles (5 days after settlement) were detached from settlement plates using a gentle spray of 1-µm filtered seawater to be used in the early juvenile culture trial (Chapter 6).

Biochemical composition of micro-algae concentrates was analysed at the commercial laboratory of Deakin University, Geelong, Australia, School of Life and Environmental Sciences, Warrnambool Campus, Princes Hwy, Sherwood Park, PO Box 423, Warrnambool, Victoria 3280, Australia.

CHAPTER 3

INGESTION AND DIGESTION OF LIVE MICRO-ALGAE AND MICRO-ALGAE CONCENTRATES BY SANDFISH, *HOLOTHURIA SCABRA*, LARVAE[2]

3.1. Introduction

The major bottlenecks to further development and expansion of hatchery-based production of tropical sea cucumbers include our lack of knowledge of their nutritional requirements and appropriate foods for larval stages. Some commonly used live micro-algae considered to be suitable foods for sandfish, *Holothuria scabra*, larvae include *Chaetoceros* spp. and *Isochrysis* aff. *galbana* (TISO) used either singly or in combination (Agudo, 2006; Battaglene, 1999; Duy, 2012; Gamboa et al., 2012; James et al., 1994; Knauer, 2011). However, there have been contrasting reports on the nutritional value of live TISO for sandfish larvae which is considered to be a good diet for auricularia larvae by some authors (e.g. Duy, 2010; James et al., 1994; Morgan, 2001) and a poor diet by others (e.g. Battaglene, 1999; Knauer, 2011). The reasons for these contrasting results are unclear but this phenomenon highlights our limited knowledge in this field and lack of consensus towards development of appropriate diets for larval culture of sandfish.

Mass culture of adequate volumes of high quality cultured micro-algae is both labour and resource demanding (Coutteau and Sorgeloos, 1992) and is often inappropriate for small-scale hatcheries in developing countries that generally lack the required resources and technical capacity. This issue has prompted research to investigate alternative food sources for larvae such as phototrophically grown, highly-concentrated marine micro-algae that is now available commercially (Reed and Henry, 2014). Such products have considerable potential for use in hatchery culture of invertebrates (Rikard and Walton, 2012; Reed and Henry, 2014) and preliminary results suggest some success in their use as a substitute for live dietary micro-algae when rearing sandfish larvae (Hair et al., 2011).

[2] Published as: Duy, N.D.Q., Pirozzi, I., Southgate, P.C., 2015. Ingestion and digestion of live micro-algae and micro-algae concentrates by sandfish, *Holothuria scabra*, larvae. *Aquaculture* 448, 256-261.

The nutritional values of micro-algae determined experimentally in growth trials not only reflect the nutrient compositions of micro-algae but also the ability of larvae to digest them and the efficiency with which their nutrients are assimilated. Not all micro-algae can be ingested and digested by invertebrate larvae. For example, only seven out of ten micro-algae species tested were ingested by larvae of the scallop, *Agropecten ventricosus*, and of those, only five were digested (Lora-Vilchis and Maeda-Martinez, 1997). Similarly, larvae of the winged pearl oyster, *Pteria sterna*, were reported to digest only two of three ingested species of micro-algae from a total of ten species tested (Martínez-Fernández et al., 2004). On this basis, determination of whether a given microalga is ingested and digested by larvae of the target species is an important first step in assessing its suitability as a larval food source and important in developing effective diets for hatchery production. The aim of this study was therefore to assess ingestion and digestion of selected live and concentrated micro-algae by sandfish auriculariae of different ages, to provide a basis for optimising hatchery feeding protocols for this species.

3.2. Materials and Methods

Ingestion and digestion of micro-algae were determined directly using epifluoresence microscopy which has been used extensively in similar studies with larval molluscs (e.g. Aldana-Aranda et al., 1997; Lora-Vilchis and Maeda-Martinez, 1997; Martínez-Fernández et al., 2004; Patino-Suarez et al., 2004). This method can be more accurate than cell counting using a high power microscope because the photosynthesizing pigments of algal cells fluoresce under blue light illumination close to the excitation maximum at 490 nm (long wavelength blue) which can be seen at different intensities indicating various stages of ingestion and digestion.

3.2.1. Broodstock and larvae

The broodstock used in this study were collected near Limanak Island in the Kavieng lagoon at the northern end of New Ireland Province in Papua New Guinea (2°34′S 150°48′E). They were held in raceways at the National Fisheries Authority (NFA) Nago Island Mariculture Research Facility (NIMRF) at Kavieng for a week prior to spawning. Broodstock were induced to spawn using thermal shock followed by the addition of dried *Spirulina* sp. to the culture water (Agudo, 2006; Duy, 2010). Eggs were hatched in a 500 L tank filled with 1 µm filtered seawater (FSW) at a density of 1 egg mL^{-1}. After 2 days, larvae in the main larval culture tanks were fed only live cultured *Isochrysis* aff. *galbana* (TISO) at a cell density of 10,000 cells mL^{-1} day^{-1} during the auricularia stage (Morgan, 2010).

Auricularia larvae were removed from the main larval culture tanks to be used in experiments on day 2 (D2), day 6 (D6) and day 10 (D10) after fertilization when they had mean (± SD, n = 90) lengths of 497.16 ± 37.21 µm, 728.06 ± 38.27 µm and 904.24 ± 44.33 µm, respectively. Larvae were retained on a 90 µm mesh screen and washed gently with FSW prior to transfer to the 20 L plastic aquaria used for feeding trials where they were established at a density of 0.3 larva mL^{-1}. Larvae from D6 and D10 were unfed for 24 h prior to each experiment. Larvae from D2, D6 and D10 were fed rations of 20,000, 30,000 and 40,000 cells mL^{-1}, respectively, during the experiments. Water temperature in aquaria used for feeding trials was maintained at 29 ± 1.0°C and the seawater was gently aerated.

3.2.2. Micro-algae cultures

Live TISO and *C. muelleri* were cultured in 20 L carboys, using f/2 medium (Guillard, 1975) in a temperature controlled (28°C) laboratory with a 16L: 8D photoperiod. Micro-algae were harvested during the exponential growth phase as a larval food source.

Commercially available micro-algae concentrates (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) purchased from an Australian distributor of the products were used in this study. Six Instant Algae® products were used: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova* sp. (Haptophyceae) (Pavlova 1800®); (3) mono-cultured *Tetraselmis* sp. (Chlorophycophyceae) (Tetraselmis 3600®); (4) mono–cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®); (5) mono-cultured *Thalassiosira pseudonana* (Bacillariophyceae) (3H 1800®); and (6) a mix of four concentrated single micro-algae species: *Isochrysis* sp., *Pavlova* sp. *Thalassiosira pseudonana* and *Tetraselmis* sp. (Shellfish Diet 1800®). Concentrates were stored in their original bottles in a refrigerator at 4°C for the duration of the study. Prior to use, a 1 mL aliquot of each concentrate was added to approximately 1 L of FSW in a clean plastic bottle and gently hand-shaken to disperse the micro-algae cells. The resulting micro-algae suspension was poured through a 90 µm mesh screen to remove any clumps before use. The cell density in each micro-algal stock suspension was then determined using a haemocytometer and the volume needed to obtain the required ration in each larval aquarium was calculated. The micro-algae used in this study and their characteristics are shown in Table 3.1.

Table 3.1. Live micro-algae and micro-algae concentrates (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) used in this study.

Group (Division)	Algae species/diet	Cell size (µm)	Status
Golden - Brown Flagellates (Haptophyta)	*Isochrysis aff. galbana* (TISO)	5-7	Live
	Isochrysis sp. *(*Isochrysis 1800®)	5-7	Concentrated
	Pavlova sp. (Pavlova 1800®)	4-7	Concentrated
Green Flagellates (Chlorophyta)	*Tetraselmis* sp. (Tetraselmis 3600®)	10-12	Concentrated
Diatoms (Bacillariophyta)	*Chaetoceros muelleri*	5-6	Live
	Thalassiosira weissflogii (TW 1200®)	7-20	Concentrated
	Thalassiosira pseudonana (3H 1800®)	4-6	Concentrated
Golden - Brown Flagellates, Green Flagellate, Diatom (Haptophyta, Chlorophyta, Bacillariophyta)	Shellfish Diet 1800®	5-12	Concentrated

3.2.3. Assessing ingestion and digestion

Larvae were fed separately with each diet for 1 hour to evaluate ingestion of the tested micro-algae. Larvae were then retained on a 90 µm mesh sieve washed gently with FSW and placed in new aquaria containing clean gently aerated 1-µm filter sea water (FSW) without food. They were examined to evaluate digestion of ingested micro-algae after 2, 4, 8, 12 and 24 h from the start of feeding.

To assess ingestion and digestion of each microalga using epiflourescence microscopy, 30 larvae from each aquarium were captured on a fine mesh and fixed with 2% formaldehyde in seawater (buffered) solution prior to examination. Micro-algae cells in the larval esophagus and stomach were identified and quantitatively assessed using a method modified from that described by Martinez-Fernandez et al. (2004). The criteria used to assess the degree of micro-algae ingestion and digestion in this study are shown in Table 3.2 and illustrated in Figure. 3.1. Ingestion was characterised by well-defined fluorescence inside the stomach of the larvae and

the degree to which the larval stomach was full after the 1 hour feeding period was estimated according to five categories (0%, 1-25%, 26-50%, 51-75% and 76-100%) (Patino-Suarez et al., 2004). Apparent digestion was characterized by the presence of fluorescence in the stomach but an absence of identifiable micro-algae cells (Table 3.2, Figure. 3.1).

Table 3.2. Criteria used to assess the degree of micro-algae ingestion and digestion in this study (adapted from Martinez-Fernandez et al., 2004)

Stage	Fluorescence	Characteristics
(I) Partial Ingestion	Red	Whole micro-algae cells visible in esophagus but not in the stomach
(II) Ingestion	Red	Whole algal cells were well defined in the stomach.
(III) Digestion	Pink, Orange or Yellow	Whole and lysed algal cells mixed in the stomach or no whole cells present (lysed algae only).
(IV) Empty	No fluorescence	Empty stomach; no micro-algae cells present.

3.2.4. Statistical analyses

Differences in stomach fullness (%) of larval fed the different diets after 1 h were determined using the non-parametric Kruskal-Wallis test. The same test was also used to identify differences in the stages of ingestion and digestion of D2, D6 and D10 auriculariae fed the different diets after 1 h and 2 h. Analyses were not conducted beyond 2 h because most diets were digested completely within two hours of feeding by all ages of larvae. Categories used to describe the degree of larval stomach fullness (i.e. 0%, 1-25%, 26-50%, 51-75% and 76-100%) were assigned different scores where '1' represented and empty stomach (0%) and 2, 3, 4, 5 were assigned to the increasing levels of stomach fullness (e.g. 76-100% = 5). Similarly, the stages of ingestion and digestion described in Table 3.2 were assigned different scores with '1' representing stage I (partial ingestion) and 2, 3, 4 representing stages II, II, IV of ingestion/digestion, respectively (Table 3.2). Mann-Whitney U tests were then performed to determine which pairs of diets differ significantly from one another. Statistical analyses were carried out using IBM SPSS Ver. 22 statistical software.

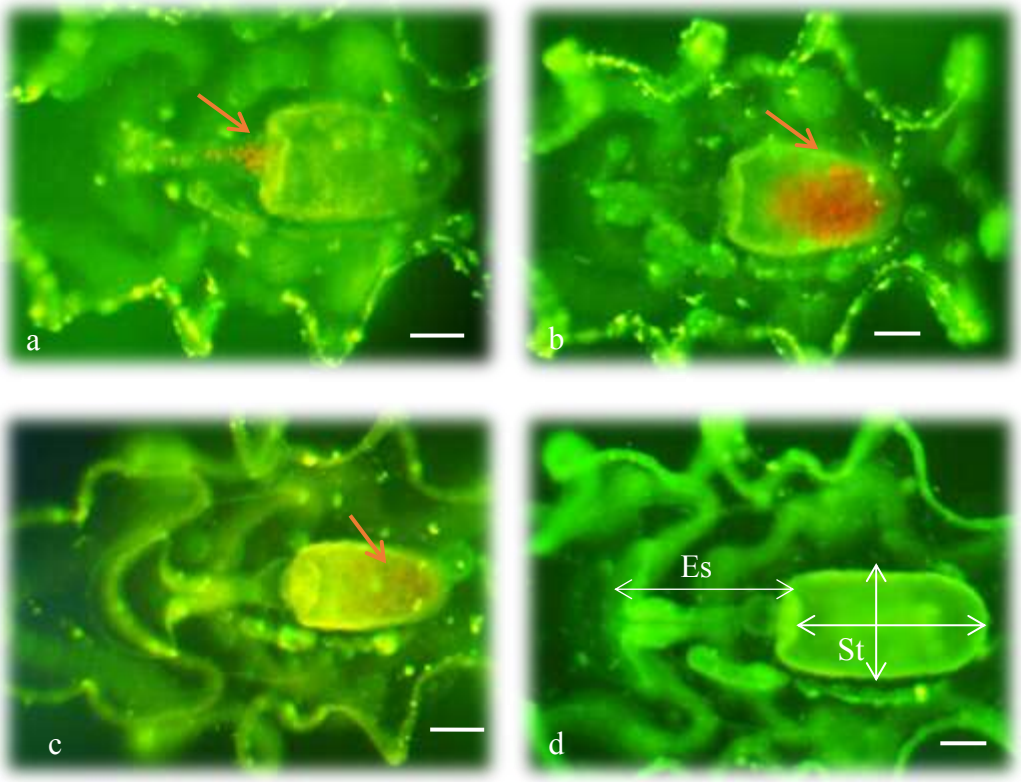

Figure 3.1. Photosynthesising pigments of algal cells in the stomach of sandfish auriculariae fluorescing under blue-light illumination representing different stages of ingestion and digestion: (a) red fluorescence of whole micro-algae cells in the esophagus but not in the stomach (arrows) (Stage I); (b) red fluorescence of ingested but undigested micro-algal cells in the stomach (Stage II); (c) pink and orange fluorescence of digested micro-algae cells in the stomach (Stage III); and (d) empty stomach without fluorescence (Stage IV). St: stomach. Es: esophagus. Scale bar 100 µm.

3.3. Results

Seven of the eight micro-algal diets assessed in this study were ingested by early to late sandfish auriculariae (Table 3.3). The exception was the micro-algae concentrate 3H 1800® which was not ingested by larvae of any of the three ages tested (Table 3.3). There was a highly significant difference between the different larval diets in terms of larval stomach fullness after 1 hour of feeding (Kruskal-Wallis, $\chi 2$ (6) = 209, $p < 0.050$) for each of the three auricularia ages. The amount of TISO ingested by D2 larvae was significantly greater than that of Isochrysis 1800® and *Chaetoceros muelleri* but lower than that of Tetraselmis 3600® (Mann-Whitney, U = 0, $p < 0.05$). However, the amount of TISO ingested did not differ from that of Pavlova 1800®, TW 1200® or Shellfish Diet 1800® (Mann-Whitney, U = 450, $p > 0.05$). The D2 larvae with

the greatest stomach fullness (51-75%) were those fed Tetraselmis 3600® while the stomachs of those fed TISO, Pavlova 1800® and Shellfish Diet 1800® were only 26-50% full of micro-algae (Table 3.3). For D6 larvae the greatest level of micro-algae ingestion was shown by those fed the Shellfish Diet 1800® and this was significantly higher than that of larvae fed TISO, Isochrysis 1800®, Tetraselmis 3600® and TW 1200® (51-75%) followed by Pavlova 1800® (26-50%) and *C. muelleri* (0%) (Mann-Whitney, $U = 0$, $p < 0.05$). The oldest larvae tested (D10) showed the highest level of ingestion when fed with Shellfish Diet 1800®, TW 1200®, Tetraselmis 3600® and TISO (76-100%) which were ingested at a significantly higher level than all other diets (Mann-Whitney, $U = 0$, $p < 0.05$; Table 3.3). Ingestion of TW 1200®, Tetraselmis 3600® and TISO was considerably higher in D10 larvae than in D6 larvae indicating higher rates of ingestion with increasing larval age. All micro-algae concentrates, with the exception of 3H 1800®, were relatively well ingested even by early (D2) auricularіae. Small cells of *Chaetoceros muelleri,* 3H 1800® and Pavlova 1800® (from 4-7 µm) were poorly ingested by D2, D6 and D10 larvae, whereas the relatively large cells of Tetraselmis 3600® (5-12 µm), TW 1200® (7-20µm**)** and Shellfish Diet 1800® (5-12 µm) were the most ingested micro-algae among those tested (Table 3.3).

Table 3.3. Relative of stomach fullness (%) in three ages of *Holothuria scabra* larvae (D2, D6 and D10) after feeding for 1 hour on one of eight micro-algae diets. Significant differences between diets in columns are indicated by different superscripts ($p < 0.050$).

Diets	D2	D6	D10	Observations
C. muelleri	0^{a}	0^{a}	0^{a}	All cells in esophagus.
TISO	$26\text{-}50^{c}$	$51\text{-}75^{c}$	$76\text{-}100^{d}$	All cells in stomach.
Isochrysis 1800®	$1\text{-}25^{b}$	$51\text{-}75^{c}$	$51\text{-}75^{c}$	All cells in stomach.
Pavlova 1800®	$26\text{-}50^{c}$	$26\text{-}50^{b}$	$26\text{-}50^{b}$	Most cells in stomach.
Tetraselmis 3600®	$51\text{-}75^{d}$	$51\text{-}75^{c}$	$76\text{-}100^{d}$	All cells in stomach.
TW 1200®	$26\text{-}50^{c}$	$51\text{-}75^{c}$	$76\text{-}100^{d}$	All cells in stomach.
3H 1800®	0	0	0	No cells observed.
Shellfish Diet 1800®	$26\text{-}50^{c}$	$76\text{-}100^{d}$	$76\text{-}100^{d}$	All cells in stomach.

There were significant differences in the ingestion rates of different micro-algae after 1 hour by D2 larvae (χ^2 (6) = 164.7, $p < 0.05$) and by D6 and D10 larvae (χ^2 (6) = 209, $p < 0.05$). Ingestion of the micro-algae pastes, with the exception of Pavlova 1800®, was significantly higher than that of *C. muelleri* (Mann-Whitney, $U = 225$, $p < 0.05$). For D10 larvae, there were also highly significant differences in ingestion between diets, with *C. muelleri* being ingested

at a significantly lower rate (Mann-Whitney, U = 0, $p < 0.05$) than all other diets which did not differ significantly from one another (Mann-Whitney, U = 450, $p > 0.050$).

There were also significant differences between diets in the levels of ingested micro-algae that was digested by the three ages of auriculariae (Kruskal-Wallis, χ^2 (6) = 209, $p < 0.05$) after 2 h of feeding. Digestion of TISO by D2 larvae was not significantly different to that of Isochrysis 1800® (Mann-Whitney, U = 450, $p > 0.05$) but both diets were digested at a significantly higher rate than the remaining micro-algae pastes (Mann-Whitney, U = 0, $p < 0.05$) that did not differ significantly from each other and were digested at a higher rate than *C. muelleri*. In contrast, digestion of *C. muelleri* by D10 larvae did not differ significantly to that of Pavlova 1800® and TW 1200®, but these were digested at a significantly higher rate than TISO, Isochrysis 1800®, Tetraselmis 3600® and Shellfish Diet 1800® (Mann-Whitney, U = 0, $p < 0.05$).

Table 3.4. Stages of ingestion and digestion[1] by two-day old (D2) sandfish (*Holothuria scabra*) larvae over a 24-hour period when fed for the first hour on one of eight micro-algae diets.

Diets	**Time (h)**					
	1	2	4	8	12	24
C. muelleri	I	I	I	I	I,II	I,II
TISO	II	III	III	III	IV	-
Isochrysis 1800®	II	III	III	III	IV	-
Pavlova 1800®	I, II	II	III	III	III	IV
Tetraselmis 3600®	II	II	II	II	III	III
TW 1200®	II	II	II	II	III	IV
3H 1800®	NI	-	-	-	-	-
Shellfish Diet 1800®	II	II	III	III	III	IV

[1]as defined in Table 2; NI = not ingested.

The relative digestive capacity of D2 sandfish larvae was low for all micro-algae tested (Table 3.4). For example, *C. muelleri* cells were first observed in the stomach of D2 larvae 12 hour after feeding (Table 3.4). In contrast, *C. muelleri* was digested completely within two hours of ingestion by mid (D6) and late (D10) stage auriculariae (Tables 3.5 and 3.6). Furthermore, it took at least 12 h for D2 larvae to completely digest TISO and Isochrysis 1800®, and 24 h for complete digestion of the other ingested micro-algae, except Tetraselmis 3600®. However, digestibility of ingested micro-algae generally increased as the larvae grew, and micro-algae were entirely digested within 4 h by D6 and D10 larvae. The exception to this was Tetraselmis

3600® (Tables 3.5 and 3.6) which was only partially digested after 1 h of feeding by D10 larvae, and digestion was still incomplete after 24 h.

Table 3.5. Stages of ingestion and digestion[1] by six-day old (D6) sandfish (*Holothuria scabra*) larvae over a 24-hour period when fed for the first hour on one of eight micro-algae diets.

Diets	**Time (h)**					
	1	2	4	8	12	24
C. muelleri	I	IV	-	-	-	-
TISO	III	III	IV	-	-	-
Isochrysis 1800®	III	III	IV	-	-	-
Pavlova 1800®	II	IV	-	-	-	-
Tetraselmis 3600®	II	II	III	III	III	III
TW 1200®	III	IV	-	-	-	-
3H 1800®	NI	-	-	-	-	-
Shellfish Diet 1800®	II	III	IV	-	-	-

[1]as defined in Table 2; NI = not ingested.

3.4. Discussion

This study is the first to use epifluorescence microscopy as a direct method for assessing ingestion and digestion of micro-algae by larval echinoderms. Prior studies with echinoderm larvae were conducted using compound microscopy to quantify ingested cells or food particles (e.g. George, 2006; Hart, 1991; Pedrotti, 1995; Schiopu et al., 2006; Strathmann, 1971); however, this method may result in errors in estimating ingested and/or digested micro-algae cells which may be difficult to discern within the larval body and difficult to differentiate from larvae body parts; this is well illustrated in the Figures of our study. Accordingly, the use of epifluorescence microscopy was considered to be a more accurate alternative method for assessing ingestion and digestion of micro-algae by sea cucumber larvae because the photosynthesising pigments of micro-algae cells fluoresce with blue-light illumination and this can be used to differentiate various stages of their ingestion and digestion.

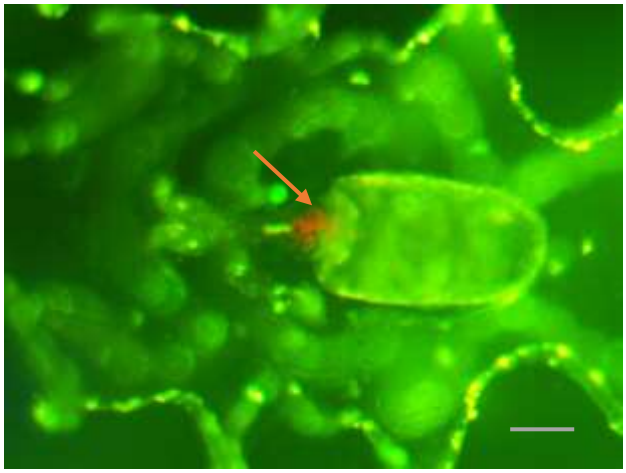

Figure 3.2. Intact *Chaetoceros muelleri* cells in red (arrow) accumulated in the esophagus of a late (D10) *Holothuria scabra* auricularia. Scale bar 100 µm.

Chaetoceros muelleri has been used as a monospecific diet for sandfish larvae (Battaglene, 1999; Duy, 2012; Knauer, 2011). It is surprising therefore that ingestion of *C. muelleri* in this study was relatively low by all ages of sandfish larvae when compared to other micro-algae. *C. muelleri* was easily captured within the oral cavity and esophagus (Figure 3.2) of D2 sandfish larvae within 1 hour of feeding and remained in the esophagus until 12 h after feeding when cells were first observed in the stomach. This suggests that early sandfish larvae require at least 8 h (prior observation interval) for the transport of ingested *C. muelleri* from the mouth and esophagus into the stomach for digestion. *Chaetoceros* is a unicellular microalga with a siliceous cell wall that often forms long filaments (Patino-Suarez et al., 2004). This characteristic could affect its rate of transport through the esophagus and into the stomach of sandfish larvae. *C. muelleri* was, however, rapidly digested by D6 and D10 sandfish larvae once transported into the stomach where digestion was complete within two hours. Our results indicate that *C. muelleri* is unsuitable as a food for D2 sandfish larvae despite the fact that this species is considered to be a rich source of essential fatty acids which are crucial for the normal development and survival of marine invertebrate larvae (Carboni et al., 2012; Reitan, 2011; Scholtz et al., 2013). More research is required to optimise the use of *C. muelleri* for feeding later auricularia stages as it has been reported to be a more effective diet for sandfish auricularia larvae than TISO and *P. salina* (Knauer, 2011). Micro-algae ration relative to larval stocking density, for example, is likely to influence rates of ingestion and digestion of *C. muelleri*.

Table 3.6. Stages of ingestion and digestion[1] by ten-day old (D10) sandfish (*Holothuria scabra*) larvae over a 24-hour period when fed for the first hour on one of eight micro-algae diets.

Diets	**Time (h)**					
	1	2	4	8	12	24
C. muelleri	I	IV	-	-	-	-
TISO	III	III	IV	-	-	-
Isochrysis 1800®	III	III	IV	-	-	-
Pavlova 1800®	III	IV	-	-	-	-
Tetraselmis 3600®	III	III	III	III	III	III
TW 1200®	III	IV	-	-	-	-
3H 1800®	NI	-	-	-	-	-
Shellfish Diet 1800®	III	III	IV	-	-	-

[1]as defined in Table 2; NI = not ingested

TISO was well ingested by sandfish larvae in both live and concentrate form, and ingestion increased in older larvae fed TISO and Isochrysis 1800®. However, TISO was better ingested than Isochrysis 1800®. This observation may relate to differences in the motility of these micro-algae. For example, although intact, individual cells of micro-algae concentrates are non-motile and negatively buoyant. This may reduce their availability to larvae in the water column compared to live micro-algae. Live TISO cells and those from the micro-algae concentrate were digested at similar rates and both completed digestion within 4 h after ingestion. Larvae completed digestion of both live and concentrated TISO after 4 h from feeding while the rest of tested micro-algae were totally digested after 24 h. Our results confirm that, of the diets tested, TISO is the most suitable for sandfish larvae in terms of ingestion and digestibility. However, increasing the ration of TISO from a density of 4 x 10^4 cell mL^{-1} to 8 x 10^4 cell mL^{-1} was reported to negatively affect growth and development of sandfish larvae, possibly because of resulting changes in pH and levels of un-ionised ammonia (Morgan, 2001). Two other studies have reported that TISO supported poor larval competence at metamorphosis when fed to sandfish auriculariae (Battaglene, 1999; Knauer, 2011) and this may relate to the nutrient composition of this species. Although TISO contains relative high levels of docosahexaenoic acid (DHA, 22:*6n-3*), it has relatively low levels of eicosapentaenoic acid (EPA, 20:5*n*-3). Both are considered to be essential dietary components for marine invertebrates (Knauer and Southgate, 1999) and the low EPA content of TISO has been suggested as a reason for its poor food value in some nutritional studies with mollusc larvae (e.g. Helm and Laing, 1987; Southgate et al., 1998).

Larval ingestion only occurs when food particles are of an appropriate size to fit into the mouth and esophagus of the target larvae (Lora-Vilchis and Maeda-Macedo, 1997). It has been suggested that echinoderm larvae can ingest cells of <50 μm more effectively during the early larvae stages (Strathmann, 1971) and, in advanced larval stages, larvae can ingest particles up to 70-80 μm, which is about the diameter of their esophagus (Strathmann, 1975). However, cell size alone does not explain the lack of ingestion of 3H 1800® (4-6 μm) reported in this study which was not ingested by any of the three ages of auricularia used in this study. Pedrotti (1995) studied food selection (size and flavour) of plutei of two echinoderms, *Paracentrotus lividus* and *Arbacia lixula*, and found that young larvae ingested larger particles (18.5 μm and 26.0 μm) at a greater rate than smaller particles (2.9 μm and 9.7 μm). The retention of these small-sized particles was also very low during the experiments (Pedrotti, 1995). Similarly, sandfish auriculariae in this study ingested greater quantities (75-100%) of Tetraselmis 3600® (cell size 10-12 μm), TW 1200® (7-20 μm) and Shellfish Diet 1800® (5-12 μm**),** which have relatively large cell sizes, and the digestive capabilities of D6 and D10 larvae were also higher than that of D2 larvae.

Shellfish Diet 1800® is comprised of four micro-algae species (*Isochrysis* sp., *Pavlova* sp. *Thalassiosira pseudonana* and *Tetraselmis* sp.) and was well ingested and digested by D6 and D10 auricularie. However, this study did not quantify the relative ingestion and digestion of the individual components of Shellfish Diet 1800®. It is possible that *Isochrysis* sp. and *Pavlova* sp. were more selectively ingested than *Tetraselmis* sp. and *Thalassiosira pseudonana* from the Shellfish Diet 1800® mixture as our results showed that *Tetraselmis* sp. cells were still incompletely digested after 24 hours and that *Thalassiosira pseudonana* cells were not observed in the larval stomach. This assumption is supported by the fact that Isochrysis 1800® and Pavlova 1800® (components of Shellfish Diet 1800®), as well as the ingested cells from Shellfish Diet 1800®, required a maximum of 4 hours to be entirely digested by D10 auriculariae.

The use of epifluorescence microscopy to investigate ingestion and digestion of various micro-algae by sandfish larvae has revealed that a variety of both live and concentrated micro-algae can be ingested by sandfish auriculariae and that the digestibility of these micro-algae improved with increasing larval age. Our results have confirmed the potential of micro-algae concentrates as alternatives to live micro-algae in hatchery culture of sandfish as first proposed by Hair et al.

(2011). However, further research should be conducted to assess the relative nutritional values of digestible micro-algae as a basis for optimising a diet for hatchery culture of sandfish, and to provide further information on the nutritional requirements of the sandfish larvae. The promising use of micro-algae concentrates in this study may also indicate that a high quality diet for sandfish larvae may not necessarily have to include live micro-algae. Such a development would facilitate simpler larval culture protocols for sandfish and expansion of hatchery production of sandfish and other tropical sea cucumbers.

CHAPTER 4

USE OF MICRO-ALGAE CONCENTRATES FOR HATCHERY CULTURE OF SANDFISH, *HOLOTHURIA SCABRA*[3]

4.1. Introduction

Sea cucumbers (Echinodermata: Holothuroidea) have been exploited for human consumption and used for their perceived medicinal properties for centuries (Hamel et al., 2001). Over 50 species are now utilised commercially within the global dried sea cucumber, 'trepang' or 'bêche-de-mer' market (Purcell et al., 2012). Sandfish, *Holothuria scabra,* is one of the most valuable sea cucumber species, selling in Hong Kong for US$115-640 kg^{-1} (dried) (Purcell et al., 2012). The increasing value of dried sea cucumber products in Asian markets, especially in China, as well as over-exploitation of many sea cucumber fisheries (Purcell et al., 2012), has prompted interest in sea cucumber aquaculture programs in numerous Indo-Pacific countries (Jimmy et al., 2012; Mills et al., 2012). Sandfish are thought to be a good candidate for aquaculture in the tropics (Battaglene et al., 1999; Jimmy et al., 2012) because they are amenable to hatchery production (Duy, 2012; Purcell et al., 2012). Additionally, juveniles and adults feed on organic matter and detritus in sediments and require no additional feed input during culture (Mills et al., 2012; Pitt and Duy, 2004); however, commercial grow-out systems are yet to be adequately developed for this species (Raison, 2008).

There are several stages within the hatchery production of sandfish. The first auricularia stage is planktonic and larvae are generally fed live micro-algae. They then transform into the non-feeding doliolariae stage and develop into pentactulae, before finally metamorphosing into newly settled juveniles that are generally grown on plates that are pre-conditioned with benthic diatoms (Agudo, 2006; Battaglene, 1999; James et al., 1994) or painted with dried *Spirulina* (Pitt and Duy, 2004; Duy, 2010). A source of live cultured micro-algae is generally considered to be a key requirement for successful hatchery culture (Agudo, 2006; Battaglene et al., 1999;

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Duy, 2010; James et al., 1994). With respect to the culture of sandfish larvae, suitable live micro-algae include *Chaetoceros* spp. and *Isochrysis* aff. *galbana* (T-ISO) fed both singly or in combination (Agudo, 2006; Battaglene, 1999; Duy, 2012; Gamboa, et al., 2012; James et al., 1994; Knauer, 2011; Morgan, 2001). However, developing and small-island nations often lack the technical resources and skilled personnel required for successful hatchery operation, and production of appropriate quantities of high quality live micro-algae, as a larval food source, is a common bottleneck (Southgate et al., 2016).

Commercially available micro-algae concentrates were recently reported to be well ingested and digested by different ages of sandfish auriculariae (Chapter 3), and are thought to have potential as replacements for live micro-algae during hatchery culture of this species (Hair et al., 2011; Chapter 3). Hair et al. (2011) reported successful larval culture of sandfish larvae when the majority of live micro-algae was replaced with concentrated micro-algae, while Zacarias-Soto et al. (2013) more recently reported successful larval culture of *Isostichopus badionotus* using micro-algae concentrates alone. Broad use of commercially available micro-algae concentrates as food for sea cucumber larvae would support development of simplified larval rearing protocols that are more appropriate for developing nations. Moreover, nutrient compositions of micro-algae concentrates are generally consistent, while those of live micro-algae can vary according to culture conditions such as growth phase, culture medium composition and temperature (Brown et al., 1997; Martínez-Fernández et al., 2006; Pacheco-Vega and Sanchez-Saavedra, 2009; Pernet et al., 2003). As such, the use of 'off-the-shelf' foods may support the development of simpler and more reliable culture protocols for sea cucumber hatcheries.

In addition to appropriate characteristics for ingestion and subsequent digestion, suitable foods for sea cucumber larvae must have appropriate nutritional compositions (Chapter 3). Determining relationships between dietary nutrient compositions and larval growth, development and survival is essential for optimising larval feeding protocols and deducing the nutritional requirements of larvae which, in the case of sandfish and holothurians more generally, are poorly known. Therefore, a greater understanding of the relationships between the nutritional composition of larval diets and larval development and survival is critically important for future studies in this field. The aims of this study were to determine the nutritional value of three commercially available micro-algae concentrates as a larval food source during

hatchery culture of sandfish, and to identify key dietary components supporting larval growth and survival.

4.2. Materials and Methods

4.2.1. Broodstock and larvae

The broodstock used in this study were collected at Limanak Island near Kavieng, New Ireland Province, in Papua New Guinea (2°34′S 150°48′E). They were transported to raceways at the National Fisheries Authority (NFA), Nago Island Marine Research Facility (NIMRF) in Kavieng a week prior to spawning. Broodstock were induced to spawn using thermal shock followed by the addition of dried *Spirulina* sp. to the spawning tank (Agudo, 2006; Duy, 2010). Eggs were hatched in a 2,000 L tank filled with 1 µm filtered seawater (FSW) at a density of 1 egg mL^{-1}. After 2 days, larvae were counted and distributed to the 500-L tanks used for the experiment.

4.2.2 Diets

Commercially available micro-algae concentrates (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) purchased from an Australian distributor were used in this study. Three of the most digestible products identified in previous research (Chapter 3) were used: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova* sp. (Haptophyceae) (Pavlova 1800®); and (3) mono–cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®). The three products were either fed individually or as a mixture composed of an equal ratio (dry weight) of *Isochrysis* sp., *Pavlova* sp. and *Thalassiosira weissflogii* making up a fourth ternary diet treatment. Micro-algae concentrates were stored in their original bottles in a refrigerator at 4ºC for the duration of the study.

4.2.3. Biochemical analysis of micro-algae concentrates

Proximate analysis of the three Instant Algae® products (Isochrysis 1800®, Pavlova 1800® and TW 1200®) was conducted using standard procedures (AOAC, 1990) after freeze-drying. Percentage moisture of the products was determined after freeze-drying to a constant weight;

protein (Kjeldahl nitrogen; N x 6.25) was determined using an automated Kjeltech (model 2300, Tecator, Sweden); total lipid was determined gravimetrically following dichloromethane/methanol extraction (2:1 v/v) (Folch et al., 1957); ash was determined by incineration in a muffle furnace (model WIT, C & L Tetlow, Australia) at 550 °C for 18 h. Nitrogen free extract (NFE) was calculated by difference following the summation of moisture, lipid, protein and ash. Energetic contents of micro-algae concentrates were estimated from proximate analyses using caloric equivalents of 4.0 cal. mg^{-1}, 4.5 cal. mg^{-1} and 9.4 cal. mg^{-1} for carbohydrate (NFE), protein and lipid, respectively (Marshall et al., 2010).

Following lipid extraction, fatty acids were esterified into methyl esters using the acid-catalysed methylation method (Christie, 2003) following the protocol of Conlan et al. (2014). The purified hexane supernatants of the micro-algae samples were placed in a gas chromatography (GC) vial for GC injection. Fatty acid methyl esters were isolated and identified using an Agilent Technologies 7890 GC System (Agilent Technologies, USA) equipped with a BPX70 capillary column (120 m × 0.25 mm internal diameter, 0.25 mm film thickness, SGE Analytical Science, Australia), a flame ionization detector (FID), an Agilent Technologies 7693 auto sampler, and a split ratio of 50:1. The injection volumes, temperature sequences, and flow rates followed the protocol of Conlan et al. (2014). The carrier gas was helium. Individual fatty acids were identified relative to known external standards (a series of mixed and individual standards from Sigma-Aldrich, Inc., St Louis, USA and Nu-Chek Prep Inc., USA) using the software GC ChemStation (Rev B.04.03, Agilent Technologies). The resulting peaks were corrected by theoretical relative FID response factors (Ackman, 2002) and quantified relative to the internal standard C23:0 (0.75 mg mL^{-1}, Sigma-Aldrich, Inc., USA).

4.2.4. Experiment design

Two-day auriculariae with initial mean (± SD, n = 90) length of 599.98 ± 22.67 µm and stomach diameter of 79.06 ± 4.71 µm were stocked into triplicate 500-L tanks (5° sloping bottom) filled with 300 L of 1-µm filtered sea water (FSW) at an initial density of 0.3 mL^{-1} and randomly allocated one of the four dietary treatments. A 50% tank water exchange was conducted daily. Water temperature was maintained at 27.5 ± 1.0°C and the culture water was moderately aerated.

Table 4.1. Daily rations (cells mL^{-1}) of the three Instant Algae® products fed to *Holothuria scabra* auriculariae in this study. Daily rations for each product provided larvae with the same dry weight of micro-algae.

	Product		
Day	**'Isochrysis 1800®'**	**'Pavlova 1800®'**	**'TW 1200®'**
2	10,000	10,391	3,926
3	11,000	11,431	4,319
4	12,000	12,470	4,712
5	13,000	13,509	5,104
6	14,000	14,548	5,497
7	15,000	15,587	5,890
8	16,000	16,626	6,282
9	17,000	17,666	6,675
10	18,000	18,705	7,068
11	19,000	19,744	7,460
12	20,000	20,783	7,853
13	21,000	21,822	8,246
14	22,000	22,861	8,638
15	23,000	23,900	9,031
16	24,000	24,940	9,424
17	25,000	25,979	9,816
18	26,000	27,018	10,209
19	27,000	28,057	10,601
20	28,000	29,096	10,994
21	29,000	30,135	11,387
22	30,000	31,174	11,779

The dry weights of individual micro-algae concentrates used in this study were determined from triplicate 1-mL aliquots removed from their original bottles after gentle agitation. They were filtered through pre-weighed glass-fibre micro filters (Whatman GF/F, 25 mm), washed with 0.5 M ammonium formate solution (20 mL) and dried at 100°C for 16 h to volatilise the ammonium formate (Epifanio, 1979). Filters were then re-weighed to determine micro-algae dry weights (Brown et al., 1998) per mL of product. The number of cells per mL of Isochrysis

1800® was also estimated using triplicate hemocytometer counts and the mean dry weight per cell was determined. For larvae fed Isochrysis 1800®, the initial ration was 10,000 cells mL^{-1} (Morgan, 2010) and this was increased by 1,000 cells mL^{-1} of Isochrysis 1800® per day as larval development proceeded. Thus, Isochrysis 1800® was fed at a rate of 10,000 cells mL^{-1} on day 1, 11,000 cells mL^{-1} on day 2, 12,000 cells mL^{-1} on day 3, 13,000 cells mL^{-1} on day 4, and so on (Table 4.1). The dry weight of each of these daily rations was calculated. All other micro-algae diets were administered at volumes that provided the same dry weight of micro-algae as the Isochrysis 1800® rations, so that larvae in all treatments received the same daily ration based on dry weight. This approach was necessary because the micro-algae used in this study vary considerably in cell size (Chapter 3), and rations administered on the basis of an equal number of micro-algae cells would differ considerably in the mass or volume of micro-algae available to the larvae across treatments.

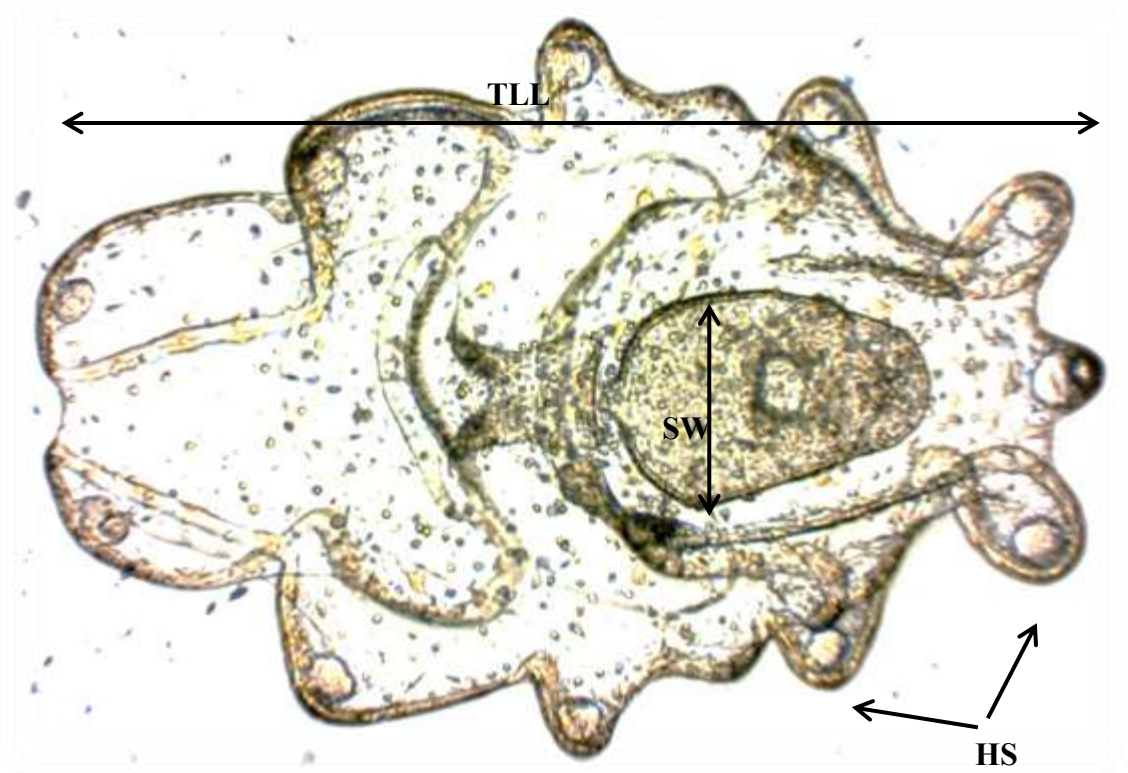

Figure 4.1. Late auricullariae with hyaline spheres. **TLL**: total larval length. **$\overline{\text{SW}}$**: stomach width. **HS**: hyaline spheres. Scale bar 100 µm.

Prior to use, micro-algae concentrates were gently hand-shaken and the required amount of each concentrate was added to a graduated container filled with 1 µm filtered seawater, prior to feeding. The daily ration was divided into three equal proportions fed three times a day with

at least 4 h between feeds to ensure the ingestion and digestion by larvae (Chapter 3) and minimise the possibility of overfeeding (Morgan, 2001). Settlement plates, painted with a slurry of dry *Spirulina* sp., were added to the culture tanks once >70% of competent doliolariae were visible during microscopic inspection (Duy, 2010).

Thirty larvae were randomly sampled from each rearing tank every two days to determine larval length and stomach width until competent doliolariae appeared. Competent doliolariae were defined as being barrel-shaped with prominently developed hyaline spheres (Ramofafia et al., 2003; Figure 4.1). The percentage of larvae with hyaline spheres was determined at the late auricularia stage. Five days after settlement, the lengths of thirty live post-settled larvae from each tank were measured using a dissecting microscope and micrometer. Larval survival was estimated at day 5 (early-mid auriculariae), day 13 (late auriculariae) and day 25 (post-settled larvae) after fertilisation. Larval development and survival were compared to that of unfed larvae.

4.2.5. Data analyses

Total larval length, stomach width, percentage of auriculariae with hyaline spheres, percentage of competent doliolariae, post-settled larval length and survival of auriculariae were compared between treatments using one-way ANOVA followed by Tukey's multiple range test to evaluate all pair-wise treatment comparisons ($P < 0.05$). All percentage data were arcsin-transformed prior to analyses. Pearson's correlation test was used to identify correlations between nutrient content of the micro-algae concentrates (total lipid, NFE, protein, total saturated, monounsaturated and polyunsaturated fatty acids, and specific fatty acids) and larval development, competency and survival. Statistical analyses were conducted using the software IBM SPSS VERSION 22.0.

4.3. Results

4.3.1. Biochemical compositions of micro-algae concentrates

Water made up between 867.0 and 880.0 mg g^{-1} of the wet weight of the Instant Algae® products used in this study. The proximate compositions of the micro-algae concentrates are shown in Table 4.2. Lipid content was highest in Isochrysis 1800® (95.36 ± 2.09 mg g^{-1} dry weight) and lowest in TW 1200® (44.37 ± 0.57 mg g^{-1} dry weight) while Pavlova 1800® (75.71 ± 0.00 mg g^{-1} dry weight) and the ternary diet (71.82 ± 0.88 mg g^{-1} dry weight) had intermediate

lipid contents. This pattern was similar for protein contents that ranged from 255.22 ± 0.77 mg g^{-1} dry weight to 208.17 ± 0.53 mg g^{-1} dry weight. However, the NFE content of TW 1200® (332.69 ± 1.77 mg g^{-1} dry weight) was significantly higher than that of the ternary diet (314.98 ± 1.47 mg g^{-1} dry weight) and Isochrysis 1800® and Pavlova 1800® (317.02 ± 0.36 and 295.21 ± 2.26 mg g^{-1} dry weight, respectively). Ash content was highest in TW 1200® (414.80 ± 0.64 mg g^{-1}) and lowest in Isochrysis 1800® (332.26 ± 1.01 mg g^{-1}). Isochrysis 1800® had the highest calculated energy content (3.66 ± 0.01 cal. g^{-1} dry weight) while TW 1200® had the lowest (2.97 ± 0.00 cal. g^{-1} dry weight) (Table 4.2).

Table 4.2. Proximate compositions (mg g^{-1} dry weight) and energy contents (cal. g^{-1} dry weight) of micro-algae concentrates used in this study.

Micro-algae	Lipid	Protein	NFE	Ash	Energy
Isochrysis 1800®	95.36 ± 2.09[a]	255.22 ± 0.77[a]	317.02 ± 0.36[b]	332.26 ± 1.01[c]	3.66 ± 0.01[a]
Pavlova 1800®	75.71 ± 0.00[b]	247.76 ± 0.75[b]	295.21 ± 2.26[c]	381.32 ± 3.02[b]	3.34 ± 0.01[b]
TW 1200®	44.37 ± 0.57[c]	208.17 ± 0.53[d]	332.69 ± 1.77[a]	414.80 ± 0.64[a]	2.97 ± 0.00[c]
Ternary diet	71.82 ± 0.88[b]	237.05 ± 0.33[c]	314.98 ± 1.47[b]	376.16 ± 0.89[b]	3.33 ± 0.01[b]

Fatty acid (FA) profiles (µg g^{-1} dry weight) of the micro-algae concentrates used in this experiment are shown in Table 4.3. Saturated fatty acids (SFA) were highest in Pavlova 1800® (3738.63 µg g^{-1} dry weight) and lowest in Isochrysis 1800® (2836.14 µg g^{-1} dry weight) and in all micro-algae concentrates, myristic acid (14:0) and palmitic acid (16:0) were the predominant SFA. The level of myristic acid (14:0) was lowest in TW 1200® (420.75 µg g^{-1} dry weight), whereas the level of palmitic acid (16:0) was highest in TW 1200® (2137.39 µg g^{-1} dry weight) compared to that of the other diets. In contrast, Isochrysis 1800® had a higher monounsaturated fatty acid (MUFA) content (3883.17 µg g^{-1} dry weight) than the ternary diet, Pavlova 1800® and Isochrysis 1800® (3002.45, 2586.86 and 2537.34 µg g^{-1} dry weight, respectively). Palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) were predominant in all micro-algae concentrates. Oleic acid was present at a much higher level in Isochrysis 1800® (2329.65 µg g^{-1} dry weight) than in Pavlova 1800® and TW 1200® (233.90 and 177.03 µg g^{-1} dry weight, respectively). Whereas, palmitoleic acid was highest in Pavlova 1800® (1979.44 µg g^{-1} dry weight) compared to that of TW 1200®, the ternary diet and Isochrysis 1800® (1700.17, 1482.59 and 768.17 µg g^{-1} dry weight, respectively).

The total polyunsaturated fatty acid (PUFA) content of Isochrysis 1800® (9330.88 µg g^{-1} dry weight) was slightly higher than that of Pavlova 1800® (9146.81 µg g^{-1} dry weight), while the PUFA concentration of TW 1200® was found to be the lowest (4298.24 µg g^{-1} dry weight) of the three products tested (Table 4.3). Isochrysis 1800® and the ternary diet contained the highest content of linoleic acid (18:2n-6) (1520.63 and 594.46 µg g^{-1} dry weight, respectively), whereas Pavlova 1800® and TW 1200® had relatively low concentrations of this fatty acid of 178.90 and 83.85 µg g^{-1} dry weight, respectively. The level of stearidonic acid (18:4n-3) was also highest in Isochrysis 1800® (2830.12 µg g^{-1} dry weight) and more than double that in the ternary diet (1391.68 µg g^{-1} dry weight) and Pavlova 1800® (1277.91 µg g^{-1} dry weight), and forty times higher than that in TW 1200® (67.03 µg g^{-1} dry weight). Arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were found in all micro-algae concentrates although ARA was found at relatively low levels (49.20 – 117.59 µg g^{-1} dry weight). The EPA level of Pavlova 1800® was highest (3478.89 µg g^{-1} dry weight) and much higher than that of TW 1200® (2085.01 µg g^{-1} dry weight) and the ternary diet (1992.78 µg g^{-1} dry weight), while that of Isochrysis 1800® was relatively low (204.44 µg g^{-1} dry weight). In contrast, Isochrysis 1800® was richest in DHA (2511.36 µg g^{-1} dry weight) compared to the ternary diet (1259.36 µg g^{-1} dry weight), Pavlova 1800® (965.63 µg g^{-1} dry weight) and TW 1200® (301.09 µg g^{-1} dry weight).

4.3.2. Larval development

Total larval lengths of auriculariae from day 3 to day 13 were significantly different between treatments (Table 4.4). Larvae fed TW 1200® had significantly greater ($P < 0.05$) mean length on day 13 (1080.87 ± 4.94 µm) than those fed the other diets, while larvae fed Pavlova 1800® recorded the lowest mean length (1035.00 ± 4.39 µm). Although the ternary diet generally supported a higher larval growth rate than Isochrysis 1800® throughout the auricularia stage, there was no significant difference in larval length on day 13 between larvae fed these two diets ($P > 0.05$). There were also significant differences in larval stomach width between treatments from day 3 to day 11 (Table 4.5).

Table 4.3. Fatty acid profiles (μg g^{-1} dry weight) of the micro-algae concentrates used in this study.

Fatty acid	Isochrysis 1800®	Pavlova 1800®	TW 1200®	Ternary diet
14:0	1084.74	2094.66	420.75	1200.05
15:0	73.51	56.87	137.40	89.26
16:0	1269.44	1289.87	2137.39	1565.56
17:0	15.69	21.57	32.24	23.17
18:0	136.63	124.01	130.80	130.48
20:0	47.33	21.50	0.00	22.94
21:0	29.93	70.23	76.48	58.88
22:0	133.96	59.93	65.12	86.34
24:0	44.92	0.00	44.27	29.73
14:1n-5	19.27	22.47	213.91	85.22
15:1n-5	142.15	136.13	125.22	134.50
16:1n-7	768.17	1979.44	1700.17	1482.59
17:1n-7	73.52	0.00	18.18	30.57
18:1n-9 t	0.00	0.00	0.00	0.00
18:1n-7 t	0.00	0.00	0.00	0.00
18:1n-9	2329.65	233.90	177.03	913.53
18:1n-7	150.14	98.75	96.43	115.11
20:1 (isomers)	26.47	21.97	31.18	26.54
22:1 (isomers)	358.00	59.74	175.22	197.66
24:1n-9	15.80	34.46	0.00	16.75
16:2n-4	45.44	70.19	391.56	169.06
16:3n-4	0.00	67.58	750.05	272.54
18:3n-4	17.85	43.80	0.00	20.55
18:2n-6 t	0.00	27.18	0.00	9.06
18:2n-6	1520.63	178.90	83.85	594.46
18:3n-6	301.59	21.35	36.17	119.70
20:2n-6	38.89	44.38	0.00	27.76
20:3n-6	30.25	0.00	0.00	10.08
20:4n-6 (ARA)	95.29	117.59	49.20	87.36
22:2n-6	0.00	0.00	0.00	0.00
22:4n-6	20.49	1522.63	90.61	544.57
22:5n-6	394.70	1051.58	204.20	550.16
18:3n-3	1207.23	216.64	31.50	485.12
18:4n-3	2830.12	1277.91	67.03	1391.68
20:3n-3	0.00	0.00	0.00	0.00
20:4n-3	0.00	0.00	0.00	0.00
20:5n-3 (EPA)	204.44	3478.89	2085.01	1922.78
22:3n-3	30.53	0.00	61.98	30.84
22:5n-3	53.56	62.56	64.49	60.20
22:6n-3 (DHA)	2511.36	965.63	301.09	1259.36
24:5n-3	0.00	0.00	0.00	0.00
24:6n-3	28.52	0.00	81.52	36.68
SFA	2836.14	3738.63	3044.45	3206.41
MUFA	3883.17	2586.86	2537.34	3002.45
PUFA	9330.88	9146.81	4298.24	7591.98
n-3 PUFA	6865.76	6001.62	2692.61	5186.66
n-6 PUFA	2401.83	2963.62	464.03	1943.16
n-3 LC PUFA	2828.41	4507.08	2594.09	3309.86
n-6 LC PUFA	540.73	2691.80	344.01	1192.18

The mean (± SE) larval stomach width of larvae fed TW 1200® was greatest on day 11 (160.85 ± 0.73 μm) and was significantly larger than that of larvae fed the ternary diet, Isochrysis 1800® and Pavlova 1800® (157.26 ± 0.22, 156.80 ± 0.60 and 156.06 ± 0.77 μm, respectively). However, there was no significant difference ($P > 0.05$) in larval stomach width between treatments on day 13 (Table 4.5).

Table 4.4. Changes in mean (± SE) total length of sandfish auriculariae fed different micro-algae concentrates during this study. Means within columns sharing different superscripts are significantly different ($P < 0.05$).

Micro-algae	Total larval length (µm)					
	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
Isochrysis 1800®	706.81 ± 1.30^{b}	811.08 ± 4.22^{b}	908.22 ± 4.25^{b}	985.43 ± 2.75^{b}	1023.61 ± 2.28^{b}	1045.83 ± 2.06^{b}
Pavlova 1800®	699.97 ± 3.45^{bc}	793.13 ± 2.56^{c}	896.83 ± 3.50^{c}	970.33 ± 2.48^{bc}	1013.64 ± 2.74^{bc}	1035.00 ± 4.39^{bc}
TW 1200®	722.91 ± 6.50^{ab}	829.03 ± 3.00^{a}	918.20 ± 3.36^{a}	1011.64 ± 5.93^{a}	1050.67 ± 5.07^{a}	1080.87 ± 4.94^{a}
Ternary diet	710.51± 1.51^{b}	813.36 ± 1.99^{b}	910.79 ± 3.74^{b}	990.84 ± 3.01^{ab}	1034.43 ± 3.29^{ab}	1058.08 ± 2.75^{ab}

The numbers of late auriculariae with hyaline spheres differed significantly between treatments (Table 4.6). From day 10 to day 13, the proportion of late auriculariae with hyaline spheres was significantly greater ($P < 0.05$) for larvae fed TW 1200® (46.7 ± 3.3 - 74.3 ± 1.3 %) than for those fed Isochrysis 1800®, Pavlova 1800® or the ternary diet, that did not differ from each other ($P > 0.05$) with the exception of those fed Pavlova 1800® on day 11. On day 11, the proportion of late auriculariae with hyaline spheres was lowest in the Pavlova 1800® treatment (31.0 ± 2.0 %) and this differed significantly from those in other treatments.

The proportions of competent doliolariae were measured as they were first observed in the larval rearing tanks from day 14 to day 16 before they settled (Table 4.7). On day 14, doliolariae were only present in tanks fed TW 1200® where more than 75% of larvae transformed into doliolariae on day 15 and settled on day 16. On day 15, there was a significant difference in the proportion of competent doliolariae between the TW 1200® treatment (75.6 ± 7.3 %) and larvae fed the other diets ($P < 0.05$), but there was no significant difference in the proportion of competent doliolariae between those fed Isochrysis 1800® (10.0 ± 1.9 %), Pavlova 1800® (6.7 ± 0.0 %) and the ternary diet (10.0 ± 0.0 %) (Table 4.7).

Table 4.5. Changes in mean (± SE) stomach width of sandfish auriculariae fed different micro-algae concentrates during this study. Means within columns sharing different superscripts are significantly different ($P < 0.05$).

Micro-algae	Stomach width (µm)					
	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13

Isochrysis 1800®	86.64 ± 0.18^{b}	118.23 ± 0.94^{c}	146.94 ± 0.32^{b}	155.44 ± 0.59^{bc}	156.80 ± 0.60^{b}	157.94 ± 1.07^{a}
Pavlova 1800®	84.82 ± 0.56^{b}	124.98 ± 0.55^{b}	145.61 ± 0.35^{c}	154.07 ± 0.27^{c}	156.06 ± 0.77^{b}	158.11 ± 0.40^{a}
TW 1200®	88.49 ± 0.63^{a}	129.03 ± 1.32^{a}	148.66 ± 0.10^{a}	159.54 ± 0.97^{a}	160.85 ± 0.73^{a}	160.31 ± 0.25^{a}
Ternary diet	87.79 ± 0.24^{a}	122.08 ± 0.59^{c}	148.23 ± 0.25^{a}	157.15 ± 0.70^{b}	157.26 ± 0.22^{b}	158.83 ± 0.84^{a}

Table 4.6. Mean (± SE) percentage of late auriculariae with hyaline spheres fed different micro-algae concentrates during this study. Means within columns sharing different superscripts are significantly different ($P < 0.05$).

Micro-algae	% Late auriculariae with hyaline spheres			
	Day 10	Day 11	Day 12	Day 13
Isochrysis 1800®	27.7 ± 2.9^{b}	38.7 ± 3.0^{b}	46.7 ± 5.2^{b}	59 ± 4.2^{b}
Pavlova 1800®	25.7 ± 1.3^{b}	31.0 ± 2.0^{c}	47.7 ± 2.3^{b}	55.3 ± 2.3^{b}
TW 1200®	46.7 ± 3.3^{a}	58.0 ± 1.0^{a}	71.0 ± 1.0^{a}	74.3 ± 1.3^{a}
Ternary diet	34.3 ± 1.3^{b}	41.0 ± 1.0^{b}	53.3 ± 2.0^{b}	61.0 ± 2.0^{b}

Consequently, the mean (± SE) lengths of 21 day post-settled larvae differed significantly between treatments. Those in tanks fed TW 1200® (1.19 ± 0.14 mm) were significantly larger than those fed the ternary diet (0.65 ± 0.20 mm), Isochrysis 1800® or Pavlova 1800® (0.59 ± 0.13 and 0.49 ± 0.07 mm, respectively). However, there was no significant difference between the mean lengths of post-settled larvae fed Isochrysis 1800® and the ternary diet ($P > 0.05$).

4.3.3. Survival

There were significant differences in the mean (± SE) survival of auriculariae and post-settled larvae between treatments (Table 4.8). There were significant differences in survival of early-mid and late auriculariae (day 5 and day 13) between the TW 1200® (84.7 ± 0.8 % and 64.0 ± 0.6 %) and Pavlova 1800® (88.0 ± 0.6 % and 68.7 ± 1.0 %) treatments, whereas survival of larvae fed Isochrysis 1800® (85.0 ± 0.7 % and 84.9 ± 0.5%) did not differ significantly ($P > 0.05$) from those fed the ternary diet (84.9 ± 0.5% and 64.3 ± 1.2 %). In the auricularia stage, Pavlova 1800® supported a slightly higher rate of survival compared to the other diets (Table 4.8); however, survival of post-settled larvae fed TW 1200® (13.7 ± 0.7 %) was significantly greater ($P < 0.05$) and almost double that of larvae fed the other diets, that did not differ significantly from each other ($P > 0.05$).

Table 4.7. Mean (± SE) percentage of competent doliolariae fed different micro-algae concentrates during this study. Means within columns (day 15) sharing different superscripts are significantly different ($P < 0.05$).

Micro-algae	% Competent doliolariae		
	Day 14	Day 15	Day 16
Isochrysis 1800®	N/A	10.0 ± 1.9^{b}	52.2 ± 5.1
Pavlova 1800®	N/A	6.7 ± 0.0^{b}	37.8 ± 5.1
TW 1200®	8.9 ± 1.9	75.6 ± 7.3^{a}	ST
Ternary diet	N/A	10.0 ± 0.0^{b}	41.1 ± 8.4

N/A: Not Available. ST: settled

4.3.4. Relationships between nutrient compositions of micro-algae concentrates and larval growth, development and survival

Table 4.8. Mean (± SE) survival (%) of sandfish larvae fed different micro-algae concentrates during the experiment. Survival of auriculariae was determined on day 5, day 13 and that of post-settled larvae were estimated on day 25 after fertilisation. Means within columns sharing different superscripts are significantly different ($P < 0.05$).

Micro-algae	Survival (%)		
	Day 5	Day13	Day 25
Isochrysis 1800®	85.0 ± 0.7^{b}	67.8 ± 0.8^{b}	6.7 ± 0.3^{b}
Pavlova 1800®	88.0 ± 0.6^{ab}	68.7 ± 1.0^{ab}	6.7 ± 0.6^{b}
TW 1200®	84.7 ± 0.8^{bc}	64.0 ± 0.6^{bc}	13.7 ± 0.7^{a}
Ternary diet	84.9 ± 0.5^{b}	64.3 ± 1.2^{b}	8.5 ± 1.3^{b}

Larval development, growth, competency and survival were significantly correlated with dietary levels of total protein, lipid, nitrogen-free extract (NFE), total energy, total dietary polyunsaturated fatty acid (PUFA) concentration, and certain specific fatty acids (FA) (Table 4.9). Dietary total lipid was negatively correlated with total larval length on day 13 ($R = -0.767$, $P < 0.05$), the proportion of late auriculariae with hyaline spheres (HS) on day 13 ($R = -0.917$, $P < 0.01$), the proportion of competent doliolariae on day 15 ($R = -0.842$, $P < 0.01$), post-settled larvae length on day 21 ($R = -0.844$, $P < 0.01$) and post-settled survival on day 25 ($R = -0.836$, $P < 0.05$). These relationships held the same for dietary protein, total energy, total PUFA and arachidonic acid (ARA) content.

Total larval length (day 13) was positively correlated with dietary NFE ($R = 0.827$, $P < 0.01$) and palmitic acid (16:0) content ($R = 0.931$, $P < 0.01$). There were positive correlations between survival of late auriculariae on day 13 and dietary lipid content ($R = 0.859$, $P < 0.01$), protein content ($R = 0.854$, $P < 0.01$), total energy ($R = 0.855$, $P < 0.01$), total PUFA content ($R = 0.939$, $P < 0.01$), ARA content ($R = 0.861$, $P < 0.01$), DHA content ($R = 0.761$, $P < 0.05$) and

the level of stearidonic acid ($R = 0.835$, $P < 0.01$). Whereas, survival of post-settled larvae on day 25 was significantly correlated with dietary NFE content ($R = 0.805$, $P < 0.01$), and palmitic acid ($R = 0.898$, $P < 0.01$) content.

There were positive correlations between the proportions of the late auriculariae with HS (day 13) and dietary NFE ($R = 0.730$, $P < 0.05$), EPA: DHA ratio ($R = 0.785$, $P < 0.05$), and palmitic acid ($R = 0.954$, $P < 0.01$) contents. The number of competent doliolariae was positively correlated with dietary NFE ($R = 0.798$, $P < 0.05$), EPA: DHA ratio ($R = 0.827$, $P < 0.05$) and palmitic acid ($R = 0.913$, $P < 0.01$) contents. These same relationships also held for the total length of post-settled larvae on day 21.

Table 4.9. Pearson's correlation between the levels of nutritional components of micro-algae concentrates (total protein, lipid, NFE, energy, total saturated, monounsaturated and polyunsaturated fatty acids and specific fatty acids) and development, competency and survival of sandfish larvae.

Nutritional components	Total larval length day 13	Stomach width day 13	Larval survival day 5	Larval survival day 13	Settled larvae survival day 25	% Late auriculariae with HS day 13	% Competent doliolariae day 15	Length of post-settled larvae day 21
Protein	-0.872**	-0.697	0.180	0.929**	-0.912**	-0.961**	-0.915**	-0.940**
Lipid	-0.767*	-0.593	-0.016	0.891**	-0.836**	-0.917**	-0.842**	-0.844**
NFE	0.827*	0.537	-0.564	-0.694	0.805*	0.730*	0.798*	0.875**
SFA	-0.427	-0.242	0.692	0.182	-0.351	-0.204	-0.337	-0.426
MUFA	-0.281	-0.396	-0.346	0.590	-0.454	-0.551	-0.451	-0.388
PUFA	-0.898**	-0.690	0.264	0.939**	-0.925**	-0.950**	-0.929**	-0.967**
ARA	-0.934**	-0.658	0.511	0.861**	-0.838**	-0.834*	-0.857**	-0.945**
DHA	-0.528	-0.509	-0.205	0.761*	-0.635	-0.740*	-0.642	-0.612
EPA	-0.116	0.061	0.578	-0.192	0.011	0.147	0.020	-0.063
EPA:DHA	0.588	0.636	0.211	-0.728*	0.674	0.785*	0.827*	0.772*
EPA:ARA	0.583	0.504	0.183	-0.762*	0.573	0.703	0.631	0.630
14:0	-0.846**	-0.552	0.585	0.678	-0.782*	-0.724*	-0.781*	-0.866**
16:0	0.931**	0.655	-0.246	-0.924**	0.898**	0.954**	0.913**	0.963**
18:4n-3	-0.642	-0.568	-0.111	0.835**	-0.732*	-0.826*	-0.739*	-0.724*
Energy	-0.721*	-0.595	-0.063	0.867**	-0.795*	-0.886**	-0.801*	-0.796*

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

4.4. Discussion

This study is the first comprehensive assessment of the nutritional value of commercially available micro-algae concentrates for sea cucumber larvae and of the relationships between dietary nutrient composition and growth, development and survival of sandfish larvae. Of particular significance, our results confirm the feasibility of using micro-algae concentrates as the sole nutrient source during hatchery culture of sandfish, and it is the first to report successful hatchery culture of *H. scabra* larvae without the use of live micro-algae. Our results indicate that all micro-algae concentrates used in this study proved nutritious for *H. scabra* larvae and supported normal growth and development, and relatively high survival through settlement compared to previous reports (Purcell et al., 2012). The micro-algae concentrates chosen for this study were previously reported to be readily digested by sandfish larvae (Chapter 3). This follow-on study therefore allowed evaluation of the nutritional requirements of sandfish larvae by assessing relationships between the nutrient compositions of micro-algae concentrates and larval growth, development and survival.

Development of *H. scabra* larvae through the feeding auricularia and non-feeding doliolaria stages, to the pentactula stage, generally occurs over approximately two weeks at 26-28ºC (Duy, 2010; Knauer, 2011; Ramofafia et al., 2003). Despite water temperature being maintained within the same range to that of prior studies, larval development in this study was slightly longer, with development into competent doliolariae occurring earliest on day 17 for larvae fed TW 1200®. Total length and stomach width of auriculariae steadily increased regardless of diet, reflecting the suitability of these micro-algae concentrates for hatchery culture of sandfish. In contrast unfed larvae showed reduction in stomach size, high mortality prior to metamorphosis and were unable to develop into competent doliolariae as reported in prior studies (Knauer, 2011; Morgan, 2008; Sun and Li, 2012). Despite a slight increase in the larval development period compared to other studies that have used live micro-algae as the larval food source (e.g. Knauer, 2011; Morgan, 2001), the total larval length of auriculariae recorded in this study was greater than that of larvae fed the best live micro-algae in some previous studies. For example, auriculariae reached mean total lengths of 918.20 µm on day 7 and 1011.64 µm on day 9 when fed TW 1200® in this study compared to 890.8 ± 12.9 µm (day 8) (Knauer, 2011) and 899 µm (day 10) (Morgan, 2001) in other studies. Moreover, settlement success appeared to be positively influenced by higher proportions of late auriculariae with hyaline spheres which consequently transformed into higher numbers of competent doliolariae

prior to metamorphosis and settlement. Hyaline spheres that are present in auriculariae of *H. scabra*, disappear during the doliolaria stage and their function as nutrient reserves through the non-feeding perimetamorphic period has been suggested (Ramofafia et al., 2003). Further research is required into the influence of hyaline spheres on metamorphic and settlement success in *H. scabra* and, if a positive relationship is proven, then the influence of diet composition on hyaline sphere formation would become a key consideration.

Little is known about the nutritional requirement of sea cucumber larvae (Purcell et al., 2012). The dietary protein contents of the Instant Algae® products used in this study ranged from 208.17 to 255.22 mg g^{-1} dry weight and the majority of the key performance indicators for *H. scabra* larvae showed negative correlation with dietary protein level. This indicates a relatively less important role for dietary protein compared to other dietary nutrients, and/or that the optimal level of dietary protein for *H. scabra* auriculariae was satisfied by all of the diets used in this study. A subordinate role for dietary protein was also indicated during larval development of the temperate sea cucumber, *Apostichopus japonicus*, where lower protease activity was reported in larvae than in juveniles (Tang et al., 2007). Dietary protein requirements for sea urchin (*Psammechinus lividus* and *P. miliaris*) larvae have been suggested to be <37% of diet dry weight (Liu et al., 2007a, 2007b) which is considerably higher than those of <26% used in this study.

Total dietary lipid contents of the Instant Algae® products used in this study ranged from 44.4 to 95.4 mg g^{-1} dry weight and were negatively correlated with larval growth and settlement success of sandfish. Liu et al. (2007a, 2007) reported that a total dietary lipid content of <7% dry weight supported development and growth of sea urchin (*P. lividus* and *P. miliaris*) larvae, while high lipid levels in formulated feeds may benefit the post-settlement growth of newly metamorphosed juveniles. In contrast, low dietary lipid content (ca. 5.6%) was found to support the best growth rates of sea urchin, *Lytechinus variegatus*, juveniles (Gibbs, 2011).

Much of the research on larval diet quality for echinoids has raised the importance of the ratio between specific long chain PUFA (EPA: DHA and EPA: ARA) to larval development. Higher dietary EPA: DHA and EPA: ARA ratios have been generally associated with improved larval performance in the sea urchins *Psammechinus lividus* and *P. miliaris* (Carboni et al., 2012; Liu et al., 2007a, 2007b). Our results indicate that a higher dietary higher EPA: DHA ratio supported significantly better larval growth, superior hyaline sphere (HS) development in

auricularlae, higher competence in doliolariae and better settlement success. A combination of optimised fatty acid nutrition combined with appropriate settlement cues may promote even better settlement in echinoderms (Dworjanyn and Pirozzi, 2008). The level of dietary palmitic acid (16:0), a readily catabolised source of energy, that was highest in TW 1200®, was also positively correlated to the presence of HS in late auricularlae and to settlement success. Our results therefore indicate that improved performance of sandfish larvae is associated with a relatively high dietary EPA: DHA ratio and a relatively high level of dietary palmitic acid.

Dietary PUFA, particularly EPA, DHA and ARA, are considered essential for the normal development and survival of marine larvae (Carboni et al., 2012; Holme et al., 2009; Knauer and Southgate, 1999). Higher dietary total lipid, ARA and DHA contents supported higher survival of late auricularlae in this study. However, TW 1200® which contains a relatively high level of EPA, and a relatively low level of DHA, supported high larval performance and relatively high survival of post-settled larvae, whereas Isochrysis 1800®, which contains a relatively low level of EPA and a high level of DHA, did not. Relatively high dietary EPA: DHA and EPA: ARA ratios have been associated with higher growth rates and survival of sea urchin (*Psammechinus lividus* and *P. miliaris*) larvae (Carboni et al., 2012; Liu et al., 2007a, 2007b). The low EPA content of T-ISO has also been suggested as a reason for its relatively poor food value in some nutritional studies with mollusc larvae (e.g. Helm and Laing, 1987; Southgate et al., 1998). Despite this, Pavlova 1800® is rich in both EPA and DHA but still supported relatively poor larval performance in this study. Although the products used in this study were presented to larvae at the same dry weight ration, their rates of ingestion and digestion by sandfish larvae differ (Chapter 3); this may have influenced the results obtained including those for Pavlova 1800®. Knauer (2011) reported that neither live *Pavlova salina* nor *Isochrysis* (T-ISO) fed separately to sandfish larvae supported development to competent doliolariae. Our results, in contrast, show that both Pavlova 1800® and Isochrysis 1800® supported normal development of sandfish larvae and production of juveniles. Both would be an appropriate food source for sandfish larvae when used either singly or in combination with the other products tested.

Our results showed a strong correlation between dietary carbohydrate (as NFE) and performance of sandfish larvae. Diatoms generally contain relatively high levels of easily digestible carbohydrate (Orozco et al., 2014) and this was the case in this study where TW 1200® (a diatom) had the highest NFE content of the three micro-algae concentrates tested. Dietary NFE was highly correlated with survival and size (growth) of sandfish larvae to day

25 and our results indicate that dietary carbohydrate is more important than dietary protein in determining the nutritional value of micro-algae for sandfish larvae. This might be a reasonable explanation for the relatively high nutritional value of the diatom *Chaetoceros muelleri* (fed live) reported during hatchery culture of sandfish from auriculariae to competent doliolariae (Knauer, 2011).

Zacarias-Soto et al. (2013) reported that a mixture of concentrated micro-algae from the Instant Algae® range (Tetraselmis and Isochrysis) can be used to raise the larvae of *Isostichopus badionotus*. In the current study, all commercially available micro-algae concentrates tested proved nutritious for *H. scabra* larvae when fed as single species, and all supported normal growth and development, and relatively high survival, through settlement. Hatchery culture of sandfish using these products, without the use of live micro-algae, is a significant finding that supports development of cheaper, simpler larval rearing protocols for this species. While acknowledging that the diets used in this study were not *iso*-lipidic or *iso*-energetic, and that they varied in their rates of ingestion and digestibility (Chapter 3) and nutrient compositions, our results help to identify some of the key dietary nutrients for *H. scabra* larvae, and provide a basis for development of more appropriate diets supporting improved hatchery culture methods.

CHAPTER 5

DEVELOPMENT OF HYALINE SPHERES IN LATE AURICULARIAE OF SANDFISH, *HOLOTHURIA SCABRA*: IS IT A RELIABLE INDICATOR OF SUBSEQUENT PERFORMANCE?[4]

5.1. Introduction

There are several stages during larval development of sea cucumbers. The first auricularia stage is planktonic and the larvae feed on phytoplankton and small suspended particles or detritus (Strathmann, 1975). Larvae then transform sequentially into the non-feeding doliolaria and pentactula stages, before finally metamorphosing into newly settled juveniles that consume benthic diatoms and bacterial films (Ito and Kitamura, 1997; Yanagisawa, 1998). Towards the end of the auricularia stage, larvae develop hyaline spheres (HS) at the tip of the lateral processes (Figure 5.1) (Chen and Chian, 1990; Smiley et al., 1991; Sewell and McEuen, 2002). HS are composed of connective tissue cells and matrix (Burke, 1987; Dautov, 1997), and histochemical examination has shown that they contain lipids (Chen et al., 1991; Dautov and Kashenko, 1995). They have been thought to play a role in larval buoyancy (Mortensen 1938; Dautov and Kashenko, 1995) and to function as a nutrient or energy store for the non-feeding peri-metamorphic period, before juveniles are competent to feed (Ramofafia et al., 2003). In larvae of the tropical sea cucumber *Holothuria scabra* (sandfish), HS are prominent in early doliolaria but subsequently disappear as larvae approach metamorphosis (Ramofafia et al., 2003). On this basis, competent doliolariae are generally considered to have prominent HS (Ramofafia et al., 2003) and their presence and size is thought to be an indicator of larval competence (Chen et al., 1991; Battaglene, 1999; Smiley et al., 1991; Ramofafia et al., 2003).

HS formation is influenced by the diet of auriculariae, and both diet composition and ration are likely to be important in this regard. Auriculariae of the temperate *Parastichopus californicus*, for example, did not develop HS when fed single species of micro-algae, but did develop them

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when fed a multi-species micro-algal diet (Smiley et al., 1991), that presumably provided a better balance of nutrients. Sandfish larvae developed HS when fed micro-algae such as *Chaetoceros muelleri, C. calcitrans* and *Rhodomonas salina* but did not complete development when fed *Isochyrsis* aff. *galbana* or *Tetraselmis chuii* (Ramofafia et al., 2003). Similarly, Knauer (2011) reported that neither live *Pavlova salina* nor *Isochrysis* (T-ISO) fed separately to *H. scabra* larvae, supported development to competent doliolaria, whereas *Chaetoceros* spp. did. Knauer (2011) noted the superiority of diatoms (*Chaetocertos* spp.) as a food for *H. scabra* auricularia, compared to flagellates (*P. salina* and T-ISO); the latter supported no development, or poor development of HS in late auricularia. The results of Ramofafia et al. (2003) and Knauer (2011) highlight considerable differences between the nutritional values of diatoms (Bacillariophyceae) and flagellates (Haptophycaea) to *H. scabra* larvae, and strongly indicate that HS formation is influenced by the nutrient composition of the larval diet.

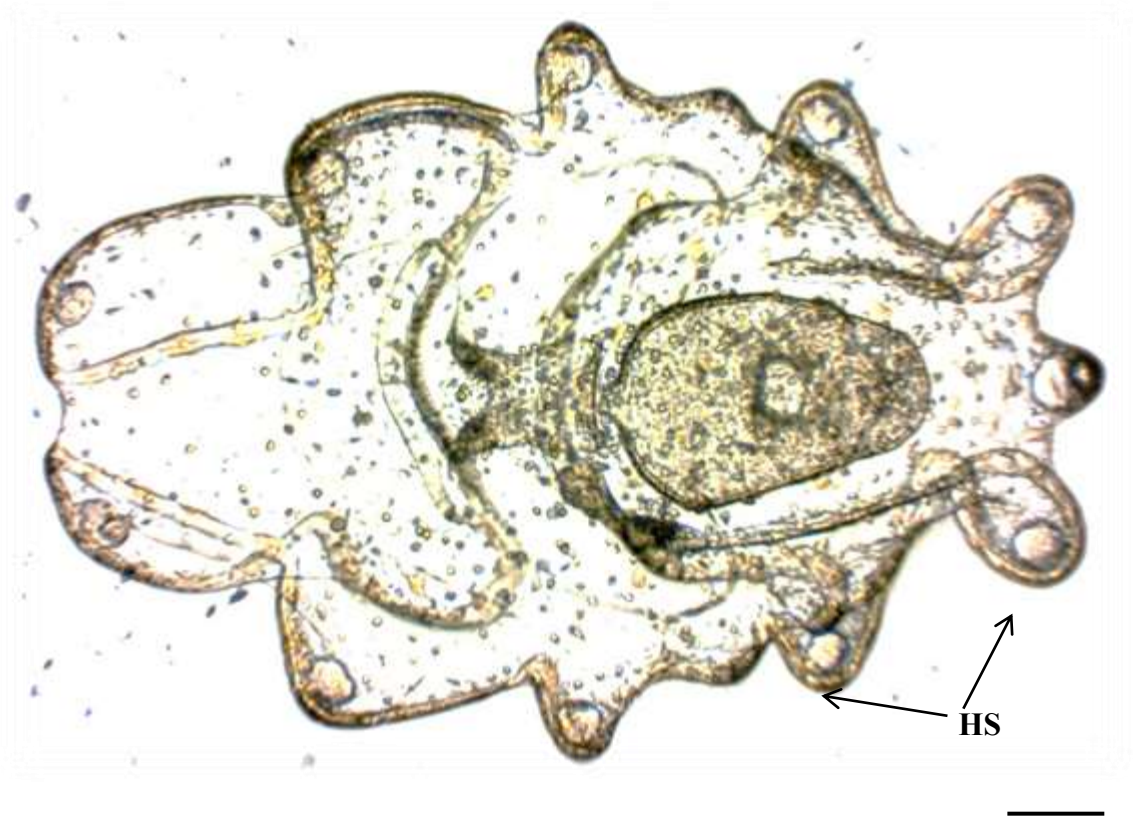


Figure 5.1. Late sandfish auriculariae with hyaline spheres. **HS**: hyaline spheres. Scale bar 100 µm.

Recent research in the laboratory has shown that commercially available micro-algae concentrates are readily ingested and digested by *H. scabra* auricularia (Chapter 3) and that

some are able to support larval growth and development through metamorphosis as a sole food source (Chapter 4). Furthermore, both diatoms and flagellates are available in concentrated form providing a broad range of nutrient compositions that can be assessed for their nutritional efficacy (Chapter 4). This study used commercially available micro-algae concentrates to examine the influence of diet composition on HS development in late *H. scabra* auriculariae. It further investigates relationships between the presence and size of HS in late auriculariae, larval competence through settlement, and early juvenile performance.

5.2. Materials and Methods

5.2.1. Broodstock and larvae

Broodstock were collected at Limanak Island near Kavieng in Papua New Guinea (2°34′S 150°48′E). They were induced to spawn using thermal shock followed by the addition of dried *Spirulina* sp. to the spawning tank (Agudo, 2006; Duy, 2010). Eggs were hatched in a 2,000 L tank filled with 1 µm filtered seawater (FSW) at a density of 1 egg mL^{-1}. After 2 days, larvae were counted and distributed to the 500-L tanks used for the experiment.

5.2.2. Diets

Three commercially available micro-algae concentrates (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) purchased from an Australian distributor were used in this study. They were: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova* sp. (Haptophyceae) (Pavlova 1800®); and (3) mono–cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®). The three products were either fed individually or as a mixture composed of an equal ration (dry weight) of *Isochrysis* sp., *Pavlova* sp. and *T. weissflogii* making up a fourth ternary diet treatment. Micro-algae concentrates were stored in their original bottles in a refrigerator at 4ºC for the duration of the study.

5.2.3. *Biochemical analysis of micro-algae concentrates*

Proximate analysis of the three Instant Algae® products (Isochrysis 1800®, Pavlova 1800® and TW 1200®) was conducted using standard procedures (AOAC, 1990) after freeze-drying. Percentage moisture was determined after freeze-drying to a constant weight; protein (Kjeldahl nitrogen; N x 6.25) was determined using an automated Kjeltech (model 2300, Tecator, Sweden); total lipid was

determined gravimetrically following dichloromethane/methanol extraction (2:1 v/v) (Folch et al., 1957); ash was determined following incineration at 550 °C for 18 h.

Table 5.1. Daily rations (cells mL^{-1}) of the three Instant Algae® products fed to *Holothuria scabra* auricularіae in this study. Daily rations for each product provided larvae with the same dry weight of micro-algae.

	Product		
Day	**'Isochrysis 1800®'**	**'Pavlova 1800®'**	**'TW 1200®'**
2	10,000	10,391	3,926
3	11,000	11,431	4,319
4	12,000	12,470	4,712
5	13,000	13,509	5,104
6	14,000	14,548	5,497
7	15,000	15,587	5,890
8	16,000	16,626	6,282
9	17,000	17,666	6,675
10	18,000	18,705	7,068
11	19,000	19,744	7,460
12	20,000	20,783	7,853
13	21,000	21,822	8,246
14	22,000	22,861	8,638
15	23,000	23,900	9,031
16	24,000	24,940	9,424
17	25,000	25,979	9,816
18	26,000	27,018	10,209
19	27,000	28,057	10,601
20	28,000	29,096	10,994
21	29,000	30,135	11,387
22	30,000	31,174	11,779

Nitrogen free extract (NFE) was calculated by difference following the summation of moisture, lipid, protein and ash. Energetic contents of micro-algae concentrates were estimated from proximate analyses using caloric equivalents of 4.0 cal. mg^{-1}, 4.5 cal. mg^{-1} and 9.4 cal. mg^{-1} for carbohydrate (NFE), protein and lipid, respectively (Marshall et al., 2010).

Following lipid extraction, fatty acids were esterified into methyl esters using the acid-catalysed methylation method (Christie, 2003) following the protocol of Conlan et al. (2014).

Fatty acid methyl esters were isolated and identified using an Agilent Technologies 7890 GC System (Agilent Technologies, USA) equipped with a BPX70 capillary column (120 m × 0.25 mm internal diameter, 0.25 mm film thickness, SGE Analytical Science, Australia), a flame ionization detector (FID), an Agilent Technologies 7693 auto sampler, and a split ratio of 50:1. The carrier gas was helium. Individual fatty acids were identified relative to known external standards (Sigma-Aldrich, Inc., St Louis, USA and Nu-Chek Prep Inc., USA) using the software GC ChemStation (Rev B.04.03, Agilent Technologies). The resulting peaks were corrected by theoretical relative FID response factors (Ackman, 2002) and quantified relative to the internal standard C23:0 (0.75 mg mL^{-1}, Sigma-Aldrich, Inc., USA).

5.2.4. *Experiment design*

Two-day auriculariae with initial mean (± SD, n = 90) length of 599.98 ± 22.67 µm and stomach diameter of 79.06 ± 4.71 µm were stocked into triplicate 500-L tanks (5º sloping bottom) filled with 300 L of 1-µm filtered sea water (FSW) at an initial density of 0.3 larvae mL^{-1} and randomly allocated one of the four dietary treatments. A 50% tank water exchange was conducted daily. Water temperature was maintained at 27.5 ± 1.0ºC and the culture water was moderately aerated.

The dry weights of individual micro-algae concentrates used in this study were determined from triplicate 1-mL aliquots removed from their original bottles after gentle agitation. They were filtered through pre-weighed glass-fibre micro filters (Whatman GF/F, 25 mm), washed with 20 mL 0.5 M ammonium formate solution and dried at 100°C for 16 h (Epifanio, 1979). Filters were then re-weighed to determine micro-algae dry weights per mL of product. The number of cells per mL of Isochrysis 1800® was also estimated using triplicate hemocytometer counts and the mean dry weight per cell was determined. For larvae fed Isochrysis 1800®, the initial ration was 10,000 cells mL^{-1} (Morgan, 2010) and this was increased by 1,000 cells mL^{-1} of Isochrysis 1800® per day as larval development proceeded. Thus, Isochrysis 1800® was fed at a rate of 10,000 cells mL^{-1} on day 2, 11,000 cells mL^{-1} on day 3, 12,000 cells mL^{-1} on day 4, 13,000 cells mL^{-1} on day 5, and so on. Because the micro-algae used in this study vary considerably in cell size (Chapter 3), and rations administered on an equal cell density basis would differ considerably in the mass or volume of micro-algae available to larvae across treatments, diets were presented to larvae on an equal dry weight basis (Knauer, 2011; Chapter 4). The dry weight of each daily Isochrysis 1800® ration was calculated and all other micro-

algae diets were administered at cell densities that provided the same dry weight (McCausland et al., 1999; Martinez-Fernandez et al., 2006; Knauer, 2011) (Table 5.1).

Prior to use, micro-algae concentrates were gently hand-shaken and the required amount of each concentrate was added to a graduated container filled with 1 μm filtered seawater, prior to feeding. The daily ration was divided into three equal proportions fed three times a day with at least 4 h between feeds to ensure ingestion and digestion by larvae (Chapter 3) and minimise the possibility of overfeeding (Morgan, 2001). Auriculariae were cultured using standard methods (Duy, 2010; Chapter 4) and settlement plates, painted with a slurry of dry *Spirulina* sp., were added to the culture tanks once >70% of competent doliolariae were visible during microscopic inspection (Duy, 2010). Competent doliolariae were defined as being barrel-shaped with prominently developed HS (Ramofafia et al., 2003).

Table 5.2. Proximate compositions (mg g^{-1} dry weight) and energy contents (cal. g^{-1} dry weight) of micro-algae concentrates used in this study.

Micro-algae	Protein	Lipid	NFE	Ash	Energy
Isochrysis 1800®	255.22 ± 0.77^{a}	95.36 ± 2.09^{a}	317.02 ± 0.36^{b}	332.26 ± 1.01^{c}	3.66 ± 0.01^{a}
Pavlova 1800®	247.76 ± 0.75^{b}	75.71 ± 0.00^{b}	295.21 ± 2.26^{c}	381.32 ± 3.02^{b}	3.34 ± 0.01^{b}
TW 1200®	208.17 ± 0.53^{d}	44.37 ± 0.57^{c}	332.69 ± 1.77^{a}	414.80 ± 0.64^{a}	2.97 ± 0.00^{c}
Ternary diet	237.05 ± 0.33^{c}	71.82 ± 0.88^{b}	$314.98 \pm 1.47^{\mathbf{b}}$	$376.16 \pm 0.89^{\mathbf{b}}$	3.33 ± 0.01^{b}

Larval survival was estimated at day 5 (early-mid auriculariae), day 13 (late auriculariae) and day 25 (post-settled larvae) after fertilization, for each treatment, by counting the number of live individual within a standard volume of water removed randomly from each culture tank. Samples were examined using a dissecting microscope. The percentage of auriculariae with HS and the diameter of HS (Figure 5.1) were determined for larvae on day 10, day 11, day 12 and day 13 after fertilization by randomly sampling thirty larvae from each rearing tank. Larvae were examined with a microscope and micrometer to measure HS diameter which was determined by measuring the largest HS of sampled auriculariae. The percentage of competent doliolariae was determined for each treatment on day 14, day 15 and day 16 according to the criteria of Ramofafia et al. (2003). The mean length of thirty live post-settled larvae from each tank was determined for each treatment on day 21 using a dissecting microscope and micrometer, and

the percentage survival of settled larvae was determined for each treatment on day 25. Larval development and survival were compared to that of unfed larvae.

5.2.5. Data analyses

The percentage of auriculariae with HS, HS diameter (Figure 5.1), percentage of competent doliolariae, post-settled larval length, and auriculariae and post-settled larval survival, were compared using one-way ANOVA followed by Tukey's multiple range test to evaluate all pair-wise treatment comparisons ($P < 0.05$). All percentage data were arcsin-transformed prior to analyses. Pearson's correlation test was used to determine correlations between hyaline sphere development and nutrient composition of the micro-algae diets (proximate components, specific fatty acids and fatty acid groups); and also to determine the relationship between competent doliolariae and settlement success. Statistical analyses were facilitated using the software IMB SPSS VERSION 22.0.

5.3. Results

5.3.1. Biochemical compositions of micro-algae diets

The proximate compositions of the micro-algae diets are shown in Table 5.2. Lipid content was highest in Isochrysis 1800® (95.36 ± 2.09 mg g^{-1} dry weight) and lowest in TW 1200® (44.37 ± 0.57 mg g^{-1} dry weight) while Pavlova 1800® (75.71 ± 0.00 mg g^{-1} dry weight) and the ternary diet (71.82 ± 0.88 mg g^{-1} dry weight) had intermediate lipid contents. This pattern was similar for protein contents that ranged from 255.22 ± 0.77 mg g^{-1} dry weight to 208.17 ± 0.53 mg g^{-1} dry weight. However, the NFE content of TW 1200® (332.69 ± 1.77 mg g^{-1} dry weight) was significantly higher than that of the ternary diet (314.98 ± 1.47 mg g^{-1} dry weight) and Isochrysis 1800® and Pavlova 1800® (317.02 ± 0.36 and 295.21 ± 2.26 mg g^{-1} dry weight, respectively). Isochrysis 1800® had the highest calculated energy content (3.66 ± 0.01 cal. g^{-1} dry weight) while TW 1200® had the lowest (2.97 ± 0.00 cal. g^{-1} dry weight) (Table 5.2).

Table 5.3. Major fatty acids (μg g^{-1} dry weight) of the micro-algae concentrates used in this study.

Fatty acid	Isochrysis 1800®	Pavlova 1800®	TW 1200®	Ternary diet
14:0	1084.74	2094.66	420.75	1200.05
16:0	1269.44	1289.87	2137.39	1565.56
16:1n-7	768.17	1979.44	1700.17	1482.59
18:1n-9	2329.65	233.90	177.03	913.53
22:1 (isomers)	358.00	59.74	175.22	197.66
18:2n-6	1520.63	178.90	83.85	594.46
18:3n-6	301.59	21.35	36.17	119.70
20:4n-6 (ARA)	95.29	117.59	49.20	87.36
22:4n-6	20.49	1522.63	90.61	544.57
22:5n-6	394.70	1051.58	204.20	550.16
18:3n-3	1207.23	216.64	31.50	485.12
18:4n-3	2830.12	1277.91	67.03	1391.68
20:5n-3 (EPA)	204.44	3478.89	2085.01	1922.78
22:6n-3 (DHA)	2511.36	965.63	301.09	1259.36
SFA	2836.14	3738.63	3044.45	3206.41
MUFA	3883.17	2586.86	2537.34	3002.45
PUFA	9330.88	9146.81	4298.24	7591.98
n-3 PUFA	6865.76	6001.62	2692.61	5186.66
n-6 PUFA	2401.83	2963.62	464.03	1943.16
n-3 LC PUFA	2828.41	4507.08	2594.09	3309.86
n-6 LC PUFA	540.73	2691.80	344.01	1192.18

The major component fatty acids of the three micro-algae concentrates used in this study are shown in Table 5.3. Myristic acid (14:0) and Palmitic acid (16:0) were the most abundant saturated fatty acids (SFA) in all micro-algae. Pavlova 1800® had the highest levels of Arachidonic acid (ARA, 20:4n-6) and eicosapentaenoic acid (EPA, 20:5n-3) among the three products tested, while Isochrysis 1800® had the highest level of docosahexaenoic acid (DHA, 22:6n-3) of 2511.36 μg g^{-1} dry weight. TW 1200® had relatively low levels of ARA and DHA compared to the two flagellates, and considerably lower levels of total polyunsaturated fatty acids (PUFA), n-3 PUFA and n-6 PUFA (Table 5.3).

5.3.2. Larval development

There were significant differences in the numbers of late auriculariae with HS between treatments (Figure 5.2). From day 10 to day 13, significantly greater numbers of late auriculariae with HS resulted from those fed TW 1200® than from those fed the other micro-

algae diets ($P < 0.05$). Larvae fed Isochrysis 1800®, Pavlova 1800® and the ternary diet showed no significant differences ($P > 0.05$) in the proportion of larvae with HS on any sampling day, except day 11, when a significant reduced proportion of late auriculariae fed Pavlova 1800® had HS.

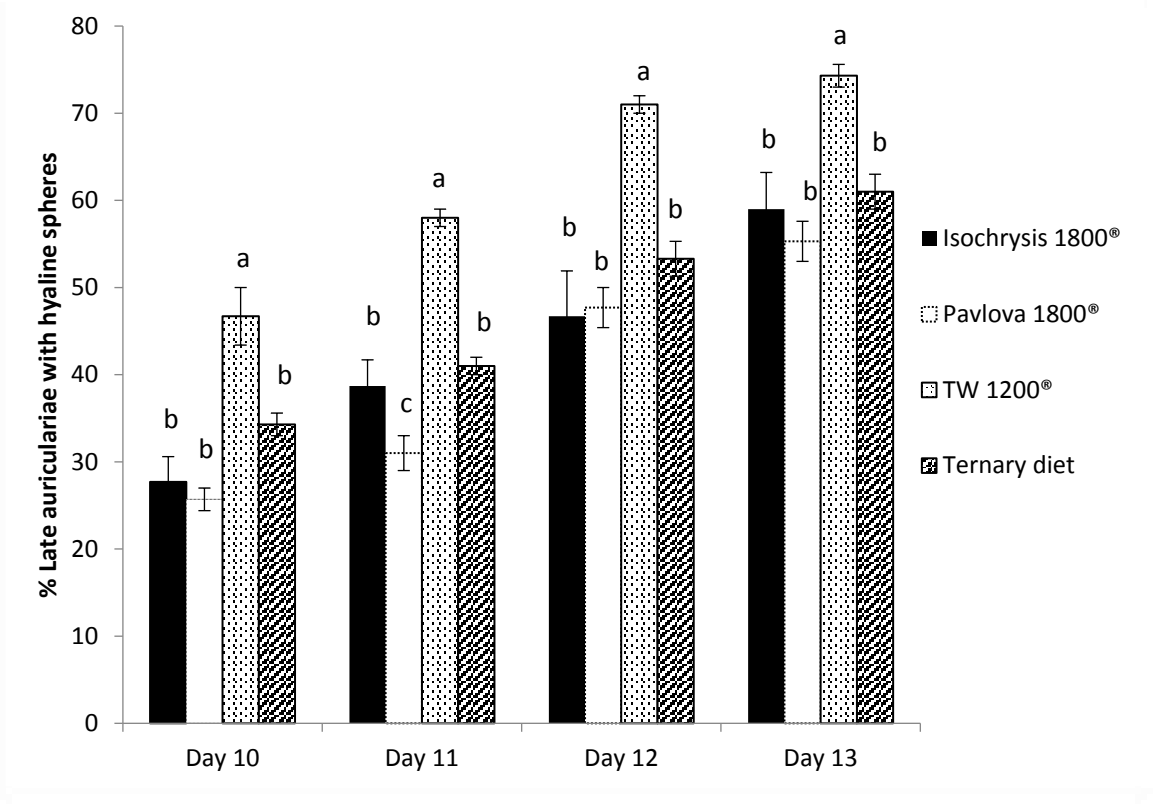


Figure 5.2. Mean (± SE) percentage of late auriculariae with hyaline spheres when fed different micro-algae concentrates during this study. Means with different superscripts at a given sampling time are significantly different ($P < 0.05$).

Similarly, there were also significant differences between treatments in the size of HS in late auriculariae (Figure 5.3). The mean diameter of HS of late auriculariae fed TW 1200® was significantly larger than that of larvae fed Isochrysis 1800®, Pavlova 1800® and the ternary diet ($P < 0.05$) on each of the sampling days. However, there was no significant difference in the mean diameter of HS of late auriculariae fed Isochrysis 1800®, Pavlova 1800® and the ternary diet from day 10 to day 13 (Figure 5. 3).

The number of competent doliolariae were measured as they were first observed in the larval rearing tanks from day 14 to day16 (Table 5.4). On day 14, doliolariae first appeared among

larvae fed TW 1200® only. More than 75% of larvae fed TW 1200® had transformed into doliolariae on day 15 and settled on day 16. On day 15, there was significant difference in the numbers of competent doliolariae fed TW 1200® (75.6 ± 7.3 %) and those fed the other micro-algae diets ($P < 0.05$), but there was no significant difference in the proportion of competent doliolariae fed Isochrysis 1800® (10.0 ± 1.9 %), Pavlova 1800® (6.7 ± 0.0 %) and the ternary diet (10.0 ± 0.0 %) ($P > 0.05$).

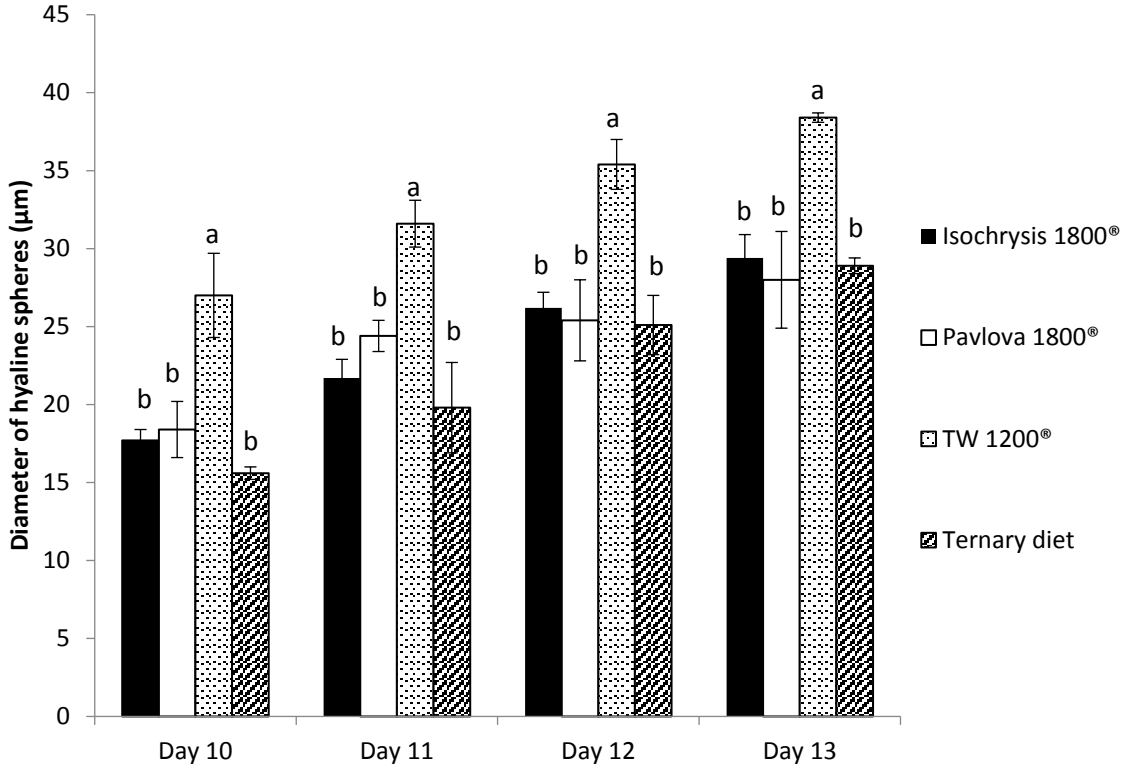


Figure 5.3. Mean (± SE) diameter of hyaline spheres in late auriculariae fed different micro-algae concentrates during this study. Means with different superscripts at a given sampling time are significantly different ($P < 0.05$).

Consequently, the length of 21 day-old post-settled larvae was significantly different between treatments ($P > 0.05$). The mean length of post-settled larvae was significantly larger in tanks fed TW 1200® (1.19 ± 0.14 mm) than that of fed the ternary diet (0.65 ± 0.20 mm) followed by Isochrysis 1800® and Pavlova 1800® (0.59 ± 0.13 and 0.49 ± 0.07 mm, respectively). However, there was no significant difference in post-settled larvae length between Isochrysis 1800® and the ternary diet ($P > 0.05$).

Table 5.4. Mean (± SE) percentage of competent doliolariae fed different micro-algae concentrates during this study. Means within columns (day 15) with different superscripts are significantly different ($P < 0.05$).

Micro-algae	% Competent doliolariae		
	Day 14	Day 15	Day 16
Isochrysis 1800®	N/A	10.0 ± 1.9^{b}	52.2 ± 5.1
Pavlova 1800®	N/A	6.7 ± 0.0^{a}	37.8 ± 5.1
TW 1200®	8.9 ± 1.9	75.6 ± 7.3^{b}	ST
Ternary diet	N/A	10.0 ± 0.0^{b}	41.1 ± 8.4

N/A: Not Available. ST: settled

5.3.3. *Larval survival*

There were significant differences in the mean (± SE) survival of auriculariae and post-settled larvae between treatments (Figure 5.4). There were significant differences in survival of early-mid and late auriculariae (day 5 and day 13) between the TW 1200® (84.7 ± 0.8 % and 64.0 ± 0.6 %) and Pavlova 1800® (88.0 ± 0.6 % and 68.7 ± 1.0 %) treatments. whereas survival of larvae fed Isochrysis 1800® (85.0 ± 0.7 % and 67.8 ± 0.8%) did not differ significantly ($P > 0.05$) from those fed the ternary diet (84.9 ± 0.5% and 64.3 ± 1.2 %). In the auricularia stage, Pavlova 1800® supported a slightly higher rate of survival compared to the other diets (Figure 5.4); however, survival of post-settled larvae fed TW 1200® (13.7 ± 0.7 %) was significantly greater ($P < 0.05$) and almost double that of larvae fed the other diets, that did not differ significantly from each other ($P > 0.05$).

5.3.4. Correlations between hyaline sphere development and nutrient compositions of diets

There were significant negative correlations between the proportions of the late auriculariae with HS on day 10 and total dietary lipid ($R = -0.714$, $p < 0.05$) and dietary protein ($R = -0.840$, $p < 0.01$) (Table 5.5). These relationships held true for the proportions of the late auriculariae with HS on day 11, 12 and 13 (Table 5.5). There were also significant negative correlations between the proportions of the late auriculariae with HS on days 11, 12 and 13 with total energy contents of the diets. In contrast, there were significant positive correlations between the proportions of late auriculariae with HS and dietary carbohydrate (as NFE) contents on day 10 ($R = 0.823$, $p < 0.05$), day 11 ($R = 0.891$, $p < 0.01$) and day13 ($R = 0.730$, $p < 0.05$). Proportions of late auriculariae with HS on both day 12 and day 13 were positively correlated to the EPA: DHA ratio ($R = 0.889$ and $R = 0.785$, respectively), while that of day 12 was only correlated to the EPA: ARA ratio (Table 5.5).

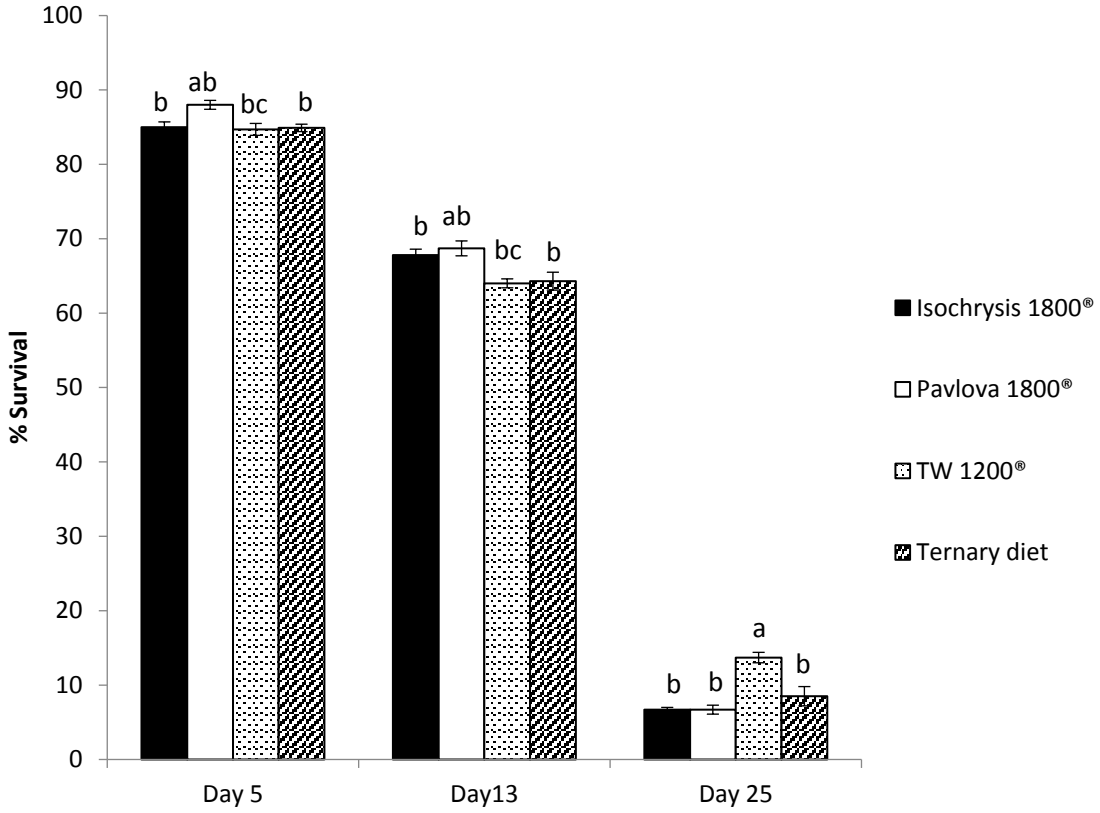


Figure 5.4. Mean (± SE) survival (%) of sandfish larvae fed different micro-algae concentrates during the experiment. Survival of auriculariae was determined on day 5, day 13 and that of post-settled larvae was estimated on day 25 after fertilisation. Means with different superscripts at a given sampling time are significantly different ($P < 0.05$).

Table 5.5. Pearson's correlation between the hyaline spheres development in late auriculariae and proximate and specific fatty acids compositions of micro-algae concentrates used in this study.

Hyaline sphere development	Lipid	Protein	NFE	Total energy	ARA	DHA	EPA	EPA/DHA	EPA/ARA
HS larvae (%) day 10	-0.714*	-0.840**	0.823*	-0.674	-0.967**	-0.505	-0.135	0.589	0.591
HS larvae (%) day 11	-0.783*	-0.907**	0.891**	-0.736*	-0.981**	-0.556	-0.128	0.669	0.586
HS larvae (%) day 12	-0.982**	-0.984**	0.623	-0.969**	-0.788*	-0.884**	0.351	0.889**	0.844**
HS larvae (%) day 13	-0.917**	-0.961**	0.730*	-0.886**	-0.834*	-0.740*	0.147	0.785*	0.703
HS diameter (µm) day 10	-0.619	-0.682	0.502	-0.612	-0.713*	-0.498	0.066	0.722*	0.659
HS diameter (µm) day 11	-0.576	-0.613	0.381	-0.579	-0.612	-0.535	0.247	0.745	0.652
HS diameter (µm) day 12	-0.717*	-0.821*	0.700	-0.689	-0.806*	-0.530	-0.035	0.680	0.543
HS diameter (µm) day 13	-0.646	-0.759*	0.643	-0.629	-0.749*	-0.481	-0.042	0.643	0.506

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Table 5.6. Pearson's correlation between the hyaline spheres development in late auriculariae, and competent doliolariae and settlement success of sandfish.

Hyaline spheres development	% Competent doliolariae day 15	Post-settled larvae size day 21	Settled larvae survival day 25
HS larvae (%) day 10	0.805**	0.892**	0.822**
HS larvae (%) day 11	0.880**	0.938**	0.849**
HS larvae (%) day 12	0.867**	0.887**	0.873**
HS larvae (%) day 13	0.853**	0.874**	0.842**
HS diameter (μm) day 10	0.775**	0.790**	0.677*
HS diameter (μm) day 11	0.770**	0.741**	0.587
HS diameter (μm) day 12	0.816**	0.813**	0.783**
HS diameter (μm) day 13	0.838**	0.846**	0.811**

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

The diameter of HS of late auriculariae on day 10 was negatively correlated to ARA content ($R = -0.713$, $P < 0.05$) but positively correlated to the EPA: DHA ratio ($R = 0.722$, $R < 0.05$) (Table 5.5). There were negative correlations between HS diameter on day 12 and dietary lipid ($R = -0.717$, $P < 0.05$), dietary protein content ($R = -0.821$, $P < 0.05$) and ARA content ($R = -0.806$, $P < 0.05$). On day 13, HS diameter was only significantly correlated to dietary protein ($R = -0.759$, $P < 0.05$) and ARA ($R = -0.749$, $P < 0.05$) contents (Table 5.5).

5.3.5. Correlations between hyaline spheres development, doliolaria competence and settlement success

There were highly positive significant correlations between HS development on days 10, 11, 12 and 13, and the proportion of competent doliolariae on day 15, post-settlement size (day 21) and post-settlement survival (day 25) (Table 5.6). Similarly, there were highly significant positive correlations between HS diameters of late auriculariae on day 10 and the proportion of competent doliolariae on day 15 ($R = 0.775$, $p < 0.01$), post-settled larvae size on day 21 ($R = 0.790$, $p < 0.01$) and survival of settled-larvae on day 25 ($R = 0.677$, $p < 0.05$). These relationships held true for HS diameter on day 11, 12 and 13 with the exceptions of HS diameter on day 11 and survival of settled-larvae on day 25 (Table 5.6).

5.4. Discussion

Formation of HS and their size varied between diets and both were clearly influenced by diet composition in this study. Our results show that: (1) HS development was strongly linked to the nutrients available to sandfish larvae during the auricularia stage; (2) larval competence

during settlement and the early post-settlement period was strongly correlated with the degree of HS development in late auricullariae; and (3) the nutritional components most strongly influencing this relationship were carbohydrates and levels of the n-3 PUFAs ARA, EPA and DHA. Development of HS in late auricullariae has been linked to subsequent larval competence in a number of sea cucumber species (Chen et al., 1991; Morgan, 2008, 2009; Smiley et al., 1991; Sun and Li, 2013) including sandfish (Battaglene, 1999; Ramofafia et al., 2003). Our results confirm this relationship for sandfish and, perhaps more importantly, have identified some of the key dietary components that influence HS development in this species. It may be possible for example, to manipulate the composition of diets used for hatchery culture of sandfish to improve hatchery production and efficiency, and improve the quality of resulting juveniles.

Hyaline sphere development was positively correlated with dietary carbohydrate content (as NFE), and with the ratios between specific LC-PUFA (EPA: ARA and EPA: DHA). Diatoms, such as *Thalassiosira* spp., generally contain relatively high levels of easily digestible carbohydrate (Orozco et al., 2014) and this was the case in this study where TW 1200® (a diatom) had the highest NFE content of the three micro-algae diets tested. Dietary NFE was strongly correlated with HS formation and HS size, while similar relationships with dietary protein and lipid contents were not positive. On this basis our results indicate that dietary carbohydrate is more important than dietary protein and lipid in determining the nutritional value of micro-algae for sandfish larvae. Despite our findings of no positive relationships between total dietary lipid content and larval performance, there were significant positive correlations between specific component fatty acids and larval performance. Specifically, higher dietary EPA: DHA and EPA: ARA ratios supported significantly better HS development in late auricullariae. Similar findings have been reported for the larvae of other echinoderms where, for example, higher dietary EPA: DHA and EPA: ARA ratios were associated with improved larval performance in the sea urchins *Paracentrotus lividus* and *Psammechinus miliaris* (Carboni et al., 2012; Liu et al., 2007a, 2007b). Our results, therefore, indicate that improved HS development in sandfish auricullariae is associated with relatively high dietary EPA: DHA and EPA: ARA ratios and a relatively high level of carbohydrate.

The strong association between HS development and subsequent larval performance demonstrated in this study suggests that a HS diameter of between 25-40 µm is required in late auricullariae to support acceptable levels of subsequent survival and growth. Larvae fed TW 1200® had a mean HS diameter of 38.4 ± 0.3 µm on day 13 compared to means of 28.0 ± 3.1 µm and 29.4 ± 1.5 µm for those fed Pavlova 1800® and Isochrysis 1800®, respectively. Survival

to day 25 was 13.7 ± 0.7 % for larvae fed TW 1200®, but was less than half this value for larvae fed Pavlova 1800® (6.7 ± 0.6 %) and Isochrysis 1800® (6.7 ± 0.3 %). Our results indicate that auriculariae with smaller HS are less able to meet the nutrient requirements of metamorphosis and settlement, and they support the contention that HS function as a nutrient and energy store for the non-feeding peri-metamorphic period (Ramofafia et al., 2003). Furthermore, the mean length of 21 day post-settled larvae fed TW 1200® (1.19 ± 0.14 mm) was around double that of larvae fed Isochrysis 1800® (0.59 ± 0.13 mm) and Pavlova 1800® (0.49 ± 0.07 mm) in the current study. This may simply reflect the more advanced rate of development in the former but may also reflect greater availability of reserves to fuel more rapid early post-settlement growth.

Knauer (2011) highlighted considerable differences between the nutritional values of diatoms and flagellates for *H. scabra* larvae, and reported that neither of the flagellates *Pavlova salina* nor *Isochrysis* (T-ISO), supported development of competent doliolaria; HS were either not present or poorly developed. Ramofafia et al. (2003) similarly reported that sandfish larvae did not complete development when fed *Isochyrsis* aff. *galbana.* Our results, in contrast, show that both Pavlova 1800® and Isochrysis 1800® supported normal HS and larval development in sandfish, and success through settlement and metamorphosis. Both Pavlova 1800® and Isochrysis 1800® were appropriate foods for sandfish larvae when used as the sole nutrient source in the current study. The reason for this discrepancy between studies is unclear although both Ramofafia et al. (2003) and Knauer (2011) used live micro-algae cultures as the larval food source. Cultured live micro-algae is known to vary in its nutrient content according to culture conditions and age, and is prone to contamination with bacteria and protozoans (Coutteau, 1996; Knauer & Southgate, 1999) which may inadvertently affect the health and/or development of recipient larvae.

In summary, our results have confirmed that for sandfish larvae, the presence and size of HS at the end of the auricularia stage, is indeed a reliable indicator of subsequent performance. Our results have also confirmed that HS formation and size is significantly influenced by the nutrient composition of the auricularia diet, and that there are significant positive correlations between HS formation and dietary levels of carbohydrate, EPA: DHA and EPA: ARA ratios. This information provides a strong basis for development of more appropriate diets for sandfish larvae that will improve HS formation and larval performance during hatchery culture and in turn improve hatchery production of this species. Further research may address the effects of varying levels of key dietary nutrients on HS formation in sandfish larvae with the aim of determining optimal levels.

CHAPTER 6

THE NUTRITIONAL VALUE OF LIVE AND CONCENTRATED MICRO-ALGAE FOR EARLY JUVENILES OF SANDFISH, *HOLOTHURIA SCABRA*[5]

6.1. Introduction

Increasing global demand for dried sea cucumber or 'bêche-de-mer' has resulted in major declines in natural sea cucumber stocks in many countries in the Asia-Pacific region (Jimmy et al., 2012; Mills et al., 2012). Although some sea cucumber fisheries have existed for centuries, they generally follow 'boom-and-bust' patterns of exploitation (Anderson et al., 2011; Hair et al., 2016a) and this has stimulated growing interest in the development of techniques for hatchery propagation, mariculture and stock enhancement of sea cucumbers in a number of countries (Purcell et al., 2012; Hair et al., 2016b). This interest has been particularly focused on sandfish, *Holothuria scabra*, which is high value species among tropical sea cucumbers (Robinson, 2013).

Hatchery culture of sandfish has been conducted in several countries, including Vietnam, India, Australia, Philippines, New Caledonia and other Pacific islands, and in eastern Africa (Agudo, 2006; Bowman, 2012; Duy, 2010; James et al., 1994; Juinio-Meñez et al., 2012; Pitt and Duy, 2004; Robinson, 2013). However, limited knowledge of the nutritional requirements of larvae and post-larvae has hindered development and hatchery production of sandfish, which is characterised by poor survival to the juvenile stage (Purcell et al., 2012). Methods for growing and feeding newly-settled sea cucumbers have generally been adopted from those used for gastropods like abalone (Battaglene, 1999). Generally, benthic diatoms (e.g. *Nitzschia* spp., *Navicula* spp.) are used to condition settlement plates, providing both a settlement induction cue and a supplemental food source for post-settlement larvae (Agudo, 2006). Although benthic diatoms and materials such as dry algae, seaweed powder and even shrimp starter feeds are commonly used as a food source for *H. scabra* juveniles during hatchery production (Pitt

[5] Published as: Duy, N.D.Q., Francis, D.S., Southgate, P.C., 2016. The nutritional value of live and concentrated micro-algae for early juveniles of sandfish, *Holothuria scabra*. *Aquaculture* 473, 97-104.

and Duy, 2004a), the relative efficacy of these foods is not well understood (Pitt and Duy, 2004a; Watanabe et al., 2012a). Micro-algae such as *Chaetoceros muelleri* and *Skeletonema* spp. have been used as a food for early juveniles of sandfish (Duy, 2012; Juinio-Meñez et al., 2012) but little research has examined the effect of individual micro-algae species on growth and survival of early post-settled juveniles. As a result, our knowledge of key dietary components for early juvenile sea cucumbers is scant and this has hindered development of more appropriate diets for this stage of culture.

Determining relationships between dietary nutrient composition and juvenile growth and survival is essential to optimise feeding protocols that, in turn, support development of more appropriate hatchery procedures. An important part of this is to generate an understanding of the relative rates of ingestion and digestion of particular diets that provide information on nutrient availability and the subsequent nutritional value of a diet. Appropriate diets should be easily ingested, readily digested and should have an appropriate nutritional composition. Although these factors have been used to assess the suitability of diets for sandfish larvae (Chapter 3 and Chapter 4), there is a lack of such information for early juveniles of this species. Early juvenile culture of sandfish is considered a bottleneck to further large-scale development of sandfish mariculture (Purcell et al., 2012). This is due, in part, to relatively slow growth rates of early juveniles to a size at which handling and husbandry is easier. Along with development of more appropriate husbandry measures, more appropriate nutrition for early juvenile sandfish is likely to improve growth rates and contribute towards addressing this issue. A greater understanding of the relationships between the nutritional composition of foods and the resulting growth and survival of early juvenile sandfish is therefore critically important for future studies in this field.

Recent research has reported successful replacement of live cultured micro-algae with commercially available micro-algae concentrates as a food source during hatchery culture of invertebrate larvae (Reed and Henry, 2014; Southgate et al., 2016; Wassnig and Southgate, 2016), including those of sandfish (Chapter 4 and Chapter 5). Micro-algae concentrates are readily ingested and their cell walls efficiently digested by sandfish larvae (Chapter 3), supporting high rates of growth and survival through settlement when used as the sole food source (Chapter 4 and Chapter 5). The same products also supported good growth rates and high survival of newly settled juveniles (Chapter 4), indicating potential for the use of such products to simplify feeding regimes for early juvenile sandfish. In this study we assessed the

relative ingestion and cell wall digestion of selected micro-algae concentrates and live micro-algae for early juveniles of sandfish. The most highly digestible of these were then assessed for their nutritional value for juvenile sandfish in a subsequent growth trial. Nutrient analysis of these micro-algae allowed investigation of relationships between nutrient composition and juvenile performance.

6.2. Materials and methods

6.2.1. Micro-algae

Live TISO and *C. muelleri* were cultured in 20 L carboys, using f/2 medium (Guillard, 1975) in a temperature controlled (28°C) laboratory with a 16L: 8D photoperiod. Micro-algae were harvested during the exponential growth phase as a larval food source. Commercially available micro-algae concentrates (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) were purchased from an Australian distributor of the products. Six Instant Algae® products were used: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured *Pavlova* sp. (Haptophyceae) (Pavlova 1800®); (3) mono-cultured *Tetraselmis* sp. (Chlorophycophyceae) (Tetraselmis 3600®); (4) mono–cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®); (5) mono-cultured *Thalassiosira pseudonana* (Bacillariophyceae) (3H 1800®); and (6) a mixture of four micro-algae species: *Isochrysis* sp., *Pavlova* sp. *Thalassiosira pseudonana* and *Tetraselmis* sp. (Shellfish Diet 1800®).

Concentrates were stored in their original bottles in a refrigerator at 4ºC for the duration of the study. Prior to use, a 1 mL aliquot of each concentrate was added to approximately 1 L of 1-µm filtered sea water (FSW) in a clean plastic bottle and gently hand-shaken to disperse the micro-algae cells. The resulting micro-algae suspension was poured through a 90 µm mesh screen to remove any clumps before use. The cell density in each micro-algal stock suspension was then determined using a haemocytometer and the volume needed to obtain the required ration in each larval aquarium was calculated. The micro-algae used in this study and their characteristics are shown in Table 6.1.

6.2.2. Biochemical analysis of micro-algae

Proximate analysis was conducted using standard procedures (AOAC, 1990). Percentage moisture of the Instant Algae® products was determined after freeze-drying to a constant

weight; protein (Kjeldahl nitrogen; N x 6.25) was determined using an automated Kjeltech (model 2300, Tecator, Sweden); total lipid was determined gravimetrically following dichloromethane/methanol extraction (2:1 v/v) (Folch et al., 1957), and ash weight was determined following incineration in a muffle furnace (model WIT, C & L Tetlow, Australia) at 550 °C for 18 h.

Table 6.1. Live micro-algae and micro-algae concentrates (Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA, 95008) used in this study.

Group (Division)	species/diet	Cell size (µm)	Status
Golden - Brown Flagellates (Haptophyta)	*Isochrysis aff. galbana* (TISO)	5-7	Live
		5-7	Concentrated
	Isochrysis sp. (Isochrysis 1800®)		
	Pavlova sp. (Pavlova 1800®)	4-7	Concentrated
Green Flagellates (Chlorophyta)	*Tetraselmis* sp. (Tetraselmis 3600®)	10-12	Concentrated
Diatoms (Bacillariophyta)	*Chaetoceros muelleri*	5-6	Live
	Thalassiosira weissflogii (TW 1200®)	7-20	Concentrated
	Thalassiosira pseudonana (3H 1800®)	4-6	Concentrated
Golden - Brown Flagellates, Green Flagellate, Diatom (Haptophyta, Chlorophyta, Bacillariophyta)	Shellfish Diet 1800®	5-12	Concentrated

Nitrogen free extract (NFE) was calculated as that remaining following summation of moisture, lipid, protein and ash values. Energetic contents of micro-algae concentrates were estimated from proximate data using caloric equivalents of 4.0 cal. mg^{-1}, 4.5 cal. mg^{-1} and 9.4 cal. mg^{-1} for carbohydrate (NFE), protein and lipid, respectively (Marshall et al., 2010). A technical problem unfortunately prevented analysis of samples of *Chaetoceros muelleri*, and

representative proximate compositional values for this species were calculated as means from data reported by Martínez-Fernández et al. (2006), Rivero-Rodríguez et al. (2007) and Jamali et al. (2015).

Following lipid extraction, fatty acids were esterified into methyl esters using the acid-catalysed methylation method (Christie, 2003) following the protocol of Conlan et al. (2014). The purified hexane supernatants of the micro-algae samples were placed in a gas chromatography (GC) vial for GC injection. Fatty acid methyl esters were isolated and identified using an Agilent Technologies 7890 GC System (Agilent Technologies, USA) equipped with a BPX70 capillary column (120 m × 0.25 mm internal diameter, 0.25 mm film thickness, SGE Analytical Science, Australia), a flame ionization detector (FID), an Agilent Technologies 7693 auto sampler, and a split ratio of 50:1. The injection volumes, temperature sequences, and flow rates followed the protocol of Conlan et al. (2014). The carrier gas was helium. Individual fatty acids were identified relative to known external standards (a series of mixed and individual standards from Sigma-Aldrich, Inc., St Louis, USA and Nu-Chek Prep Inc., USA) using the software GC ChemStation (Rev B.04.03, Agilent Technologies). The resulting peaks were corrected by theoretical relative FID response factors (Ackman, 2002) and quantified relative to the internal standard C23:0 (0.75 mg mL^{-1}, Sigma-Aldrich, Inc., USA). Fatty acid compositional data used for *C. muelleri* was that reported by Martínez-Fernández et al. (2006).

6.2.3. Juvenile sandfish

Early juveniles of sandfish (*Holothuria scabra*) were sourced from the National Fisheries Authority (NFA), Nago Island Mariculture Research Facility (NIMRF) in Kavieng, Papua New Guinea that undertakes routine hatchery culture of this species (Hair et al., 2016a; 2016b). Larvae were cultured according to the methods described in Chapter 4 and Chapter 5. Five days after settlement, with a mean (± SD, n = 60) length of 1.23 ± 0.10 mm, juveniles were counted, washed gently with FSW and transferred to the 5-L aquaria used for experiments. Aquaria had a floor area of 314 cm^2 and juveniles were stocked at a density of 0.3 juvenile cm^{-2}. Water temperature in the aquaria was maintained at 27.5 ± 1.0 ºC and the seawater was gently aerated during experiments. Juveniles were unfed for 24 h prior to the start of each experiment.

6.2.4. Experiment 1 - Assessing ingestion of micro-alga

All micro-algae (live and concentrated, Table 6.1) were fed to juveniles at a daily density of 40,000 cells mL^{-1} based on that recommended for late auricularіae stage sandfish larvae by Pitt (2001). Juveniles were fed for 24 h, when five randomly chosen individuals were removed from each aquarium, placed into petri-dishes and examined using a compound microscope to determine whether micro-algae had been ingested. Since ingested micro-algae cannot be seen through the body wall of juveniles, ingestion was simply assessed by the production of faeces. Juveniles were observed for faeces production and the time taken between their removal from aquaria and faeces production was recorded for all individuals tested.

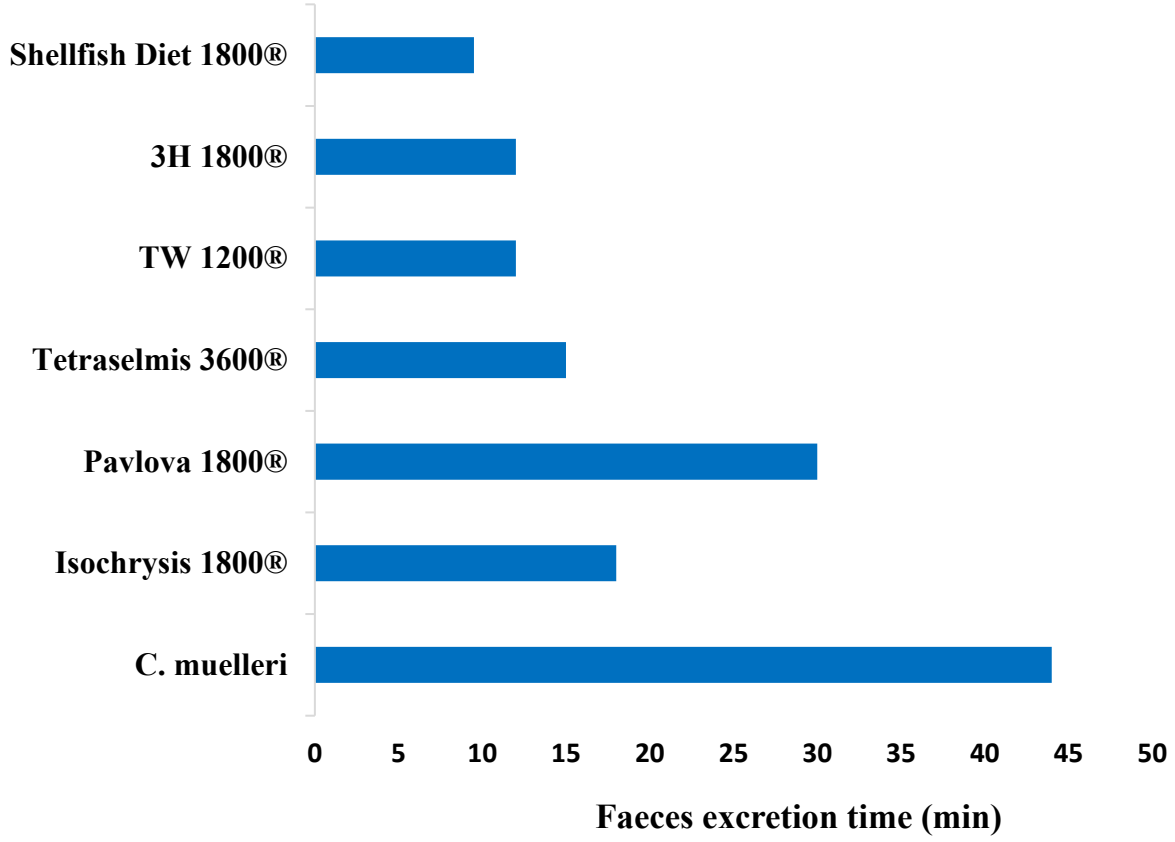


Figure 6.1. Faeces excretion time (min) of early juveniles of *Holothuria scabra* fed six commercial micro-algae concentrates and live *Chaetoceros muelleri* for 24 hours.

6.2.5. Experiment 2 - Assessing cell wall digestion of micro-algae

Based on the results of the ingestion trial, seven micro-algae were selected for assessment of their cell wall digestion by early juvenile sandfish; *C. muelleri*, Isochrysis 1800®, Pavlova 1800®, Tetraselmis 3600®, TW 1200®, 3H 1800® and Shellfish Diet 1800®. Cell wall digestion

of micro-algae was determined directly using epi-fluorescence microscopy, which has been used to describe ingestion and digestion of micro-algae by sandfish auricularia (Chapter 3) and allows intact micro-algae cells to be differentiated from lysed cells. Again, juveniles were fed for 24 h when 30 randomly chosen individuals were removed from each aquarium and placed into separate petri-dishes. After 1 hour, faeces from each petri-dish were collected and examined using both compound (yellow background) and epi-fluorescence microscopy (green background) to assess the cell wall digestion of micro-algae within the faeces. Cell wall digestion was ranked at three levels (high, medium and indigestible) depending on the proportion of cell walls remaining intact. Diets with medium and high cell wall digestion rates were further assessed for their relative nutritional values.

6.2.6. Growth and survival trial with selected digestible micro-algae for early juveniles

Based on the results of the cell wall digestion trial, five micro-algae diets were assessed for their nutritional value for early juvenile sandfish; *C. muelleri*, Isochrysis 1800®, Pavlova 1800®, TW 1200®, and a ternary combination composed of a mixture (equal dry weight) of Isochrysis 1800®, Pavlova 1800® and TW 1200®. All micro-algae were assessed against an unfed control treatment. Shellfish Diet 1800®, used in the cell wall digestion trial, was replaced by the ternary diet in this growth trial because *Thalassiosira pseudonana* and *Tetraselmis* sp. are major components of Shellfish Diet 1800® but both were undigested by early juvenile sandfish.

Juveniles fed *C. muelleri* again received a daily ration of 40,000 cells mL^{-1} and, because the micro-algae used in this study vary in cell size, daily rations of other micro-algae were varied so that each presented the same dry weight ration (Knauer, 2011; Chapter 4). Thus, Isochrysis 1800®, Pavlova 1800® and TW 1200® were fed at daily rations of 28,000, 29,000 and 10,990 cells mL^{-1}, respectively. There were three replicate aquaria per treatment and each received a 100% daily water exchange. The growth trial was terminated after two weeks. Survival was determined by counting the number of live juveniles in each aquarium and means were calculated for each treatment. Growth during the experiment was determined by measuring the lengths of 10 randomly selected juveniles from each aquarium using a dissecting microscope and micrometer, before calculating treatment means.

6.2.7. Statistical analyses

Growth and survival of early juveniles were compared between treatments using one-way ANOVA followed by Tukey's multiple range test to evaluate all pair-wise treatment comparisons ($P < 0.05$). Pearson's correlation test was used to identify key correlations between the levels of specific nutrients in the micro-algae diets (total lipid, NFE, protein, total saturated, monounsaturated and polyunsaturated fatty acids, and specific fatty acids) and juvenile performance (growth and survival). Statistical analyses were conducted using the software IBM SPSS VERSION 22.0.

6.3. Results

6.3.1. Biochemical compositions of micro-algae

Table 6.2. Proximate compositions (mg g^{-1} dry weight) and energy contents (cal. g^{-1} dry weight) of micro-algae concentrates used in a 14-day growth trial with early juvenile *Holothuria scabra*. Values are means ± SE (*n=2*) and means within columns with different superscripts are significantly different ($P < 0.05$).

Micro-algae	Lipid	Protein	NFE	Ash	Energy
Isochrysis 1800®	95.36 ± 2.09^{b}	255.22 ± 0.77^{b}	317.02 ± 0.36^{b}	332.26 ± 1.01^{c}	3.66 ± 0.01^{a}
Pavlova 1800®	75.71 ± 0.00^{c}	247.76 ± 0.75^{c}	295.21 ± 2.26^{c}	381.32 ± 3.02^{b}	3.34 ± 0.01^{ab}
TW 1200®	44.37 ± 0.57^{d}	208.17 ± 0.53^{e}	332.69 ± 1.77^{a}	414.80 ± 0.64^{a}	2.97 ± 0.00^{b}
Ternary diet	71.82 ± 0.88^{c}	237.05 ± 0.33^{d}	314.98 ± 1.47^{b}	376.16 ± 0.89^{b}	3.33 ± 0.01^{ab}
C. muelleri[1]	131.80 ± 15.88^{a}	399.20 ± 32.22^{a}	89.46 ± 34.99^{d}	379.53 ± 7.83^{b}	3.39 ± 0.12^{a}

[1]Values are means (± SE) of data from Martínez-Fernández et al. (2006), Rivero-Rodríguez et al. (2007) and Jamali et al. (2015).

The proximate compositions of the micro-algae diets used in this study are shown in Table 6.2. Lipid content was highest in *C. muelleri* (131.80 ± 15.88 mg g^{-1} dry weight), followed by Isochrysis 1800® (95.36 ± 2.09 mg g^{-1} dry weight) and was lowest in TW 1200® (44.37 ± 0.57 mg g^{-1} dry weight). This pattern was similar for protein contents that ranged from 399.20 ± 32.22 mg g^{-1} dry weight to 208.17 ± 0.53 mg g^{-1} dry weight.

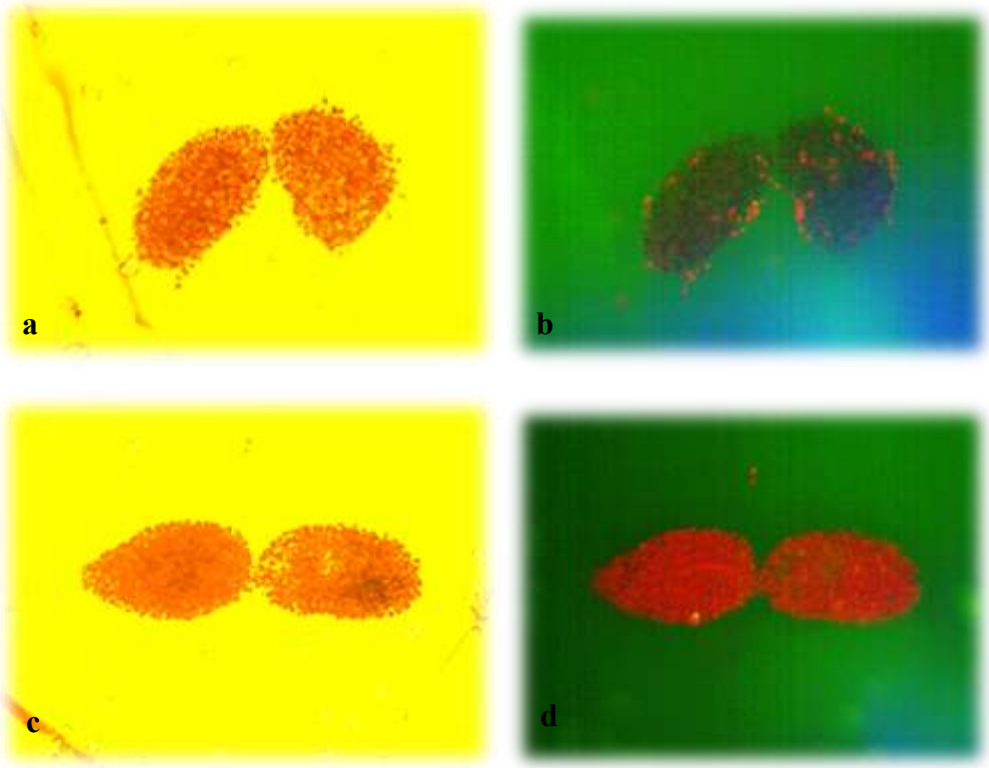
However, the NFE content of *C. muelleri* was lowest (89.46 ± 34.99 mg g^{-1} dry weight) while that of TW 1200® (332.69 ± 1.77 mg g^{-1} dry weight) was significantly higher than that of the ternary diet (314.98 ± 1.47 mg g^{-1} dry weight) and Isochrysis 1800® and Pavlova 1800® (317.02 ± 0.36 and 295.21 ± 2.26 mg g^{-1} dry weight, respectively). Isochrysis 1800® had the highest calculated energy content (3.66 ± 0.01 cal. g^{-1} dry weight) while TW 1200® had the lowest (2.97 ± 0.00 cal. g^{-1} dry weight) (Table 6.2).

Table 6.3. Fatty acid profiles (μg g^{-1} dry weight) of the micro-algae concentrates used in this study.

Fatty acid	Isochrysis 1800®	Pavlova 1800®	TW 1200®	Ternary diet	*C. muelleri*[1]
14:0	1084.74	2094.66	420.75	1200.05	667.70
16:0	1269.44	1289.87	2137.39	1565.56	327.78
16:1n-7	768.17	1979.44	1700.17	1482.59	1274.70
18:1n-9	2329.65	233.90	177.03	913.53	72.84
22:1 (isomers)	358.00	59.74	175.22	197.66	-
18:2n-6	1520.63	178.90	83.85	594.46	60.70
18:3n-6	301.59	21.35	36.17	119.70	48.56
20:4n-6 (ARA)	95.29	117.59	49.20	87.36	169.96
22:4n-6	20.49	1522.63	90.61	544.57	-
22:5n-6	394.70	1051.58	204.20	550.16	-
18:3n-3	1207.23	216.64	31.50	485.12	-
18:4n-3	2830.12	1277.91	67.03	1391.68	36.42
20:5n-3 (EPA)	204.44	3478.89	2085.01	1922.78	1262.56
22:6n-3 (DHA)	2511.36	965.63	301.09	1259.36	60.70
SFA	2836.14	3738.63	3044.45	3206.41	1080.46
MUFA	3883.17	2586.86	2537.34	3002.45	1481.08
PUFA	9330.88	9146.81	4298.24	7591.98	1638.90
n-3 PUFA	6865.76	6001.62	2692.61	5186.66	1359.68
n-6 PUFA	2401.83	2963.62	464.03	1943.16	279.22
n-3 LC PUFA	2828.41	4507.08	2594.09	3309.86	1323.26
n-6 LC PUFA	540.73	2691.80	344.01	1192.18	169.96

[1]Data from Martínez-Fernández et al. (2006).

The major component fatty acids of the micro-algae used in the growth trial are shown in Table 6.3. Myristic acid (14:0) and palmitic acid (16:0) were the most abundant saturated fatty acids (SFA) in both live and concentrated micro-algae. The level of myristic acid was much lower in TW 1200® (420.75 μg g^{-1} dry weight) and *C. muelleri* (667.70 μg g^{-1} dry weight) than in Pavlova 1800® (2094.66 μg g^{-1} dry weight) and Isochrysis 1800® (1084.74 μg g^{-1} dry weight) while *C. muelleri* had the lowest palmitic acid content (327.78 μg g^{-1} dry weight).



Figur 6.2. Photographs of faeces from *Holothuria scabra* early juveniles after feeding on TW 1200® (a, b) and Tetraselmis 3600® (c, d) when observed using both compound (yellow background) and epifluorescence (green background) microscopy. Photosynthesizing pigments of intact micro-algae cells in the faeces fluoresce under blue-light illumination; (b) shows high cell wall digestibility with few intact cells in the faeces and (d) shows low cell wall digestibility with mainly intact cells in the faeces. Individual faecal packets are 240-250 µm in length.

C. muelleri had the highest levels of arachidonic acid (ARA, 20:4n-6) (169.96 µg g^{-1} dry weight), followed by Pavlova 1800® (119.59 µg g^{-1} dry weight) while Isochrysis 1800® had the lowest level of eicosapentaenoic acid (EPA, 20:5n-3) (204.44 µg g^{-1} dry weight). However, Isochrysis 1800® had the highest level of of docosahexaenoic acid (DHA, 22:6n-3) (2511.36 µg g^{-1} dry weight) while that of *C. muelleri* was the lowest (60.70 µg g^{-1} dry weight). TW 1200® had relatively low levels of ARA and DHA compared to the two flagellates, and considerably lower levels of total polyunsaturated fatty acids (PUFA), n-3 PUFA and n-6 PUFA (Table 6.3).

Table 6.4. Relative digestibility (high, medium or indigestible) of seven micro-algae diets after feeding to early juveniles of *Holothuria scabra* for 24 hours. Cells were observed using both compound (yellow background) and epifluorescence microscopy (green background) to assess the level of relative digestibility (see Figure 6.2).

Diets	Digestibility level	Observations
C. muelleri	high	Mostly lysed algal cells found in faeces
Isochrysis 1800®	medium	Intact algal cells and lysed algal cells mixed in the faeces
Pavlova 1800®	medium	Intact algal cells and lysed algal cells mixed in the faeces
Tetraselmis 3600®	indigestible	Mostly intact algal cells in the faeces (Figure 6.2)
TW 1200®	high	Mostly lysed algal cells found in the faeces (Figure 6.2)
3H 1800®	indigestible	Mostly intact algal cells in the faeces
Shellfish Diet 1800®	medium	Intact algal cells and lysed algal cells mixed in the faeces

6.3.2. Ingestion and cell wall digestion of micro-algae

All micro-algae diets, with the exception of live T-ISO, were ingested by newly settled early sandfish juveniles. The faeces excretion times after removal from aquaria varied between ingested diets with juveniles fed the Shellfish Diet 1800® having the shortest excretion time of 9.5 min while those fed live *C. muelleri* had the longest excretion time of 44 min (Figure 6.1). Cell wall digestion by juvenile sandfish was evident in five of the ingested diets (TW1200®, live *C. muelleri*, Pavlova 1800®, Isochryis 1800® and Shellfish 1800®), while cell walls in Tetraselmis 3600® and 3H 1800® were not digested (Table 6.4). Figure 6.2 illustrates differences between faeces excreted by juveniles fed TW 1200® and that excreted by juveniles fed Tetraselmis 3600® when observed using epifluorescence microscopy. Photosynthesising pigments of intact micro-algae cells in the faeces fluoresce under the blue-light illumination of the epi-fluorescence microscope while micro-algae with digested cell walls do not. It is clear that there are few intact cells in the faeces of juveniles fed TW 1200® (Figure 2b), while the majority, if not all cells in the faeces of juveniles fed Tetraselmis 3600® appear to be intact (Figure 2d).

Table 6.5. Mean (± SE, *n*=3) final length (mm) and survival (%) of early juveniles fed five different micro-algae for two weeks. Means within columns with different superscripts are significantly different (P < 0.05).

Diet	Length (mm)	Survival (%)
Isochrysis 1800®	2.99 ± 0.06^{d}	67.00 ± 3.79^{ab}
Pavlova 1800®	3.20 ± 0.06^{c}	67.33 ± 1.86^{ab}
TW 1200®	3.49 ± 0.05^{b}	78.33 ± 1.20^{a}
Ternary diet	3.46 ± 0.02^{b}	68.33 ± 6.06^{ab}
C. muelleri	4.10 ± 0.03^{a}	79.33 ± 6.12^{a}
Control (unfed)	1.54 ± 0.04^{e}	58.33 ± 3.71^{b}

6.3.3. Growth and survival trial with selected digestible micro-algae for early juveniles

Table 6.6. Pearson's correlations between levels of dietary component of micro-algae used in this study and survival and growth of early juvenile *Holothuria scabra*.

Nutrient component	Survival	Growth
Protein	0.433	0.714*
Lipid	0.232	0.499
NFE	-0.51	-0.799**
Total energy	-0.362	-0.277
SFA	-0.487	-0.781**
MUFA	-0.583	-0.885**
PUFA	-0.732*	-0.937**
ARA (20:4n-6)	0.119	0.481
DHA (22:6n-3)	-0.644*	-0.827**
EPA (20:5n-3)	-0.024	-0.011
EPA: DHA	0.651*	0.901**
EPA: ARA	0.231	0.004
16:0	-0.09	-0.466
18:4n-3	-0.686*	-.0.830**

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Survival and final mean lengths of early juveniles fed digestible micro-algae are shown in Table 6.5. Survival during the experiment ranged from 58.33 ± 3.7 % for unfed juveniles to 79.33 ± 6.1 % for those fed *C. muelleri*; however, there were no significant differences in juvenile survival between any of the micro-algae treatments (Table 6.5). Juveniles fed all

micro-algae diets tested (*C. muelleri*, Isochrysis 1800®, Pavlova 1800®, TW 1200® and the ternary diet) had significantly greater mean length than unfed controls at the end of the growth trial. Mean length was greatest for juveniles fed *C. muelleri* (4.10 ± 0.03 mm) while mean lengths of juveniles fed TW 1200® and the ternary diet (3.49 ± 0.05 and 3.46 ± 0.02 mm, respectively) were the highest among those fed concentrated micro-algae (Table 6.5). This difference between juveniles fed live *C. muelleri* and those fed the micro-algae concentrates was significantly different ($P < 0.05$). Mean (± SE) increases in the length of juveniles during the experiment ranged from 0.32 ± 0.03 µm for unfed juveniles to 2.90 ± 0.04 µm for those fed *C. muelleri* (Figure 6.3). Juveniles fed TW 1200® increased in length by 2.27 ± 0.06 µm during the growth trial (Figure 6.3) and performed the best of the four treatments receiving the micro-algae concentrates.

6.3.4. Relationships between nutrient composition of micro-algae and growth and survival of early juveniles of sandfish

Relationships between nutrient compositions of micro-algae and growth and survival of early juvenile sandfish are shown in the Table 6.6. Early juvenile length at the end of the growth trial was positively correlated with dietary levels of crude protein ($R = 0.714$, $P < 0.05$) and EPA: DHA ratio ($R = 0.901$, $P < 0.01$). Early juvenile survival was only positively, and significantly, correlated with dietary EPA: DHA ratio ($R = 0.651$, $P < 0.05$). In contrast, there were significant negative correlations between early juvenile growth and dietary NFE ($R = -0.799$, $P < 0.01$), total SFA ($R = -0781$, $P < 0.01$), total MUFA ($R = -0885$, $P < 0.01$) and total PUFA ($R = -0.937$, $P < 0.01$) contents.

Correlations between juvenile length and dietary lipid ($R = 0.499$) and energy ($R= -0.277$) contents were not significant and the same relationships held true for juvenile survival. Similarly, there were no significant correlations between juvenile growth or survival and dietary levels of specific fatty acids including ARA ($R = 0.481$ and 0.119, respectively), EPA ($R = -0.011$ and -0.024, respectively), EPA: ARA ratio ($R = 0.004$ and 0.231, respectively) and palmitic acid ($R = -0.466$ and -0.09, respectively).

6.4. Discussion

This study is the first comprehensive investigation of the nutritional values of various micro-algae for early juvenile sandfish, *H. scabra*. Cell wall digestion of these diets was measured directly using epi-fluourecense microcopy, a method previously used to determine ingestion

and cell wall digestion of various micro-algae by sandfish larvae (Chapter 3). The method is relatively simple and well suited to provisional assessment of different micro-algae diets, prior to more exhaustive growth trials (Chapter 3 and Chapter 4), and this was the approach used in the current study. Early sandfish juveniles grew at a faster rate when fed diets with greater rates of cell wall digestion (e.g. *C. muelleri* and TW 1200®) and showed reduced growth rates when fed diets with lower rates of cell wall digestion (e.g. Isochrysis 1800® and Pavlova 1800®).

This study is the first to use epifluorescence microscopy as a direct method for assessing cell wall digestion of micro-algae by sea cucumber juveniles, allowing a simple visible assessment of relative digestion of different micro-algae diets. Using this technique, the photosynthetic pigments of micro-algae cells fluoresce with blue-light illumination, permitting the identification of ingested cells and the subsequent assessment of cell wall digestion via a presence/ absence of fluorescence (Chapter 3). Few prior studies have investigated diet digestion or ingredient digestibility by sea cucumber juveniles. Slater et al. (2011) used inert markers (acid-washed sand and chromic oxide) within artificial diets to determine apparent digestibility of various carbohydrate and protein sources by *Australostichopus mollis* juveniles (20-40 g). Acid-washed sand was again used as a dietary marker, demonstrating superior apparent digestibility of animal-based nutrients compared to plant-based nutrients in diets fed to *H. scabra* juveniles (Orozco et al., 2014).

Chapter 3 reported that seven of eight micro-algae tested (two live micro-algae: TISO and *C. muelleri*; and five micro-algae concentrates: Isochrysis 1800®, Pavlova 1800®, Tetraselmis 3600®, TW 1200® and Shellfish Diet 1800®) were ingested and digested by auriculariae of *H. scabra* with cell wall digestion occurring more rapidly in older larvae. For example, *Chaetoceros muelleri* was found to be rapidly digested by 6-day and 10-day old larvae but was unsuitable as a food source for 2-day old sandfish larvae (Chapter 3). In the present study, however, the cell walls of *C. muelleri* demonstrated the greatest rate of digestion of the micro-algae tested for early juvenile sandfish. *Chaetoceros muelleri* was also the most nutritious of the micro-algae tested and supported the greatest growth and survival at the end of the growth trial among treatments. *Chaetoceros muelleri* has been reported to be a highly nutritious micro-alga for *H. scabra* in prior studies and has been recommended as a monospecific diet during the hatchery culture of this species (Duy, 2012; Knauer, 2011; Juinio-Meñez et al, 2012). Use of micro-algae concentrates, in place of cultured live micro-algae, greatly simplifies the hatchery procedure for sea cucumbers (Chapter 4 and Chapter 5), but unfortunately, neither *C.*

muelleri nor any other species of *Chaetoceros*, is currently available in concentrated form. Beside TW 1200® (*Thalassiosira weissflogii*), another diatom currently available in concentrated form is *Thalassiosira pseudonana* (3H 1800®). However, although ingested, the cell walls of 3H 1800® were not well digested by *H. scabra* early juveniles in the present study, while prior research has shown that 3H 1800® was not ingested by *H. scabra* auricularіae (Chapter 3). In contrast, the cell walls of TW 1200® were highly digested by *H. scabra* early juveniles and was the most nutritious of the micro-algae concentrates tested. Similar results with *H. scabra* larvae (Chapter 3 and Chapter 4) indicate high nutritional value of TW 1200® for both larvae and early juveniles of *H. scabra*.

An understanding of feeding behavior is critically important in selecting an appropriate diet, or food, for sea cucumber juveniles during hatchery culture. At the settlement stage sandfish larvae become grazers that forage detrital organic matter from substrates (Lopez, 1987). Adherence of food particles to tentacle nodules, following secretion of an adhesive substance at their surface, is the most important method of food collection by sea cucumber juveniles (Cameron and Fankboner, 1984). Extended tentacles are placed against the substrate and attached detrital particles are then drawn into the mouth. The results of this study confirm the feasibility using micro-algae as a food source for early juveniles of sandfish, with the exception of TISO which was not ingested. TISO is a golden-brown flagellate and its motility, when live, likely resulted in few cells settling to the aquarium floor where they could be ingested. Cells of Isochrysis 1800®, in contrast, are intact but non-motile and this facilitated settlement of cells and their ingestion by sandfish juveniles. Unlike live TISO, live *C. muelleri* is immotile and readily settled onto the floor of aquaria where it was easily captured and ingested by the early juveniles.

Survival and growth of *H. scabra* juveniles in this study was not significantly correlated to dietary lipid content, or to dietary SFA, MUFA or long chain PUFA (LC-PUFA) content. Much of the research on diet quality for larval echinoids has raised the importance of the ratio between specific LC-PUFA to larval development. For example, higher dietary EPA:DHA and EPA:ARA ratios have been generally associated with improved performance of the larvae of sea urchins *Paracentrotus lividus* and *Psammechinus miliaris* (Carboni et al., 2012; Liu et al., 2007a, 2007b). Similarly, Chapter 4 and Chapter 5 reported that a higher dietary EPA: DHA ratio supported significantly better performance of sandfish larvae, including superior hyaline sphere development in auricularіae, improved competence of doliolariae and better settlement

success. The results of this study also confirm that improved performance of sandfish juveniles is associated with a relatively high dietary EPA: DHA ratio, where both juvenile length and survival at the end of the growth trial were positively and significantly correlated with dietary EPA: DHA ratio.

Our results showed a strong correlation between dietary protein level and performance of early sandfish juveniles. Prior studies have reported on the nutrient requirements and the efficacy of various feed ingredients and food sources for juveniles of a number of sea cucumber species including *Apostichopus japonicus* (Gao et al., 2011; Liu et al., 2009; Seo & Lee, 2011; Seo et al., 2011; Zhou et al., 2006), *A. mollis* (Maxwell et al., 2009; Slater et al., 2009; Slater et al., 2011; Zamora & Jeffs, 2011) and *H. scabra* (Oroco et al., 2014), all demonstrating the importance of organic matter, protein and carbohydrate for growth. Sun et al. (2004) and Oroco et al. (2014) reported that the growth rate and digestion efficiency of cultured *A. japonicus* and *H. scabra* juveniles, respectively, increase with dietary protein content. Similarly, mixed commercial dried algal diets (Algamac 2000 and Algamac Protein Plus fed at a 1:1 ratio) with high protein contents of 39.0-42.9% supported better growth rates and survival in *H. scabra versicolor* (Giraspy and Ivy, 2008). Moreover, research with *Apostichopus japonicus* (Tang et al., 2007) found that protease activity decreased from the auricularia to doliolaria larval stages, but then increased during the later stages of larval development in the pentactula stage; protease activity was higher in juveniles than in the larvae, potentially indicating increased importance of dietary protein in juveniles. A similar situation may occur in *H. scabra* as prior research in this laboratory has shown that the performance of *H. scabra* auriculariae was not significantly (or positively) correlated with dietary protein content, and that dietary carbohydrate was a more important dietary component for auriculariae (Chapter 4 and Chapter 5). However, the results of the current study clearly indicate increased importance of dietary protein in *H. scabra* juveniles. High dietary carbohydrate content supported rapid growth of *Stichopus (Apostichopus) japonicus* and *A. mollis* juveniles (Slater et al., 2009; Zhou et al., 2006), and similar results were reported for *H. scabra* juveniles by Orozco et al. (2014). In contrast, the results of the present study indicate a highly significant negative relationship between early juvenile performance of *H. scabra* and dietary carbohydrate content. An explanation for these differences is currently unclear, particularly considering the strong positive correlation between dietary carbohydrate content and the formation of hyaline spheres in sandfish larvae, and subsequent correlation between hyaline sphere development and settlement success and post-settled larval size (Chapter 5). It is possible that the discrepancy between the results reported by

Orozco et al. (2014) and this study relate to the size of the juveniles used; animals used here were considerably smaller (1.23 ± 0.10 mm) than those of 4.89-19.76 g used by Orozco et al. (2014) that would have estimated lengths of 3-5.5 cm based on the data of Pitt and Duy (2004b).

Although previous nutritional studies have been conducted with *H. scabra* juveniles, this is the first to investigate nutrition of newly settled juveniles of this species. The juveniles used in this study were considerably smaller than those used in similar research with *H. scabra*, such as those of 4.89-19.76 g used by Orozco et al. (2014) and those of 2.2-8.3 g and 20 cm in length used by Watanabe et al. (2012a; 2012b). They were also smaller than the juveniles of other species of sea cucumbers used in similar nutritional studies (e.g. Gao et al., 2011; Liu et al., 2009; Seo and Lee, 2011; Seo et al., 2011; Zhou et al., 2006). Sandfish larvae show preferential settlement onto seagrass where they remain for 4-5 weeks before migrating to sand at a length of around 6 mm to become deposit-feeders (Mercier et al., 2000). In culture systems, conditioned settlement plates and similar surfaces provide appropriate substrates for newly settled sandfish, where they remain until they reach a size of around 1-2 g when they are transferred to culture systems containing sand (Duy, 2010). On this basis, it is likely that the feeding behaviour of the animals used in the present study differed from that of the larger individuals used in prior studies. The suitability of the experimental system used in the present study is demonstrated by the relatively high rates of survival of juveniles across treatments which ranged from 67-79%. An important contributing factor to high survival in this study may be daily complete water exchanges carried out on culture vessels during the experiment. This protocol resulted in no contamination of cultures by predators and food competitors (i.e. ciliates and copepods) which can have a major influence on mortality of early juveniles in culture (Duy, 2010; Mills et al., 2012).

This study investigated ingestion, cell wall digestion and nutritional value of both live micro-algae and commercially available micro-algae concentrates for early juvenile sandfish, *H. scabra*. Results showed that live *C. muelleri* supported superior juvenile performance to three micro-algae concentrates fed either singly or in combination. Although *C. muelleri* is unsuitable as a food for 2-day old sandfish larvae (Chapter 3), it has high nutritional value for older *H. scabra* larvae (Duy, 2012; Juinio-Meñez et al, 2012) and, as demonstrated in the present study, for early juveniles of this species. Our results also highlight the potential application of micro-algae concentrates in hatchery culture of *H. scabra*. All micro-algae concentrates used in this study were ingested and their cell walls digested by *H. scabra* larvae,

supporting larval development through settlement when used as a sole food source (Chapter 3 Chapter 4 and Chapter 5). The three micro-algae concentrates (Isochrysis 1800®, Pavlova 1800® and TW 1200®) used successfully with *H. scabra* larvae (Chapter 4) were also shown to support good rates of growth and survival of juveniles in this study. These results demonstrate that micro-algae concentrates can be used as the sole food source for both larvae and early juveniles of *H. scabra* and, of those assessed in this study and in previous studies investigating sandfish larvae, TW 1200® was found to be the most nutritious for *H. scabra* larvae and early juveniles (Chapter 4 and Chapter 5). As such, the results of this study provide a basis for developing more simplified hatchery culture methods for *H. scabra* that do not require the culture of live micro-algae and are therefore more appropriate for regional hatcheries that often lack the technical resources and skilled personnel required to produce appropriate quantities of high quality live micro-algae.

CHAPTER 7

GENERAL DISCUSSION

7.1. Introduction

This project used both live and concentrated micro-algae to investigate the influences of micro-algal diets and diet composition on growth, development and survival of sandfish, *Holothuria scabra,* larvae and early juveniles. The overall objective of this study was to investigate the possibility of replacing live micro-algae with commercially available micro-algae concentrates for sandfish larvae and early juveniles, with a view to simplification of hatchery culture methods for this species. This objective was addressed through a number of experiments that assessed the efficacy of commercially-available micro-algae concentrates, including their ingestion and digestion, and their relative nutritional values. The major outputs of this study and their relevance and potential application, are summarised in Table 7.1 and described below.

7.2. Ingestion and digestion of live and concentrated micro-algae

Determination of whether a given micro-alga is ingested and digested by sandfish larvae is an important first step in assessing its suitability as a larval food source. This study is the first to use epifluorescence microscopy as a direct method for assessing ingestion and digestion of micro-algae by larval echinoderms. Prior studies with echinoderm larvae were conducted using compound microscopy to quantify ingested cells or food particles (e.g. George, 2006; Hart, 1991; Pedrotti, 1995; Schiopu et al., 2006; Strathmann, 1971); however, this method may result in errors in estimating ingested and/or digested micro-algae cells which may be difficult to discern within the larval body, and difficult to differentiate from larvae body parts; this is well illustrated in the Figures of this study (e.g. Figure 3.1 and Figure 3.2). Accordingly, the use of epifluorescence microscopy was considered to be a more accurate alternative method for assessing ingestion and digestion of micro-algae by sea cucumber larvae because the photosynthesising pigments of micro-algae cells fluoresce with blue-light illumination and this can be used to monitor ingestion and to differentiate various stages of their digestion. The results of this study confirm that epifluorescence microscopy methodology can be used successfully for sea cucumber larvae as it has been for the larvae of a number of other invertebrates (e.g. Aldana-Aranda et al., 1997; Lora-Vilchis and Maeda-Martinez, 1997; Martínez-Fernández et al., 2004; Patino-Suarez et al., 2004).

Table 7.1. Major outputs from this study and their applications.

Prior knowledge/practice	Outputs of this study	Application(s) of outputs
1. Poor knowledge of relative ingestion/digestion of micro-algae by sea cucumber larvae and early juveniles.	First assessment of the relative ingestion and digestion of micro-algae by sandfish larvae and early juveniles.	Identification of the most ingested and digestible Instant Algae® products as a basis for subsequent experiments in this study.
2. No prior use of epifluorescence microscopy in nutritional studies with sea cucumber larvae and juveniles.	Demonstration of the value of epifluorescence microscopy as a tool to investigate nutritional physiology of sea cucumber larvae and early juveniles. Knowledge of the relative ingestion and digestion of micro-algae by sandfish larvae and early juveniles.	Information on the relative ingestion and digestion of micro-algae, and on the use of epifluorescence microscopy that can benefit broader nutritional studies with sea cucumber larvae and early juveniles.
3. No prior assessment of the nutritional value of commercially-available micro-algae concentrates for larvae and early juveniles of sandfish.	First assessment of the nutritional value of micro-algae concentrates for sandfish larvae and early juveniles based on their nutrient compositions.	Information on the relative nutritional value of commercially-available micro-algae concentrates that can be used in product selection and for improved diet formulation for sandfish larvae and early juveniles. New information to inform diet selection for larvae and juvenile sandfish that will help improve performance and support improved hatchery production.
4. No prior knowledge of key dietary nutrients for larvae and early juveniles of sea cucumbers.	First assessment of the relationships between dietary nutrient composition and growth, development and survival of sea cucumber larvae and early juveniles. Identification of key dietary nutrients for sandfish larvae and early juveniles.	Identification of the key dietary nutrients for sandfish larvae and early juveniles provides a basis for developing more appropriate diets targeting known nutrient requirements that support increased hatchery production and improved quality of progeny.
5. Hatchery culture practice involved exclusive use of live cultured micro-algae for larval rearing of sandfish.	Demonstration that live micro-algae is not obligatory for successful hatchery production of sandfish. Confirmation of the feasibility of using micro-algae concentrate as a sole food during hatchery culture of sandfish.	Use of diets without live micro-algae provides a basis for developing simplified hatchery methods for sandfish more appropriate for developing countries. Simplified and cheaper hatchery methods for sandfish support regional expansion of hatchery-based sandfish culture.
6. Prior studies hypothesized that hyaline sphere (HS) development is an indicator of subsequent larval performance in sea cucumbers.	Confirmation of the relationship between HS development and subsequent larval performance for sandfish. Identification of key dietary components that influence HS development.	It may possible to influence HS development through diet composition to improve hatchery production and efficiency, and improve the quality of resulting juveniles.

The results in Chapter 3 show potential for using micro-algae concentrates as alternatives to live micro-algae in hatchery culture of sandfish based on ingestion and digestion. All commercially available micro-algae concentrates tested were readily ingested and digested by *H. scabra* larvae with the exception of *Thalassiosira pseudonana* (3H 1800®) which was not ingested by larvae of any of the three ages tested (2, 6 and 10 days after fertilisation). Further experiments in Chapters 4, 5 and 6 were conducted to assess the relative nutritional values of digestible micro-algae as a basis for developing an appropriate diet for hatchery culture of sandfish, based on commercially-available micro-algae concentrates, and to provide further information on the nutritional requirements of sandfish larvae.

7.3. Use of micro-algae concentrates for hatchery culture of sandfish, *Holothuria scabra*

In addition to appropriate characteristics for ingestion and subsequent digestion, suitable foods for sea cucumber larvae must have appropriate nutritional compositions (Chapter 3). Determining relationships between dietary nutrient compositions and larval growth, development and survival is essential for optimising larval feeding protocols and deducing the nutritional requirements of larvae which, in the case of sandfish and holothurians more generally, are poorly known. Therefore, a greater understanding of the relationships between the nutritional composition of larval diets and larval performance is critically important for future studies in this field. Chapter 3 showed that all commercially available micro-algae concentrates tested were readily ingested and digested by sandfish larvae with the exception of *Thalassiosira pseudonana* (3H 1800®) and research in Chapter 4 assessed the nutritional value of three of the ingestible commercially available micro-algae concentrates for sandfish larvae. Assessment of larval performance and comparison with nutrient compositions of tested diets, helped identify key dietary components for sandfish larvae. This study was the first comprehensive assessment of the nutritional value of commercially available micro-algae concentrates for sandfish larvae, and of the relationships between dietary nutrient composition and growth, development and survival of the larvae of any sea cucumber species. Of particular significance, results confirmed the feasibility of using micro-algae concentrates as the sole nutrient source during hatchery culture of sandfish and, as such, is the first to report successful hatchery culture of sandfish larvae without the use of live micro-algae. Results indicate that all micro-algae concentrates used in this study proved nutritious for sandfish larvae and supported normal growth and development, and relatively high survival through settlement compared to previous reports using live cultured micro-algae (Purcell et al., 2012).

Zacarias-Soto et al. (2013) reported that a mixture of concentrated micro-algae from the Instant Algae® range (*Tetraselmis* and *Isochrysis*) can be used to raise the larvae of *Isostichopus badionotus*. But in the current study, all commercially available micro-algae concentrates tested proved nutritious for sandfish larvae when fed as single species, and all supported normal growth and development, and relatively high survival, through settlement. Hatchery culture of sandfish using these products, without the use of live micro-algae, is a significant finding that supports development of cheaper, simpler larval rearing protocols for this species. The diets used in this study were not *iso*-lipidic or *iso*-energetic, and they varied in their rates of ingestion and digestibility and nutrient compositions, however, the results help to identify some of the key dietary nutrients for sandfish larvae, and provide a basis for development of more appropriate diets supporting improved hatchery culture methods.

7.4. Development of hyaline spheres in late auricularіae of sandfish

Results of Chapter 3 showed that commercially available micro-algae concentrates are readily ingested and digested by sandfish auricularіae and some are able to support larval growth and development through metamorphosis as a sole food source (Chapter 4). Furthermore, both diatoms and flagellates are available in concentrated form providing a broad range of nutrient compositions that allow comparison of larval performance relative to diet composition (Chapter 4). Chapter 5 used different micro-algae concentrates to examine the influence of diet composition on hyaline sphere (HS) development in late sandfish auricularіae and to further investigate the relationships between the presence and size of HS in late auricularіae, larval competence through settlement, and early juvenile performance.

Formation of HS and their size varied between diets and both were clearly influenced by diet composition in this study. Results showed that HS development was strongly linked to the nutrients available to sandfish larvae during the auricularia stage, that larval competence during settlement and the early post-settlement period was strongly correlated with the degree of HS development in late auricularіae, and that the dietary components most strongly influencing this relationship were carbohydrates and levels of the n-3 PUFAs ARA, EPA and DHA. The results confirm the strong relationship between development of HS in late auricularіae of sandfish and subsequent larval competence. Results also identified some of the key dietary components that influence HS development in this species. It may be possible for example, to

manipulate the composition of diets used for hatchery culture of sandfish to improve hatchery production and efficiency, and improve the quality of resulting juveniles. This would be an appropriate basis for future research.

7.5. The nutritional value of live and concentrated micro-algae for early juveniles of sandfish, *Holothuria scabra*

Determining relationships between dietary nutrient composition and juvenile growth and survival is essential to optimise feeding protocols that, in turn, support development of more appropriate hatchery procedures. An important part of this is to generate an understanding of the relative rates of ingestion and digestion of particular diets that provide information on nutrient availability and the subsequent nutritional value of a diet. Appropriate diets should be easily ingested, readily digested and should have an appropriate nutritional composition. Although these factors were used to assess the suitability of diets for sandfish larvae in Chapter 3 and Chapter 4, there is a lack of such information for early juveniles of sandfish, a stage in the hatchery culture cycle that is considered a bottleneck to further large-scale development of sandfish mariculture (Purcell et al., 2012). Chapter 6 assessed ingestion, cell wall digestion and relative nutritional values of two live micro-algae (TISO and *Chaetoceros muelleri*) and six concentrated micro-algae products (Instant Algae®, Reed Mariculture Inc.) (eight micro-algae in total) for early juveniles of sandfish.

Live *C. muelleri* supported superior juvenile performance to three micro-algae concentrates fed either singly or in combination (Isochrysis 1800®, Pavlova 1800® and TW 1200®). Although *C. muelleri* is unsuitable as a food for 2-day old sandfish larvae (Chapter 3), it has high nutritional value for older sandfish larvae (Duy, 2012; Juinio-Meñez et al, 2012; Chapters 4 and 5) and, as demonstrated in the Chapter 6, for early juveniles of this species. Nevertheless, the three concentrates tested with sandfish juveniles also supported good rates of growth and survival and, similar to the results with auricularia (Chapters 4 and 5), TW 1200® was the best performing of the tested micro-algae concentrates.

Assessment of the nutritional value of micro-algae concentrates for sandfish larvae (Chapters 4 and 5) and early juveniles (Chapter 6) showed quite clearly that Instant Algae® products are able to totally replace live cultured micro-algae through the entire hatchery production process, and are able to support high rates of growth and survival. Of the Instant Algae® products tested

in this study, TW 1200® was clearly superior as a sole food source for larvae and early juveniles. The results provide a basis for developing more simplified hatchery culture methods for sandfish that do not require the culture of live micro-algae and are therefore more appropriate for regional hatcheries that often lack the technical resources and skilled personnel required to produce appropriate quantities of high quality live micro-algae. A logical next step towards this development is optimisation of diets composed of micro-algae concentrates for sandfish larvae and juveniles to maximise hatchery production. Live micro-algae diets are normally composed of a mixture of species to provide a better balance of nutrients (Southgate, 2012) to larvae or juveniles. Although the potential benefits of multi-species diets compared to single species diets of live micro-algae has not yet been assessed for the larvae of sea cucumbers, the same principle is likely to apply to micro-algae concentrates and future research should address the potential benefits of mixed species micro-algae concentrate diets for sandfish, with a view to optimising diet composition.

7.6. Application of the results of this study

The three micro-algae concentrates (Isochrysis 1800®, Pavlova 1800® and TW 1200®) proved nutritious for *H. scabra* larvae when fed as single species, and all supported normal growth and development, and relatively high survival, through settlement. Hatchery culture of sandfish using these products, without the use of live micro-algae, is a significant finding that supports development of cheaper, simpler larval rearing protocols for this species. The results of this study also identify key dietary nutrients and provide a basis for development of more appropriate diets for sandfish larvae and early juveniles that better target nutritional needs and support improved hatchery production.

The major potential applications of the results of this study are outlined in Table 7.1. They are:

- Replacement of live micro-algae with commercially available micro-algae concentrates allowing simpler more technically appropriate hatchery culture protocols to be developed for sandfish;
- Simplified hatchery culture methods for sandfish will support improved production that will support expansion of sandfish culture operations throughout the tropical Indo-Pacific; and
- Development and use of more and greater quality of progeny.

As a direct result of the outputs of this study, routine use of micro-algae concentrates in hatchery culture of sandfish now occurs in government hatcheries in Fiji, PNG and the Philippines, and is being developed for commercial sandfish hatchery culture in Australia.

The results of this study allow development of existing hatchery protocols for sandfish and a recommended larval culture protocol for sandfish, based on micro-algae concentrates as a larval food source in place of live micro-algae, is shown in Table 7.2.

7.7. Future research

This study has focused on assessment of the ingestion, digestion and relative nutritional values of certain products within the Reed Mariculture Instant Algae® range, for larvae and early juveniles of sandfish. This research was conducted at small scale and generally focused on assessing the nutritional value of single products. For optimal application of the results of this study, further research is recommended as a basis for developing a micro-algae concentrate-based culture protocol for large-scale hatchery culture of sandfish.

7.7.1. *Optimising micro-algae diet composition*

It is broadly acknowledged that diets composed of mixed species of live micro-algae provide a better balance of nutrients for invertebrate larvae than single species diets (Brown et al., 1989; Knauer and Southgate, 1999). Exhaustive examination of combinations of all available products from the Instant Algae® range was beyond the scope of this study, but a number of products have been identified as appropriate nutrient sources for sandfish larvae. Further research should consider the potential of varying combinations of these products within mixed species diets for sandfish larvae. While there is obvious advantage in simplification of hatchery culture protocols for sandfish in regional hatcheries, where a single food product would be preferred, it is possible that a combination of products may support improved survival or growth/development of sandfish larvae that could justify the additional costs associated with a more complex feeding regime.

7.7.2 *Optimising micro-algae ration*

The micro-algae products assessed in this study varied greatly in their cell sized and volume and this is likely to affect relative rates of ingestion and digestion for different species. Furthermore, the buoyancy of cells from the various products is likely to vary affecting their availability to larvae within culture systems. All these factors are likely to influence the optimal ration for sandfish larvae, with optimal ration itself likely to vary according to the age of larvae. Once an optimised diet has been established for sandfish larvae (see 7.7.1), future research should also determine an age-specific optimal ration for sandfish that will support maximal survival and growth, and minimise the cost of food provision by reducing over-feeding.

7.7.3. *Assessing micro-algae concentrates for large scale hatchery culture*

The results of this study have allowed development of a recommended larval culture protocol for sandfish based on micro-algae concentrates (Table 7.2). However, research in this study was conducted at a relatively small-scale. The cells of the micro-algae products used in this study are intact but are inert and, in the case of flagellates, are non-motile. All are negatively buoyant. These differences to the cells of some live micro-algae, are likely to result in behavioural differences (between live and concentrated micro-algae cells) when used in large-scale culture systems, particularly relating to buoyancy and their availability to sandfish larvae within the water column for extended periods between feeds. On this basis, a key area for future research will be to confirm the potential of micro-algae concentrates shown here in small-scale culture, in commercial scale hatchery culture systems. Full assessment of this potential and comparison to conventional hatchery culture methods using cultured live micro-algae, should also include cost-benefit analysis to provide an indication of potential economic as well as biological and culture advantages using micro-algae concentrates.

Table 7.2. Recommended revised larval culture protocol for sandfish based on micro-algae concentrates.

Husbandry	Feeding
Larvae	
- Two-day auriculariae: stock at 0.3 mL^{-1} - 30% water exchange per day - When > 70% larvae reach doliolaria stage add *Spirulina* coated settlement plates into the tank	- Initial daily ration of 4,000 cells mL^{-1} of TW 1200® fed three time a day (2-3 weeks) - Increase daily ration by 10% per day - Stop feeding when > 70% of larvae reach doliolaria stage
Early Juveniles	

- Five-day post settled juveniles: stock at 0.3 juvenile cm^{-2} - 50% water exchange per day - When juveniles reach 4-5 mm transfer to hapa nets or bare tanks for further nursery culture	- Daily ration of 11,000 cell mL^{-1} of TW 1200®, fed once a day

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APPENDIX 1

Duy, N.D.Q., Pirozzi, I., Southgate, P.C., 2015. Ingestion and digestion of live micro-algae and micro-algae concentrates by sandfish, *Holothuria scabra*, larvae. Aquaculture. 448(0), 256-261.

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Duy, N.D.Q., Francis, D.S., Southgate, P.C., 2016b. Development of hyaline spheres in late auricularіae of sandfish, *Holothuria scabra*: is it a reliable indicator of subsequent performance? Aquaculture. 465, 144-151.

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Ingestion and digestion of live microalgae and microalgae concentrates by sandfish, *Holothuria scabra*, larvae

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ABSTRACT

Information on the nutritional requirements and preferred diets of sea cucumber (Holothuriidae) larvae is extremely limited and this has hindered development of hatchery culture methods. This study assessed ingestion and digestion of two live (TISO and *Chaetoceros muelleri*) and six concentrated microalgae (Instant Algae®, Reed Mariculture Inc.) by sandfish (*Holothuria scabra*) auricularia larvae of different ages using epifluorescence microscopy. This is the first study to report the use of epifluorescence microscopy with larval echinoderms and experiments were conducted using 2, 6 and 10 day old auricularia larvae. Seven of the eight microalgae tested were ingested and digested by the larvae with digestion occurring more rapidly in older larvae. *C. muelleri* was rapidly digested by 6-day and 10-day old larvae but our results indicate that *C. muelleri* is unsuitable as a food for 2-day old sandfish larvae. TISO was well ingested by sandfish larvae in both live and concentrated forms and live TISO was the most suitable of the microalgae tested in terms of ingestion and digestibility. All commercially available microalgae concentrates tested were readily ingested and digested by *H. scabra* larvae with the exception of *Thalassiosira pseudonana* (3H 1800®) which was not ingested by larvae of any of the three ages tested. Our results show potential for using microalgae concentrates as alternatives to live microalgae in hatchery culture of sandfish. However, further research should be conducted to assess the relative nutritional values of digestible microalgae as a basis for optimising a diet for hatchery culture of sandfish, and to provide further information on the nutritional requirements of sandfish larvae.

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Use of micro-algae concentrates for hatchery culture of sandfish, *Holothuria scabra*

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ABSTRACT

Three Instant Algae® (Reed Mariculture Inc., Campbell, CA, USA, 95008) products: (1) mono-cultured *Isochrysis* sp. (Haptophyceae) (Isochrysis 1800®); (2) mono-cultured Pavlova sp. (Haptophyceae) (Pavlova 1800®); and (3) mono-cultured *Thalassiosira weissflogii* (Bacillariophyceae) (TW 1200®) were used to feed sandfish (*Holothuria scabra*) larvae both singly and in ternary combination to assess their nutritional efficacy. Two-day auricularlae were held at a starting density of 0.3 mL^{-1} and were initially fed a daily ration equivalent to the dry weight of 10,000 cells mL^{-1} of Isochrysis 1800®. This ration was increased by the dry weight equivalent of 1000 cells mL^{-1} of Isochrysis 1800® per day as larval development proceeded. Post-settled larvae fed TW 1200® were significantly larger than those fed the ternary diet, Isochrysis 1800® or Pavlova 1800®. There were significant differences in the mean (±SE) survival of auricularlae and post-settled larvae between treatments and survival to settlement was significantly higher ($P < 0.05$) for larvae fed TW 1200® (13.7 ± 0.7%) alone. Laval development, competency and survival were significantly correlated with dietary levels of total protein, lipid and nitrogen-free extract (NFE), and with total polyunsaturated fatty acid (PUFA) content of the diets, and the levels of some specific fatty acids (FA). The proportion of late auricularlae with hyaline spheres (day 13), numbers of competent doliolariae (day 15) and the total length of post-settled larvae (day 21) were all positively correlated with dietary NFE and palmitic acid (16:0) contents, as well as dietary EPA: DHA ratio. This study is the first comprehensive assessment of the nutritional value of micro-algae concentrates for sandfish larvae based on their nutrient compositions. Our study confirms the feasibility of using commercially available microalgae concentrates as a sole food source for hatchery culture of sandfish, and is the first to report successful hatchery culture of *H. scabra* without using live micro-algae. All micro-algae concentrates used in this study proved nutritious for *H. scabra* larvae and supported normal growth and development and relatively high survival, through settlement. Use of commercially available micro-algae concentrates as a replacement for live micro-algae in sandfish hatcheries supports development of cheaper, simpler larval rearing protocols for this species.

Statement of relevance: Hatchery culture of sandfish using micro-algae concentrates, without the use of live micro-algae, is a significant finding that supports development of cheaper, simpler larval rearing protocols for this species. Our study provides detailed nutritional compositional data for the products used and on this basis we were able to deduce new information relating to key nutrients for sea cucumber larvae.

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Development of hyaline spheres in late auriculariae of sandfish, *Holothuria scabra*: Is it a reliable indicator of subsequent performance?

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ABSTRACT

This study assessed the influence of diet composition on hyaline sphere (HS) development in auricularia larvae of the sandfish, *Holothuria scabra*, and the subsequent relationships between the presence and size of hyaline spheres and competency through settlement and early juvenile performance. Two-day old larvae were fed one of three commercially available micro-algae diets that varied in their nutrient compositions: (1) *Isochrysis* sp. (Haptophyceae); (2) *Pavlova* sp. (Haptophyceae); and (3) *Thalassiosira weissflogii* (Bacillariophyceae) or a ternary combination of the three. There were positive significant correlations between HS development in late auriculariae on days 10, 11, 12 and 13 post-fertilisation, and the proportion of competent doliolariae on day 15, post-settlement size (day 21) and post-settlement survival (day 25). The dietary components that most strongly influenced these relationships were carbohydrates and the n-3 polyunsaturated fatty acids arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Our result confirm the strong relationship between HS formation in late auriculariae of sandfish and subsequent larval competency through settlement. As such, the presence and size of HS is a reliable indicator of subsequent performance for sandfish. Given that HS development was influenced by the nutrients available to sandfish auriculariae, there is clear opportunity for development of more appropriate diets for hatchery culture of this species that will improve HS formation and larval performance supporting improved hatchery production.

Statement of relevance: Our results have confirmed that for sandfish larvae, the presence and size of HS at the end of the auricularia stage, is indeed a reliable indicator of subsequent performance. Our results have also confirmed that HS formation and size is significantly influenced by the nutrient composition of the auricularia diet, and that there are significant positive correlations between HS formation and dietary levels of carbohydrate, EPA:DHA and EPA:ARA ratios. This information provides a strong basis for development of more appropriate diets for sandfish larvae that will improve HS formation and larval performance during hatchery culture and in turn improve hatchery production of this species.

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The nutritional value of live and concentrated micro-algae for early juveniles of sandfish, *Holothuria scabra*

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ABSTRACT

Information on the nutritional requirements and preferred diets of sea cucumber juveniles is extremely limited and this has hindered development of hatchery culture methods. This study assessed ingestion, cell wall digestion and relative nutritional values of two live micro-algae (*Isochrysis* aff. *galbana* (TISO) and *Chaetoceros muelleri*) and six concentrated micro-algae products (Instant Algae®, Reed Mariculture Inc.) for early juveniles of sandfish, *Holothuria scabra*. Seven of the eight micro-algae tested were ingested by juveniles with the exception of live TISO. Faeces excretion times varied between ingested diets that passed through the juvenile gut in less than 1 h. The cell walls of five of the eight micro-algae tested were partially or mostly digested (*Chaetoceros muelleri*, TW1200®, Palova 1800®, Isochrysis 1800® and Shellfish 1800®), while the cell walls of Tetraselmis 3600® and 3H 1800® remained intact. Juvenile growth rates were significantly different between diet treatments over the duration of a 14 day growth trial. Mean (±SE) length of early juveniles at the end of the growth trial was highest for those fed live *C. muelleri* (4.10 ± 0.03 mm) followed by TW 1200® (3.49 ± 0.05 mm). Juvenile survival did not differ significantly between diet treatments and was highest for those fed *C. muelleri* (79.33 ± 6.11%) followed by TW1200® (78.33 ± 1.20%). Pearson's correlation tests were used to identify key correlations between the levels of specific nutrients and juvenile performance (growth and survival). A significant positive correlation between growth and dietary protein content ($P < 0.05$), and a highly significant positive correlation between growth and dietary EPA:DHA ratio ($P < 0.01$) provide new information to inform diet selection for juvenile sandfish that will help improve juvenile performance and support improved hatchery production. Use of commercially available micro-algae concentrates as a replacement for live micro-algae in sandfish hatcheries supports development of cheaper, simpler post settlement rearing protocols for this species.

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