Reproductive Conditioning and Spawning of the Sea Cucumber Holothuria scabra for Hatchery Production

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Abstract

This thesis reports on an investigation of brood stock conditioning methods for the tropical sea cucumber *Holothuria scabra*. The project aims to advance the field of sea cucumber aquaculture by developing improved methods for the conditioning and spawning of brood stock.

There is great demand for sea cucumber products and that has seen the aquaculture production of the temperate species *Apostichopus japonicus* increase dramatically in the last decade, surpassing capture fisheries production. This increase in aquaculture production has not been mirrored for tropical species despite established methods for hatchery production and a large market demand. With wild harvests of sea cucumber unlikely to increase there is an opportunity to develop a substantial sea cucumber aquaculture industry in Northern Australia.

Addressing the lack of knowledge on the requirements of cultured *H. scabra* brood stock is important for the advancement of tropical sea cucumber aquaculture. In this thesis, a series of tank based brood stock conditioning experiments are analysed and the conclusions form recommendations for the culture of adult *H. scabra*. The application of anaesthetic as an aid in brood stock research is shown to have no effect on gonad development of captive female *H. scabra.* Anaesthesia, gonad sampling and a simple tagging method are combined with culture conditions to enable monitoring of the gonad condition and reproductive development in individual brood stock. Surgical biopsy and needle biopsy methods are used to assess gonad condition in individual male and female *H. scabra* during conditioning experiments. The mass and gonad development of brood stock monitored over month-long conditioning periods showed that animals both lost and gained mass while gonad development occurred in all experiments with treatments having little effect on the ratios of brood stock at each gametogenic stage. Animals frequently spawned in the tanks in response to disturbance from handling stress and also naturally with the lunar cycle, which has direct effect on hatchery production through unwanted loss of gametes.

This study also identifies and characterises a number of putative novel *H. scabra* neuropeptides. Neuropeptides are important signalling molecules involved in the regulation of physiological processes such as reproduction. A transcriptome of *H. scabra*

radial nerve was produced for the first time and *in silico* protein prediction unveiled an array of putative neuropeptide precursors. Sequence assembly of 4.9 Gb resulted in 51 874 transcripts. These transcripts were screened for putative neuropeptide precursors. Seventy seven putative *H. scabra* precursor sequences were identified and 50 of those appear to be novel precursors. Among the identified neuropeptides were homologues of sea star gonad stimulating substance (GSS) and the holothurian NGIWYamide, both of which have been shown to induce a reproductive response in echinoderms. Biological assays screening for reproductive activity identified an active component of *H. scabra* radial nerve and further purification and sequencing lead to the identification of a peptide associated with oocyte maturation and ovulation.

This thesis provides practical recommendations for husbandry and conditioning of *H. scabra* brood stock and presents evidence for a diverse array of neuropeptidergic signalling molecules in holothurians. The project therefore advanced the field of sea cucumber aquaculture by improving methods for the conditioning and spawning of brood stock and improving our understanding of the peptidergic control of reproduction in a non-model deuterostome.

Declaration of originality

I, Luke Turner certify that I am the sole author of the thesis and that the contents has not been submitted in any other form for another degree or diploma at any university or other institution. Information derived from the published or unpublished work of others has been acknowledged accordingly.

X ~Turner~

Luke Turner

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Declaration of originality

Acknowledgements

Table of contents

List of Figures

List of Tables

List of abbreviations

1MA	1-Methyladenine
aa	Amino acid
ACN	Acetonitrile
AS	Artificial seawater
BLAST	Basic local alignment sequence tool
CF	Coelomic Fluid
CNS	Central nervous system / tissues
COG	Clusters of Orthologous Groups
CRH	Corticotropin releasing hormone
CT	Calcitonin
DAC	Darwin Aquaculture Centre
df	Degrees of freedom
FET	Fishers exact test
FPKM	Fragments per kilobase per million fragments
FSW	Filtered seawater (1um filtered UV sterilised)
Gb	Giga bases
GI	Gonad index
GnRH	Gonadotropin releasing hormone
GO	Gene ontology
GPCR	G-protein Coupled Receptor
GPH	Glycoprotein hormone
GSS	Gonad stimulating substance
GSSLP	Gonad stimulating substance-like peptide
GV	Germinal vesicle
GVBD	Germinal vesicle break down
H & E	Hematoxylin and eosin
Hscnp	*Holothuria scabra* neuropeptide
IDA	Information dependant acquisition
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC-MS/MS	Liquid chromatography-tandem MS
MD	Molecular dynamics
MIF	Maturation inducing factor
MS	Mass spectrometry

n	Sample size
ORF	Open reading frame
OT	Oxytocin
PPLNP	Pedal peptide like neuropeptide
PTM	Post-translational modification
PTM	Post-translational modification
RACE	Rapid amplification of cDNA ends
RNE	Radial Nerve Extract
RNF	Radial nerve factor
RP-HPLC	Reverse phase High performance liquid chromatography
rpm	Revolutions per minute
RSW	Unfiltered seawater (Raw seawater)
SD	Standard deviation
SE	Standard Error
TFA	Trifluoroacetic acid
TOFMS	Time of flight mass spectrometry
TRH	Thyrotropin releasing hormone
VP	Vasopressin
VT	Vasotocin

1 General Introduction and literature review

Sea cucumbers, or Holothurians (class Holothuroidea), are benthic echinoderms with a cylindrical body shape and pentameric radial symmetry. Sea cucumbers are found in many different marine habitat types from coral reefs and lagoons, coastal flats and seagrass beds to the deep sea floor. In some habitats holothurians can be the dominant megafauna and occur at very high densities (Piepenburg and Schmid, 1997). The ecological roles of holothurians are largely unknown, however, there is evidence that they are a particularly important part of the benthos. For instance, they are involved in nutrient cycling on coral reefs (Uthicke, 2001b, Uthicke, 2001a) and the dissolution of $CaCO_3$ in seawater (Schneider *et al.*, 2011). Also, they probably have significant impacts in other habitats through either nutrient cycling or bioturbation (Wolkenhauer *et al.*, 2010), which may be particularly important in areas they occur at high densities. Thus, depletion through overfishing of sea cucumbers may have downstream effects on marine ecosystems.

1.1 General biology of *Holothuria scabra*

1.1.1 Taxonomy and identification of *H. scabra*

Echinodermata Holothuroidea

    Aspidochirotida

        Holothuriidae

            Holothuria (Metriatyla) *scabra* Jaeger, 1833

    Although the colouration of the body varies greatly between different sea cucumber species, identification of *H. scabra* is usually possible in the field from external observation. Two other similar species, *Holothuria lessioni* (Massin et. al., 2009), formerly *H. scabra* var. *versicolour* (Conand, 1986) *and Holothuria aculeate,* (Semper, 1868) could be confused with *H. scabra. H. aculeata* has only been identified from one specimen found in both the Philippines and one from Micronesia, suggesting it may be either uncommon or a rare hybrid of *H. scabra and H. lessoni* (Massin *et al.*, 2009, Kim *et al.*, 2014). *H. lessoni* and *H. scabra* are morphologically and behaviourally similar, however there are some key differences which enable quick species identification. First,

in *H. lessoni* their dorsal podia are darker and larger and they may possess a blotchy pattern that is never present in *H. scabra*. Second, wrinkles are common on the bivium of *H. scabra* but rare on *H. lessoni* (if present they are relatively shallow). Finally, *H. lessoni* are found in deeper waters and grow larger than *H. scabra.* These traits are often enough to distinguish between the two species. Where these two species coexist in high densities, they have been found to hybridise resulting in specimens with dissimilar spicule morphology (Massin *et al.*, 2009, Uthicke *et al.*, 2005).

1.1.2   Life cycle

H. scabra are broadcast spawning holothurians, where males and females release gametes simultaneously into the water column (Hamel *et al.,* 2001). After fertilisation embryos develop through a planktotrophic larval phase, followed by a brief non-feeding stage before settlement. Embryos develop into auricularia, a feeding stage that lasts for 10-14 days until metamorphoses into doliolaria before settling. At settlement, the larvae develop into pentacula, grow into juveniles (Ramofafia *et al.,* 2003). Settlement occurs on seagrass leaves, where newly settled juveniles remain until they reach a length of 10 mm before moving onto the sand. Juveniles of ca. 10-150 mm that have moved onto the sediment, shelter in the sea grass beds and have a pronounced daily burrowing cycle (Mercier *et al.*, 1999, Mercier *et al.*, 2000b). Adult distribution suggests that larger animals migrate away from the seagrass and onto slightly deeper habitats with lower organic matter content (Mercier *et al.*, 2000a).

1.1.3   Anatomy

H. scabra are medium-sized holothurians, with adults ranging in weight from 300-1000 g (Hamel *et al.,* 2001). Their body is generally cylindrical with a flattened trivium (ventrum) and an arched bivium (dorsum). Colour varies greatly from almost white to brown to black, although they are usually lighter or white on the underside. The bivium often has transverse wrinkles that are sometimes associated with darker markings. Dark transverse markings or stripes are common in juveniles and less common or reduced in adults. The mouth is anterio-ventral and contains 20 retractile feeding tentacles, while the anus is located terminally on the posterior. The body is covered with small papillae and the body wall is tough and thick (ca. 5-10mm). The coelomic cavity contains

coelomic fluid (CF) that bathes the body organs including the digestive and haemal systems, water vascular organs, gonad and respiratory trees (Fig. 1.1). The digestive system consists of a long looped intestine attached to the dorsal mesentery, as well as a short pharynx and stomach followed by a descending small intestine, ascending small intestine and a looped large intestine (Mary Bai, 1994). Respiration takes place in the right and left respiratory trees, which act like water lungs aspirated by cloacal pumping (Fig. 1.1). The right respiratory tree is attached to the inner body wall and is retained on evisceration (see *Auto evisceration,* 1.1.4). The inside of the body wall is lined with muscle tissue interspersed with five radial muscle bands running longitudinally, which help to define the ambulacra.

The sea cucumber nervous system consists of a circumoral nerve ring surrounding the oral parts. Five radial nerves, which stem from the oral nerve ring, run longitudinally along the inside of the body wall innervating the body. The radial nerves align with the ambulacra and longitudinal muscle bands (Mashanov *et al.*, 2006, Mashanov *et al.*, 2009). The gonad is present as a single tuft of tubules branching out from the gonad basis attached to the dorsal mesentery. Upon spawning, gametes are released through a single gonoduct that connects the gonad basis to the gonopore in the anterior bivium (Fig. 1.1).
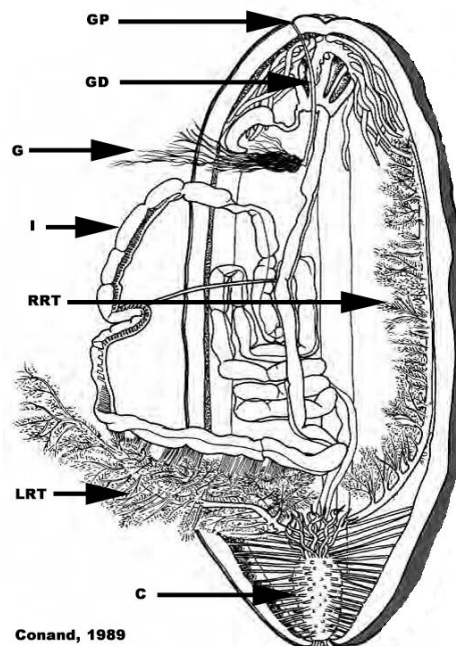
Figure 1.1: Anatomy of a generalised sea cucumber adapted from Conand (1989). GP- gonopore, GD- gonoduct, G- gonad, I- intestine, RRT- right respiratory tree, LRT- left respiratory tree and C- cloaca.

1.1.4 Auto evisceration

Autonomy is commonly observed in echinoderms and auto-evisceration and regeneration has been studied extensively in holothurians (García-arrarás and Greenberg, 2001). Their astonishing regenerative capabilities (Dolmatov *et al.*, 1996, Dolmatov and Ginanova, 2009, Mary Bai, 1971) have also generated interest from the fields of medicine and genetics (Mashanov *et al.*, 2008, Ortiz-Pineda *et al.*, 2009).

Auto-evisceration is the expulsion of some of the internal organs through the mouth, anus or a lesion in the body wall, and can be induced by numerous physical and chemical stimuli (Barker and Xu, 1993). However, distilled water injected into the coelom or mechanical stress, are the simplest methods used in experimental studies to induce auto-evisceration (Mary Bai, 1971). Poor water quality and transportation stress have also led to auto-evisceration in captive *H. scabra* (Battaglene *et al.*, 2002). Auto-evisceration in *H. scabra* involves the perforation of the cloacal wall at the junction of the respiratory trees causing coelomic fluid (CF) to flow out of the anus (Mary Bai, 1971). Contraction of the longitudinal muscles causes the lower digestive tract to be expelled first, immediately followed by the left respiratory tree and associated haemal organs. Lastly, the upper digestive tract is expelled. The right respiratory tree remains intact, as does the dorsal mesentery where the regeneration of the digestive system is initiated (Mary Bai, 1971).

In holothurians, the effect of auto-evisceration on reproduction is unknown. Gonad tubules in the later stage of growth that are large and intertwined with the organs which are expelled can detach from the gonad basis (Mary Bai, 1971). Regeneration post evisceration is more rapid in *H. scabra* than reported for other species (Garcia-Arraras *et al.*, 1998, Kille, 1935, Shukalyuk and Dolmatov, 2001). Mary Bai (1971) reported that after seven days the digestive tract was sufficiently regenerated as to allow the passage of sediments. The digestive tract, left respiratory tree and the haemal system were fully developed after 35 days in the same study, while regeneration of the gonad tubules did not occur.

Auto-evisceration is an important consideration when transporting, preserving, handling and designing experiments using *H. scabra*. The use of anaesthetics could potentially reduce the auto evisceration response of holothurians during experiments

or transportations, which unavoidably involve rough handling. There are anaesthetics and relaxants that have been used for *H. scabra* but their application on adults and effect on brood stock requires investigation (Watanabe *et al.,* 2012).

1.2 Gametogenesis

Reproductive biology varies greatly among holothurians. Asexual reproduction via binary fission is common in some species that may also reproduce sexually (Uthicke *et al.*, 1998, Lee *et al.*, 2009). Reproduction in hermaphroditic and brooding holothurians has also been described (Frick *et al.*, 1996, Kubota and Tomari, 1998, Kubota, 2000). Most commercial holothurians, however, are gonochoric broadcast spawners with gonads consisting of one or two tufts of tubules extending from a gonad basis that is attached to the dorsal mesentery (Conand, 1993, Hamel *et al.*, 2001). Gonad morphology also varies from species to species, which may exhibit elongate, globose or nodose tubules that vary in size and degree of branching (Sewell *et al.*, 1997, Conand, 1993). Male and female gonads of *H. scabra* have similar external morphology, although the female fecund gonads are larger and translucent orange in colour while the mature or growing testes appear white and opaque (Ramofafia *et al.*, 2003). The ovaries and testes can be more difficult to differentiate microscopically in early or spent stages of gametogenesis and sex cannot be determined visually in immature specimens at < 100 mm in length (Demeuldre and Eeckhaut, 2012).

In reproductive studies, it is often important to determine the stage of gonad maturity. Ramofafia *et al*. (2003) developed a maturity scale for those *H. scabra* found in the Solomon Islands (Table 1.1). The authors described the stages based on gonad morphology and histology as: Stage 1- Indeterminate, Stage 2- Growing, Stage 3- Mature, Stage 4- Partly spawned, Stage 5- Spent. This, and similar maturity scales have been used to assess the reproductive cycles of *H. scabra* across the Indo-Pacific (Rasolofonirina *et al.*, 2007, Tuwo, 1999, Che and Gomez, 1985, Purwati, 2006, Muthiga *et al.*, 2009).

Table 1.1: Maturity scale for gonad development of *Holothuria scabra.* from Ramofafia *et al*. (2003).

Maturity stage, sex	Gonad wt. (g)	Tubule Length (mm)	Tubule Diameter (mm)	Tubule Branching	Condition	Colour
I: indeterminate	< 5	<10	<0.3	0	Gametes not evident	White
II: growing						
Female	5–30	30–70	0.7–1.4	1–2	Developing oocytes (20–80 µm)	Orange
Male	5–30	20–60	0.5–1.3	1–2	Sperm developing	White
III: mature						
Female	40–200	40–160	1.0–3.0	2–3	Oocytes visible through transparent tubule (120–170 µm)	Reddish orange
Male	30–200	30–170	0.5–2.5	2–3	Tubules packed with sperm, tubules have beaded appearance	Creamy white
IV: partly spawned						
Female	30–120	30–140	0.5–2.10.	1–3	Tubules reduced, empty regions of lumen visible, phagocytes present in spawned and unspawned tubules	Reddish orange
Male	20–90	20–150	3–1.9	1–3	Spawned tubules reduced, unspawned tubules packed with sperm	Creamy white
V: spent						
Female	0.1–40	6–130	0.1–1.8	1–3	Tubules reduced and shrunken, relict oocytes present	Pale orange
Male	0.1–40	10–140	0.1–1.3	1–3	Unspawned sperm present in shrunken and wrinkled tubules	White

Quantitative methods for determining and comparing gonad development stages have been derived for some echinoderms. These methods avoid subjectivity in gonad assessment but usually require destructive sampling of the animal (Doyle *et al.*, 2012, Foglietta *et al.*, 2004, Singh *et al.*, 2001). For example, gonad indices (GI) are the most commonly used method to quantify gonad development and have been applied to

elucidate the reproductive cycle of *H. scabra* (Table 1.2). These are generally measures of the ratio of gonad to body tissue weight but there is some variation on the exact measures used. The plastic nature of the mass of sea cucumbers, given their capacity to hold varying amounts of water and sediment, requires additional thought when designing GI measures. Some researchers use drained body weight including the body organs, others remove gut contents before weighing and some use body wall weight (Morgan, 2000b, Ramofafia *et al.*, 2003). All of these methods assume that changes in GI correspond to the various stages in gametogenesis (Doyle *et al.*, 2012). The large diversity of methods used in GI studies complicate comparisons between GIs from different studies (Mercier and Hamel, 2009). Monthly sampling of GI for *H. scabra* across its distribution has been able to elucidate the peak periods for gonad growth for the species and provides a good general indication of the population level spawning periods (Che and Gomez, 1985, Tuwo, 1999, Morgan, 2000b, Ramofafia *et al.*, 2003, Muthiga *et al.*, 2009).

Table 1.2: Reproduction of *Holothuria scabra* in the Indo-Pacific (Adapted and continued from Ramofafia *et al.* (2003).

Latitude	Location	Methods	GI peak	Reproductive pattern	Spawning period	Reference
27°N	Egypt	GI	July	Biannual	April and June-August	(Hasan, 2005)
13°N	Philippines	GI and Histology	No obvious peaks	Year round	Increased activity in June and October (biannual peaks assumed)	(Che and Gomez, 1985)
9°N	India	GI	July and October	Biannual	July–August (main) October–November	(Krishnaswamy and Krishnan, 1967)
3°S	Indonesia (Ambon)	GI and Histology	October and March	Year round	March-April and October-December	Susetiono, 1996 and Kailola, 1997 Reviewed by (Purwati, 2006)
4°S	Indonesia (Lampung)	GI and Histology	November and March	Year round	March-May and November-January	Darsono et. al., 1995 Reviewed in (Purwati, 2006)
4°S	Kenya	GI	January-March and October-November	Biannual	July-September and November-December	(Muthiga *et al.*, 2009)
5°S	Indonesia	GI and Histology	April and November	Biannual	April and November	(Tuwo, 1999)
6°S	Tanzania	GI	May-September and October-January	Biannual	June-August and December-January	(Kithakeni and Ndaro, 2002)
9°S	Soloman Islands	GI and Histology	August and October (Females) August (Males)	Year round	Enhanced activity from September to December	(Ramofafia *et al.*, 2003)
20°S	New Caledonia	GI	August and December	Biannual	August-September December–January	(Conand, 1993)
23°S	Madagascar	GI, Histology, MI	November-April	Annual	November-April	(Rasolofonirina *et al.*, 2007)
27°S	Australia	GI and Histology	December	Annual	November–January	(Harriott, 1980)
27°S	Australia	GI and Histology	November	Annual	November-December	(Morgan, 2000b)

Gonad biopsy methods have been applied in some studies as a non-destructive way of observing gonad condition. In this procedure, gonads can be sampled using a hypodermic needle and syringe (Mercier *et al.*, 2000a), although using this method may be difficult to sample small gonads and can cause auto-evisceration (Morgan, 1999). Alternatively, a small incision can be made in the body wall to observe and sample gonad tissue with apparently no significant adverse effect on reproduction (Hamel and Mercier, 1999, Hamel and Mercier, 2007, Fujiwara *et al.*, 2010b). There are no established methods for biopsy of gonad tissue for *H. scabra* brood stock. The application of the previously reported biopsy methods (described previously) to this species needs to be assessed because the tough nature of the integument of the species may make biopsy by incision or needle difficult.

Oogenesis is initiated in the epithelial layer of the ovary with oogonia bulging into the gonad lumen as they develop into fully grown oocytes (Smiley, 1990). The most obvious change in oocyte size during the reproductive period occurs during vitellogenesis or yolk formation. In vertebrates vitellogenesis involves the transfer of vitellogenin yolk proteins (VTGs) into the developing oocytes (Smiley, 1990). In sea urchins and sea cucumbers VTGs have not been reported, instead, transferrin-like proteins termed major yolk proteins (MYPs) are involved in yolk formation (Fujiwara *et al.*, 2010a, Prowse and Byrne, 2012). MYP is produced in the gut and in the gonads of both sexes in sea urchins (Unuma and Walker, 2010). The precise function of MYP has not been resolved but it is a major component of nutritive phagocytes in the ovary and testis (Unuma *et al.*, 2003, Unuma *et al.*, 2010) and is abundant in the CF of sea cucumbers (Fujiwara *et al.*, 2010a). Fully-grown *H. scabra* oocytes are approximately 170-180 μm.

1.2.1 Exogenous control of reproductive timing

The timing of reproduction varies throughout the geographic distribution of *H. scabra* (Table 2). Generally, spawning output ranges from annual and biannual at higher latitudes to asynchronous, year round, in the lower latitudes (Ramofafia *et al.*, 2003).

The association between latitude and the reproductive output of *H. scabra* suggests a seasonal effect on the timing of reproduction in this species. Peak spawning periods indicated from changes in population GI, occur in the warmer parts of the year at the highest latitudes and approach year round at latitudes within 20 degrees of the equator (Table 1.2). Aside from temperature, other environmental cues that have been proposed to correlate with peak spawning times are nutrient availability, salinity, photoperiod and lunar cycle (Morgan, 2000b, Che and Gomez, 1985, Mercier *et al.*, 2000a, Rasolofonirina *et al.*, 2007).

Over exploitation of *H. scabra* populations also affects reproduction in populations. For instance, over-harvesting of larger individuals within a population resulted in a severely skewed sex ratio that favours the early maturing males and was associated with marked decline in GI (Hasan, 2005, Muthiga *et al.*, 2009). Skewed sex ratio and declines in mean population GIs are probable indicators of a reduction in population reproductive output. Thereby placing isolated, overfished populations at risk of collapse.

1.3   Maturation and spawning

In vitro fertilisation is a method sometimes used for obtaining controlled seed production in fish for aquaculture. Stripping and fertilisation of oocytes excised from holothurian ovaries is currently not a commonly used method for obtaining large numbers of embryos, due to the meiotic pause in oocyte development (Miyazaki *et al.*, 2005). In broadcast spawning holothurian species, fully grown oocytes must undergo final maturation before they become fertilisable and are released. As with some species of cultured fish, holothurians require hormonal treatment to induce final oocyte maturation prior to *in vitro* fertilisation. Maturation in sea cucumber oocytes is characterised by germinal vesicle break down (GVBD), where the germinal vesicle migrates to the oocyte polar process before breaking down (Miyazaki *et al.*, 2005, Pang *et al.*, 2010). Final oocyte maturation occurs post-ovulation, immediately prior to spawning as oocytes pass through the oviduct, as characterised in *Holothuria leucospilota* (Hamel and Mercier, 2007). The biomolecule involved in this is known as a maturation inducing factor (MIF). That biomolecule is similar to 1-methyladenine (1-MA)

identified in asteroids, yet not the same since 1-MA fails to induce oocyte maturation in those sea cucumbers that have been tested, including *H. leucospilota* and *H. pardalis* (Maruyama, 1980). Reducing agents dithiothreitol (DTT), 2, 3-dimercapto-1-propanol (BAL) and L-cysteine can induce oocyte maturation in asteroids and holothuroids, however, they are not useful for *in vitro* production of embryos because of high levels of deformity in the resultant larvae (Kishimoto and Kanatani, 1980, Kishimoto and Kanatani, 1973). Techniques for *in vitro* fertilisation of sea cucumber oocytes have been achieved through the application of various substances such as the reducing agents described above and also a factor present in sea urchin spawn which has been applied to *H. scabra* ovary to induce oocyte maturation (Léonet *et al.*, 2009). However, the endogenous MIF and gonad stimulating substance (GSS) remain uncharacterised in holothurians.

Studies of GI in *H. scabra* indicate spawning periods vary with latitude and are also temporally variable (Table 1.2). Generally, populations at lower latitudes, in warmer less seasonal climates, show aperiodic spawning inferred by mature gametes observed throughout the year and certain periods of enhanced gametogenesis (Ramofafia *et al.*, 2003). Populations at higher latitudes have shown seasonal and often biannual spawning (Morgan, 2000b). The peak reproductive periods can differ from year to year, probably in relation to local exogenous gametogenic cues (Ramofafia *et al.*, 2003). For example, spawning events have been correlated with the lunar cycle (Mercier *et al.*, 2000b, Mercier *et al.*, 2007), where males begin spawning first in the evening, raising the anterior portion of their body and swaying. The release of sperm is observed as a steady stream over an extended period whilst the females release gametes in one or several bursts about an hour after the first male begins spawning (Morgan, 2000c, Battaglene *et al.*, 2002).

Spawning induction methods used in *H. scabra* culture rely on nonspecific environmental stresses. Usually one or a combination of physical stimulation, draining, thermal stress, and water quality changes are used to induce spawning. Thermal stress is the most effective method, whereby raising the water temperature by 3-5°C for approximately one hour triggers the animals to spawn late in the evening (Morgan, 2000c, Battaglene *et al.*, 2002, Ramofafia *et al.*, 2003). However, this generic technique is time consuming and usually results in gamete release in around 10% of females, even

during peak spawning times (Battaglene *et al.*, 2002). The development of a hormonal spawning stimulant, as used in fish aquaculture with the gonadotropin-releasing hormone analogue (GnRHa), could potentially reduce time, costs and increase holothurian aquaculture production.

1.3.1 Endogenous control of reproductive timing

In sea cucumbers, like sea stars, neurological control of reproduction is moderated by a GSS that stimulates the gonad to secrete a maturation inducing factor (MIF), which in turn acts on the oocyte leading to maturation promoting factor (MPF) production, GVBD and maturation (Kalachev, 2013). GSS has recently been characterised in two sea star species (*Asterias amurensis* and *Asterina pectinifera*), showing relaxin-like neuropeptide features, stimulating the production of 1-MA by follicle cells in the ovary via G-protein coupled receptor mediated pathway (Mita *et al.*, 2009, Mita *et al.*, 2014, Mita *et al.*, 2015). Sea star GSS in radial nerve extracts (RNEs) has been reported to be active when tested in some, but not all, holothurian species (Strathmann and Sato, 1969), suggesting relatedness of GSS amongst these echinoderms. A GSS-like peptide (GSS-LP) has been identified in the sea cucumber *Apostichopus japonicus* (Katow *et al.*, 2009). GSS-LP was named GSS-like based on the peptides ability to trigger GVBD (Katow *et al.*, 2009), however, it is not structurally similar to sea star GSS. Immuno-histological investigations have shown that the GSS-LP is produced in the neural tissues and may be transported to the gonad via an unknown pathway (Ahmed *et al.*, 2011).

Recent reports of substances that can induce spawning in other species of holothurian suggest that signalling molecules involved in spawning regulation are generated from neural tissues and then transmitted via the perivisceral coelomic fluid (PCF) (Mercier and Hamel, 2002, Kato *et al.*, 2009, Fujiwara *et al.*, 2010b). Mercier and Hamel (2002) could induce spawning in three species of tropical holothurian by taking PCF from pre-spawning individuals and injecting it into non-spawning conspecifics. This suggested that a substance or substances transmitted via the PCF is acting in a spawn-signalling pathway. Furthermore, a small peptide (named cubifrin) with the amino acid sequence NGIWYamide has been identified from neural tissues of *A. japonicus* that is able to induce oocyte maturation and ovulation (Kato *et al.*, 2009). Cubifrin injections have been used as a spawn-inducing agent for *A. japonicus* demonstrating that it could

potentially be used commercially in hatchery production (Fujiwara *et al.*, 2010b). The active PCF and cubifrin-like compounds are yet to be investigated in *H. scabra*. *In vivo* and *in vitro* spawning trials are necessary to determine the efficacy of cubifrin and *H. scabra* radial nerve factors as spawning inducing agents and evaluate their potential for use in aquaculture production.

Hormonal control over the gametogenic synchrony of the Canadian sea cucumber *Cucumaria frondosa* has been demonstrated by Hamel and Mercier (1999). Experiments conducted in aquaria discovered a decrease in gametogenic synchrony among individuals when isolated from mature conspecifics. Hamel and Mercier (1999) also found that a component of the mucus shed by mature individuals could induce gonadal development in other individuals. These findings provided the first evidence for a pheromone mechanism of reproductive synchrony in holothurians, however the active component within the mucus is yet to be identified. Pheromones have also been implicated in holothurian aggregation behaviour leading up to spawning events (Mercier *et al.*, 2000a). These aggregations may be mediated by pheromone signalling involved with gametogenesis and facilitate synchronisation of reproductive events, thus improving reproductive isolation and fertilisation success.

1.3.1.1 Neuropeptides

The study of echinoderm neuropeptides began with investigations of immunolocalisation investigations related to the molluscan peptide FMRF amide. Two SALMF amides (S1 and S2) were subsequently identified in the sea stars *Asterias rubens* and *A. forbesi* (Elphick *et al.*, 1991). These peptides are now the most widely studied echinoderm neuropeptides. Following their identification, the same methods (high performance liquid chromatography and immunoassay) were used to identify the first two holothurian neuropeptides which included two sea cucumber SALMF amide like peptides; GFSKLYFamide and SGYSVLYFamide (Diazmiranda *et al.*, 1992). Later, Birenheide *et al* (1998) proposed biological activity of four holothurian neuropeptides: the peptide NGIWYamide stiffened the connective tissue of the dermis of *A. japonicus and H. leucospilota* whereas two SALMFamide-like peptides softened it, and the Stichopin peptide (DRQGWPACYDSNGNYKC) acted as an acetylcholine antagonist.

A particular focus of sea cucumber neuropeptide research has been the investigation of gonadotropic activity from nerve tissue extracts. The substance in the radial nerve that triggers oocyte maturation and spawning was initially identified as a peptide of several thousand Daltons (Maruyama, 1985). Candidate neuropeptides were subsequently sequenced from purifications of *A. japonicus* radial nerve tissue, including the *A. japonicus* gonadotropic peptides Cubifrin, QGLFSGVamide (Kato *et al.*, 2009) and the GSSL peptide VLSKQAHHHHHEGWSLPGVPAEIDDLAGNIDYNIFKEOREKIK (Katow *et al.*, 2009). Each of these peptides shows oocyte maturation activity in *in vitro* bioassays. Cubifrin can additionally induce spawning behaviour and gamete release in mature *A. japonicus* (Kato *et al.*, 2009, Fujiwara *et al.*, 2010b). Similar research into sea star spawning pathways has yielded more information.

Further advancements in the identification of echinoderm neuropeptides came with the sequencing of the sea urchin genome of *Strongylocentrotus purpuratus* (Menschaert *et al.*, 2010). Sequence data analysis enabled for the *in silico* identification of 20 neuropeptide precursors including a precursor encoding putative sea urchin GnRH, SALMFamide, thyrotropin releasing hormone, calcitonin, AN peptides, pedal peptides and other unnamed putative peptide precursors (Rowe and Elphick, 2012). NGFFFamide and a sea urchin peptide with vasopressin/oxytocin sequence similarities, termed echinotocin, have also been reported (Elphick and Rowe, 2009). As more echinoderm sequence data became available, Rowe *et al* (2014) derived the first list of holothurian neuropeptide precursors by using the previously identified sea urchin peptides as BLAST queries against published transcriptome data from *A. japonicus* (Du *et al.*, 2012).

1.4 Sea cucumber fisheries and aquaculture

1.4.1 Aquaculture

Sea cucumbers are harvested as a high value food commodity for which the largest market is in China (Purcell *et al.*, 2014). The high value and high demand for sea cucumber products has led to increased aquaculture production of sea cucumbers (Lovatelli *et al.*, 2004). The technology for aquaculture production of tropical holothurian species has lagged behind that of the temperate species *A. japonicus*. Aquaculture production of *A. japonicus* in 2012 was estimated at 171 000 t at an

estimated value of 594 million (USD), with the vast majority produced in China. By comparison, aquaculture production of *H. scabra* (the main tropical species for sea cucumber aquaculture) was 120t with a value of 0.6 million (USD) with the majority of production reportedly from Vietnam (FAO, 2012). There is great demand for sea cucumber products and that has seen the aquaculture production of *A. japonicus* increase dramatically in the last decade, surpassing production from the wild capture fisheries. This increase in aquaculture production has not been mirrored for tropical species despite established methods for hatchery production and a large market demand. With global wild fishery production of sea cucumber unlikely to increase there is an opportunity to develop a substantial sea cucumber aquaculture industry in Northern Australia.

1.4.1.1 Brood stock

Very little is known about the requirements of adult *H. scabra* held in captivity. At present *H. scabra* brood stock are most commonly held in earthen ponds or tanks or obtained from wild populations during the spawning season (Pitt, 2001, Battaglene *et al.*, 2002). Methods for the conditioning of brood stock in tanks for spawning outside of the natural spawning period have not been developed (Pitt, 2001, Morgan, 2000a).

Brood stock held in ponds is often easier to induce to spawn and ripen earlier than brood stock collected from the wild (Pitt and Duy, 2005, Agudo, 2006). However, there are little reported data for *H. scabra* brood stock conditioned in ponds. Spawning has been induced in every month of the year from a population of pond cultured brood stock in Vietnam (Pitt and Duy, 2005). Similarly, spawning from wild collected brood stock was successful in every month of the year except February in the Solomon Islands (Battaglene *et al.*, 2002). In Australia, induced spawning from wild collected and pond cultured brood stock has only been successful seasonally. Those factors which are important for the conditioning of *H. scabra* brood stock or on the timing of reproduction at the individual level is unknown. It is clear that there is a need to develop experimental systems that will allow manipulative experiments to test hypothesised cues for reproduction in *H. scabra*. The experiments will also investigate the reproductive cycle of *H. scabra* at the individual level.

Tank based brood stock maturation systems involve systems with flow through seawater or static seawater with daily water exchange. These systems generally house 15-30 animals in 1t tanks with sand or mud substrates (Table 3). Whilst conducting trials on *H. scabra* brood stock, Morgan (2000c) observed that captive animals lost up to 40% body mass over the period of captivity despite provision of food supplements. The study also found declines in fecundity of female brood stock and in hatch rate after five weeks conditioning. The animals were held in tanks with sand substrate and fed supplements including powdered kelp, lucerne pellets and prawn pellets. Moreover, after five weeks into captivity the animals showed signs of disease and did not recover (Morgan, 2000a). Such difficulties with holding brood stock long-term make conditioning experiments difficult. Other food supplements reported include chicken manure, shrimp starter feed, ground algae, rice bran, soybean meal, prawn head waste, blue crab diet, abalone diet and seagrass powder (Table 3). Duy (2012) reported the conditioning of brood stock in tanks at low densities in Vietnam. Although the methods reported enabled year round induced spawning, loss of body mass was still observed in the brood stock suggesting that the conditions in the tanks would not allow longer term culture of brood stock. There is a need to develop suitable husbandry practices and systems that enable sufficient numbers of adult *H. scabra* to experimentally test hypotheses relating to the factors regulating reproduction.

In China, *A. japonicus* brood stock are conditioned for spawning shortly prior to the natural spawning period by gradually increasing the water temperature and supplying the animals with a brown algae food supplement (Liu *et al.*, 2005). Similarly in Japan *A. japonicus* have been conditioned in ambient water temperature and supplied brown alga as food source prior to spawning (Ito and Kitamura, 1998) (Table 1.3).

Table 1.3: Methods and conditions for holothurian brood stock maturation.

Source	Number of brood stock	Tank Size (t)	Substrate	Feed	Maintenance	Species
(Agudo, 2006)	15-30	1-4	10-15cm sand or mud	50 gday^{-1} prawn head waste, soya bean powder, rice bran and Seagrass powder.	Static with aeration. Water exchanged each day	*H. scabra*
(Morgan, 1999)	15-30	12	Sand	none	Flow through	*H. scabra*
(James *et al.*, 1994, James, 1999)	20-30	1	6" of Sand	Ground fresh algae	Water changed daily sand changed fortnightly	*H. scabra*
(James, 1996)	15-20	0.8	100mm of mud	50 gday^{-1} mix of prawn head waste, soybean powder and rice bran	Aeration and water changed daily.	*H. scabra*
(Duy, 2012)	50 @ <2m^{-2}		10-15cm clean sand	1gm^{-3} Shrimp starter feed twice a day	Stable temperature below 30°C water exchange done in morning	*H. scabra*
(Laxminarayana, 2005)	15-20	1	6" sandy substratum	Ground fresh seaweed	Water changed daily sand changed fortnightly	*Bohadschia marmorata* and *Holothuria atra*
(Asha and Muthiah, 2007)	30	1	150mm fine coral sand	5% of body weight *Sargassum* spp. Powder	Water changed daily sand changed fortnightly	*Holothuria spinifera*
(Yanagisawa, 1998)	50-100			Dried powdered brown algae	4-5^{0}C below ambient water temperature	*A. japonicus*
(Liu *et al.*, 2005)	30-50/m^3			5-10% body weight brown seaweed or bottom mud	Gradual increase in water temperature	*A. japonicus*
(Ito and Kitamura, 1998)	100	2		*Undaria pinnatifida*	Ambient temperature	*A. japonicus*
(Dabbagh and Sedaghat, 2012)	25	1	Bare tank	Extract of *Sargassum sp.*, *Isocrysis sp* and *Chaetoceros sp*.	Water exchanged daily	*H. scabra*

Carbohydrates appear to be less important for gonad development than protein and lipid (Krishnan, 1968). Similarly sea cucumber juveniles show stronger growth when fed protein rich diets (Seo and Lee, 2011).

The number of contributing progenitors in induced spawning trials is usually low. For example, during a year-long study in the Solomon Islands, fertilised gametes were collected in only 46% of the spawning attempts. Additionally, at best, 35% of the induced brood stock in each attempt released gametes; with an average of 1.9 females spawning per trial (Battaglene *et al.*, 2002). Low contribution rates from brood stock make the genetic management of hatchery production difficult, since large numbers of brood stock must be held in ponds or sourced from the wild for each production run to maximise variation of the progeny. For commercial production of *H. scabra,* methods to enable controlled maturation of brood stock and spawning outside of the peak periods would be beneficial (Pitt, 2001, Agudo, 2006, Battaglene *et al.*, 2002). Current success rates in induction of spawning from wild, tank and pond-reared brood stock are low, thus restrict the commercial production of *H. scabra.*

1.4.1.2 Larval culture

Holothurian larvae are cultured in tanks, usually in static culture with no or little water exchange and fed micro algae. The planktonic larval cycle usually completes within 14 days in the hatchery but may be longer depending on environmental conditions such as temperature. Prior to settlement, competent larvae metamorphose into doliolaria. Doliolaria are barrel shaped ciliated larvae which actively swim and search for areas to settle. Doliolaria are non-feeding and develop tentacles that enable attachment to the substrates. In the natural environment, settlement is thought to occur on seagrass leaves, therefore components from seagrass leaves may act as settlement cues (Mercier *et al.*, 2000a, Mercier *et al.*, 2000b). Post settlement the doliolaria metamorphose into pentacular with more podia attaching them to the substrate. At this time, the post-larvae begin feeding on biofilms (Mercier *et al.*, 2000b). In the hatchery, post-larval sea cucumber are reared until a few millimetres in size before being detached from the substrate and transferred to nursery systems (Battaglene and Seymour, 1998).

Nursery culture utilises hapa nets, tanks or ponds. At this stage, density is an important consideration due to density-dependent growth, and maximum productivity

density has been estimated at 225 g m^{-2} (Battaglene *et al.*, 1999). In tank-based nursery systems, small juveniles up to 20 mm in length, are cultured on hard substrates such as fiberglass plates. Larger juveniles at >20 mm, grow and survive better if transferred from hard substrates onto sand (Battaglene *et al.*, 1999). Light, substrate and stocking density have been identified as major factors influencing survival and growth of juveniles in tanks, while algal-based diets added to tanks did not improve growth in tank-cultured juveniles at a hatchery in the Solomon Islands (Battaglene *et al.*, 1999).

Juvenile *H. scabra* have been stocked into shrimp ponds, pens constructed in intertidal zones and released onto sand, seagrass and crushed coral habitats in Vietnam, Solomon Islands, Fiji, Madagascar, New Caledonia, India, Philippines and Australia where reported growth rates are usually good but do vary with location (Purcell *et al.*, 2012). Predation from fish can be as high as 100% on released juveniles (Dance *et al.*, 2003). Crabs are also a major predator of released sea cucumber juveniles (pers. obs., Francour, 1997). Differences in survival and growth suggest site selection is an important consideration for stock enhancement and ranching of *H. scabra* (Dance *et al.*, 2003, Purcell and Simutoga, 2008).

1.4.1.3 Grow-out

Grow-out of cultured sea cucumber is conducted in ponds, seabed pens, suspended cages or on open sea ranches. In China, *A. japonicus* are farmed in ponds lined with rocks or tiles as substrate, but are more commonly grown in sea ranches at an extensive scale (Chen, 2003). Juveniles are reared in land based facilities before being released onto natural or artificial reef habitat, then harvested by divers once at a marketable size (Chen, 2003, Chen, 2005). Similarly, *H. scabra* are grown out in ponds and are also ranched in intertidal enclosures or ranched where they subsist on natural productivity where they do not require additional feed. Grow-out time for *H. scabra* varies with location and habitat but is approximately 18 months to reach a size of ≈400g for ranched individuals (Juinio-Meñez *et al.*, 2013). Research conducted in Vietnam has shown that *H. scabra* grow well in ponds but do not flourish in polyculture with white shrimp. However, alternating crops of sea cucumber and shrimp produces improved growth and may mediate the nutrient build up in the bottom of shrimp culture ponds (Mills *et al.*, 2012). Co-culture of temperate species has shown greater success. For example,

Australostichopus mollis are able to utilise waste produced by mussel and oyster farms (Zamora *et al.*, 2014, Slater *et al.*, 2009). Risks associated with rapid decline in salinity and/or dissolved oxygen levels are the most common causes of crop failure in ponds, while predation is the main cause of mortality for ranched sea cucumber (Purcell *et al.*, 2012).

1.4.2 Fisheries

Holothurianss have been harvested for over 1000 years and are consumed predominantly in China as a food and a health tonic (Conand and Bryne, 1993). Most of the commercially valuable holothurians belong to the order aspidochirotida, which are characterised by peltate form oral tentacles and often a thick body wall. Sea cucumbers are most notably traded as Beche-de-mer or trepang but may also be traded fresh, salted or pickled (Conand and Bryne, 1993). Processing sea cucumbers into Beche–de-mer involves gutting, boiling and drying of the body wall. The price of the traded product depends on the size, the type of holothurian species and quality of the processing. Beche-de-mer is reconstituted for consumption or can be further processed into other goods such as supplements, confectionary and cosmetics (Yeun-Suk *et al.*, 1999).

Demand for sea cucumber products has led to increased fishing pressure and overexploitation in many parts of the world, in particular, the tropical holothurian fisheries (Lovatelli *et al.*, 2004, Conand and Bryne, 1993, Aumeeruddy and Conand, 2008). Holothurian fisheries include several species ranging in commercial value (Conand and Bryne, 1993). *H. scabra,* traded as "sandfish", is one of the high value target species. *H. scabra* are fished throughout the tropical western pacific including northern Australia, as well as in the Indian Ocean including India, Sri Lanka and Madagascar. *H. scabra* is a predominantly tropical species found from eastern Africa to the central pacific; most commonly found on sand, mud and silt in coastal bays and lagoons associated with seagrass beds. Declining catches in many countries and increasing demand for sea cucumber products has created pressure on the stocks over most of its distribution (Conand and Bryne, 1993). This in turn has led to increasing interest and research into holothurian mariculture in an attempt to develop technology for stock restoration and management through aquaculture (Battaglene, 1999, Battaglene *et al.*, 2002, Kille, 1935, Morgan, 2000c). *H. scabra* has demonstrated amenability to

aquaculture and has been cultured in many countries (Battaglene *et al.*, 2002, Morgan, 2000c, Leonet *et al.*, 2009, Ramofafia *et al.*, 2003, Pitt, 2001, Dabbagh and Sedaghat, 2012, James, 1999). Leading researchers and resource managers to postulate aquaculture as a potential tool in the management of sea cucumber resources (Lovatelli *et al.*, 2004).

1.5 Summary

Holothurian fisheries have peaked globally and there is still great demand for holothurian products. Aquaculture production has grown significantly in the temperate regions of Japan and China where *A. japonicus* is the main species cultured. Tropical aquaculture, on the other hand has been slow to progress, despite *H. scabra* being a good candidate for aquaculture due to its high market value and suitability for aquaculture. A seemingly large potential exists to increase tropical holothurian aquaculture, however, there are researchable constraints that currently impede production. These constraints include lack of knowledge on, and suitable methods for, culturing, conditioning and efficiently spawning brood stock.

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2 Anaesthesia, gonad sampling and marking of the sea cucumber *Holothuria scabra* for commercial scale brood stock culture

2.1 Introduction

Brood stock husbandry and reproductive conditioning are both essential aspects of closed life cycle aquaculture production systems. The technology and tools implemented for managing brood stock for hatchery production are usually species-specific and whilst there is considerable literature on the short-term care and spawning of *H. scabra* brood stock, there is little information regarding long-term culture and reproductive conditioning for captive individuals and populations. The information that is most well established includes methods for anaesthesia, gonad sampling and tagging.

2.1.1 Anaesthesia

Anaesthesia has been applied to holothurians to enable fixation of specimens for taxonomic analysis (Massin *et al.*, 2009). Many of the distinguishing features of holothurians become retracted when the animal is disturbed or immersed in fixative. $MgCl_2$ (5%) is commonly recommended as a specimen relaxant prior to fixation (Lewbart and Mosley, 2012). Although this has been used frequently there are no data reported on the recovery of specimens post-anaesthesia, and the exposure times required to induce anaesthesia are mostly longer than practical for use in aquaculture. Anaesthesia has also been used as a method of reducing the variability of individual length measurements of sea cucumber. Whilst under anaesthesia, juvenile *H. scabra* and juvenile *A. japonicus* relax and their body becomes fully extended, enabling the measurement of the maximum body length (Yamana and Hamano, 2006). Watanabe *et al*. (2012) used similar methods to anaesthetise juvenile *H. scabra* (<20 g) within about 20 min using concentrations of 2% and 4% of menthol saturated ethanol solution (menthol/ethanol) in seawater. An effective dosage of menthol/ethanol anaesthetic has not been reported for adult *H. scabra*. Other reported marine invertebrate anaesthetics such as 5% $MgCl_2$ (Suquet *et al.*, 2009), require longer exposure times to be effective i.e. >2 h for juvenile *H. scabra* (pers. obs.). Anaesthetics requiring long exposure times are less desirable for anaesthetising large numbers of animals or for use in aquaculture (Mills *et al.*, 1997). An anaesthetic protocol for adult *H. scabra* may be a useful tool in

reducing the stress during experiments requiring biopsy of the gonad or other handling procedures.

Determining the exact time at which a sea cucumber (and other invertebrates) is actually anaesthetised can be problematic since they may already exist as a sedentary animal. A relatively simple assessment tool is to monitor the loss of righting reflex; most animals naturally respond to being turned upside-down by righting themselves, and in *H. scabra* this response is quick and consistent. Loss of righting reflex does not necessarily mean that anaesthesia has been entirely achieved. However, in conjunction with relaxation, defined by loss of muscle tone, and the loss of the contraction response to physical stimulus, it is sufficient evidence to indicate anaesthesia (Acosta-Salmón and Davis, 2007). For an anaesthetic to be utilised as a tool for the assessment of gonad tissue in brood stock culture systems it needs to meet certain criteria; it must require a short exposure time to be practical for large-scale application, it must not cause mortality and it must not cause adverse effects on subsequent growth and gonad development e.g. lead to resorption of gonad tubules or other loss of gametes.

2.1.2 Gonad sampling

Studies investigating reproduction of sea cucumbers are often conducted on commercially important species. With the primary goal to determine the maturation stages in the reproductive cycle of species, these types of studies involve the investigation of gonad indices (GI), which necessitate sacrificing the animals involved. Gonad indices and histological analysis methods applied to *H. scabra* have enabled the accurate assessment of reproductive cycles of several populations (Krishnaswamy and Krishnan, 1967, Krishnan, 1968, Che and Gomez, 1985, Conand, 1993, Tuwo, 1999, Morgan, 2000b, Ramofafia *et al.*, 2003, Purwati, 2006, Rasolofonirina *et al.*, 2007, Muthiga *et al.*, 2009). These studies show that reproductive cycles tend to vary depending on location and range from annual to biannual to asynchronous. Asynchrony is common at low latitudes, and whilst this has the advantage of providing mature animals all year round for use as brood stock in aquaculture, the proportion of mature females within the population can be low (Battaglene *et al.*, 2002, Ramofafia *et al.*, 2003). Histological investigations of *H. scabra* and other sea cucumbers have defined five maturity stages. These can be distinguished based on a several features of the gonad

such as gamete size and density, tubule length, tubule diameter, tubule wall thickness and gonad colour.

The ovary of *H. scabra* consists of many tubules branching out from a single gonad basis. The tubules that comprise a particular ovary may not be all at the same stage of gametogenesis (Ramofafia *et al.*, 2003). Therefore, a sufficiently sized sample of tubules is required to discern the dominant stage among the tubules and to obtain an accurate representation of the reproductive stage of an individual. Surgical biopsy enables a large proportion of the tubules to be sampled regardless of the size of the gonad, which can range from very small (<1 g) to large (>90 g). Therefore surgical biopsy is at this point the best method to ascertain the reproductive stage of an individual.

Non-lethal assessment of gonad stage at the individual level would be useful in captive brood stock management and improve hatchery productivity. This methodology would also enable further experimental research to be undertaken to investigate the factors involved in gametogenesis at the individual animal level.

2.1.3   Tagging

Pit tags, coded wire tags, and T-bar tags, branding and fluorescent dyes have been tested with different sea cucumber species with variable success (Kirshenbaum *et al.*, 2006, Cieciel *et al.*, 2009). The major issue is that autotomy and regeneration of damaged sea cucumber tissues makes the marking of sea cucumbers difficult; tissue that tags are attached to are often shed by the sea cucumber resulting in poor tag retention.

The most effective method for batch-marking sea cucumber juveniles is chemical marking. Spicules in the dermal layers of sea cucumbers can be marked with fluorochromes such as calcein and tetracycline (Purcell *et al.*, 2006). Juvenile *H. scabra* tagged using this method can retain marked spicules for up to a year (Purcell *et al.*, 2006). Some of the limitations of chemical marking are that laboratory analysis of samples is required before an identification can be made and there are a limited number of different fluorochromes and differences in the uptake and retention of each. Chemical marking of spicules is the most effective way to mark batches or cohorts of sea cucumbers but the limited range of fluorochromes available and the processing required for each sample to be identified make this method inappropriate for marking individuals. For individual marking, the comparatively simple methods of scarring the dorsal

epidermis have been used previously to mark sea cucumbers (Reichenbach, 1999, Mercier *et al.*, 2000). In this case, it should be noted that this tagging method can temporarily effect the behaviour of sea cucumber (Shiell, 2006, Cieciel *et al.*, 2009). Immediate identification of individuals is important for developing experiments that aim to enhance our understanding of the dynamics of reproduction at individual and group levels in aquaculture systems, and in wild populations. Marking individuals with scarring and frequent monitoring appears to be the most appropriate method for individual identification of *H. scabra.*

2.1.4 Summary of proposed research

The research presented in this chapter is an analysis of methods applied to investigate the reproductive state of *H. scabra* brood stock. First, a method for inducing anaesthesia of brood stock for handling and invasive gonad biopsy procedures is described. The effects of anaesthesia on the body metrics and gonad development are also quantified. Second, a simple tagging methodology is provided for the identification of individuals in captivity. Established maturity index scale for *H. scabra* is applied to a Northern Australian population for the first time to assess its usefulness for monitoring gonad condition for brood stock. These experiments further our knowledge of this species and provide potential application of these methods for both reproductive biology research and for use in commercial hatcheries.

2.2 Methods

2.2.1 Analysis of menthol/ethanol solution as an anaesthetic

2.2.1.1 Anaesthesia of adult *H. scabra* using Menthol

Adult *H. scabra* (mean mass 539 g ± 92 sd) were obtained from a captive population held in a pond facility in Darwin, Australia. The test animals of unknown sex were acclimatised in bare tanks (300 L) with flow-through seawater at ambient temperature for 2 days prior to the trial. During the acclimation period the sea cucumber purged their gut contents and the sediment and faeces were removed.

The animals were anaesthetised in 10 L plastic aquaria with sand filtered seawater and aeration. Anaesthetic stock solution was prepared according to Yamana

et al (2005). Menthol crystals were dissolved in AR grade ethanol (Chem-Supply, South Australia) at 5.6 g L^{-1}. The stock solution was then added to seawater (30°C) at the appropriate volume to give a final concentration of 1%, 2%, 3% or 4% in 10 L total volume. A fifth aquarium received no anaesthetic solution as a negative control. Vigorous aeration was used to thoroughly mix the solutions for ten minutes, before the animals were immersed.

Five individuals were placed into each container holding the different concentrations of anaesthetic solution. Animals in the anaesthetic solutions were picked up and manually placed upside-down on their bivium in the aquaria repeatedly after 5, 10, 20, 30, 40, 50 and 60 min and the proportion of individuals in each aquaria that were able to re-right themselves were recorded. An individual was deemed to be anaesthetised if it was unable to re-right and had ceased movement for >5 min after being overturned. To determine a natural baseline for the righting response of *H. scabra,* the time taken for an overturned animal to re-right completely was recorded for each individual used in the trial prior to the experiment.

Once individuals were determined to be anaesthetised they were transferred to recovery aquaria with 1 µm filtered flow-through seawater. Recovery was checked after 10, 20 and 30 min. Animals were deemed to be recovered once the animal was able to re-right itself. Post-recovery, the animals were transferred to outdoor tanks with sand substrate and flow through sand-filtered seawater where they remained for four weeks to monitor survival and recovery. The experiment was repeated in triplicate.

The dosages of menthol/ethanol anaesthetic were assessed for usefulness in aquaculture based on mean time taken to anaesthetise all individuals (ANOVA) with the control group removed. Relaxation properties and evisceration response was also considered when determining the effectiveness of the dosage.

2.2.1.2 Effect of menthol in sea cucumber anaesthesia

The effect of menthol in the anaesthetic solutions was assessed using the methods described above with the exceptions that i) one animal (mean mass 331 g ± 68 sd) was used per treatment in 5 L aquaria using 2% solutions of menthol saturated ethanol or ethanol only, in seawater, and ii) recovery of the animals post anaesthesia was

determined by the time taken for each individual to re-right at 10, 20 and 30 min after being transferred from anaesthetic to clean seawater.

2.2.1.3 Effect of anaesthesia on body morphology, recovery and gonad development of *H. scabra* post-biopsy

An experiment was conducted from November 2011 to January 2012 to assess surgical biopsy under anaesthesia, as a method of sampling the gonadal tissues of *H. scabra* in reproductive studies. Sixteen females that were anaesthetised for the biopsy procedure and fourteen females that were not anaesthetised for the procedure were used in the trial. Samples of ovary tissue of both anaesthetised and non-anaesthetised *H. scabra* were collected prior to the animals undergoing a six week maturation period. The maturation of the animals was assessed before and after the conditioning period and compared between animals that were anaesthetised and not anaesthetised.

2.2.2 Gonad sampling

2.2.2.1 Anaesthesia

Adult *H. scabra* were collected from ponds at a brood stock holding facility in Darwin, Australia. The animals were transferred by road (10 km) to the Darwin Aquaculture Centre (DAC) where they were held in tanks with flow-through 1 µm filtered, UV treated seawater (FSW) for 48 h to purge the sediment contained in the gut. Anaesthesia was induced in aquaria containing 10 L of 2% menthol/ethanol seawater solution prepared as described previously. Animals to be anaesthetised were placed into the anaesthetic solution with aeration for 1 h. After the animals were anaesthetised they were removed and immediately transferred to the operating table to undergo the biopsy procedure.

2.2.2.2 Surgical biopsy and sampling

Surgical biopsy and sampling procedures were the same for both anaesthetised and non-anaesthetised groups. An incision of ≈30 mm was made longitudinally in the bivium, adjacent to the gonad i.e. on the right side of the dorsal interambualcrum (Figure 2.1).
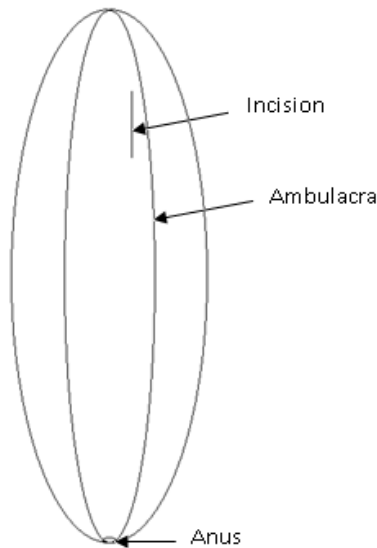
Figure 2.1: Dorsal view of a sea cucumber showing the location of the incision made to excise a sample of the gonad.

The incision was made through the body wall and coelomic epithelium to expose the gonad within the coelomic cavity. Three branched gonad tubules were excised from the gonad basis of each animal. Visual examination of tubules excised from the gonad was used to determine the sex. Males and individuals of indeterminate sex were immediately transferred to recovery tanks and were not used in the experiment. Samples were collected from females prior to them being placed into the experimental maturation system. Tubule length was measured, to the nearest millimetre, from the tip of the most distal tubule branch to the base of the tubule where it attached to the gonad basis. Tubule width was measured at the widest part of the most distal tubule branch using an Olympus CX40 compound microscope, fitted with an eyepiece graticule, at 40 x magnification. Once the tubules were measured, a 1 cm section was removed from each tubule and fixed in 10% formalin in FSW. Fixed sections were used to estimate oocyte number within the tubules. The sections of formalin fixed tubule were placed onto a glass microscope slide and the oocytes were separated from the tubule wall by gently applying pressure along the outer surface of the tubule wall forcing the oocytes out one end of the sectioned tubule. The separated oocytes were then suspended in 5 ml of FSW, a 1 ml aliquot was taken and the number of oocytes within the aliquot was determined using a Motic SMZ-140 stereoscopic microscope and a Ward counting wheel. The number of oocytes in the 1 ml aliquot was multiplied by 5 to estimate the number of oocytes for each 1 cm section of tubule. Oocyte size frequency within the

tubules was estimated by measuring the diameter of each of thirty oocytes including the jelly coat (if any). Oocytes were measured using an Olympus CX40 compound microscope and eyepiece graticule at 100 x magnification.

2.2.2.3 Conditioning

At post-biopsy, animals were transferred to assigned tanks in the experimental brood stock culture system. The system was set up in three blocks of ten tanks with each block assigned five treatment and five control animals except for the 2nd block which was assigned four treatment and six control animals. Anaesthetised (treatment) animals were assigned to a tank using a random number generator to nominate either the right or left tank for each row of each block of tanks.

The experimental maturation system was constructed using thirty 200 L polyethylene tanks containing 35 L of fine washed sand (grain size <1500 µm) per tank as substrate (Fig. 2.2). Each tank was supplied with aeration and flow-through FSW at an exchange rate of 350 L day^{-1}. The tanks were pre-conditioned for 17 days prior to the experiment. Pre-conditioning involved addition of 40 g of organic fertiliser, pelleted chicken manure (Tropigrow, Darwin) that had been ground into particle sizes of <2 mm.

Animals were monitored daily for the six week conditioning period. Each week the tanks were fertilised with 40 g of the ground fertiliser mixed with 150 ml of seawater and spread throughout each tank.

Figure 2.2: Experimental system for *Holothuria scabra* brood stock maturation trials. Each tank supplied with filtered seawater and aeration. Set up was in a temperature controlled room.

After the conditioning period all test animals were purged for two days, anaesthetised and their ovary assessed once again, as described above. The mass (g), length (mm) and width (mm) of the animals were recorded before anaesthesia and again whilst anaesthetised.

2.2.2.4 Analysis

Effects of the surgical biopsy method on ovarian condition was assessed by comparing mean oocyte size, oocyte number, tubule diameter and tubule length from both treatment groups before and after the conditioning period. The effect of each surgical biopsy method on the general health of the animals was assessed by comparing the mean survival and mass change of individuals in each group using Student's t test. Oocyte size and reproductive stage frequency histograms were used to compare the ovarian development of each treatment group before and after conditioning. The differences in proportions of females at each reproductive stage from each treatment group were tested for statistical significance using Fisher's Exact Test (FET). The body metrics (mean mass, length and width) of the animals' pre and post anaesthesia were compared using paired t-tests. Where appropriate, Student's t-test were used to

compare differences in the mean ovary size measurements and Mann-Whitney U tests were used to test for significance in the oocyte size and number between treatment groups. Paired sample t-tests and related samples Wilcoxon signed rank tests were used to assess the effect of the conditioning period within the treatment groups.

2.2.2.5 Assessment of the reproductive condition of H. scabra in the Northern Territory, Australia

One hundred adult *H. scabra* were collected, by diving, along the coast of the Northern Territory between Darwin and Croker Island (11°S) in May 2012. The obtained animals were weighed to the nearest gram using an electronic balance and then dissected. Gut contents were removed except for the right respiratory tree. The gonad, if present was collected, drained and weighed. The body wall was drained and also weighed (gutted weight). Gonad index was calculated as; *gonad weight / gutted weight x 100*.

A few tubules of each gonad were fixed in 10% formalin in FSW and transported to the laboratory at the DAC for determination of reproductive stage using a microscope. The maturity scale used in this study is based on that developed for *H. scabra* populations in the Solomon Islands by Ramofafia *et al.* (2003). The authors describe the stages based on gonad morphology and histology as; Stage 1: Indeterminate, Stage 2: Growing, Stage 3: Mature, Stage 4: Partly spawned, Stage 5: Spent (See Ch. 1). Reproductive stage was assigned by visual inspection of the gonad using the stages defined by Ramofafia *et al*. (2003). Briefly, stage 1 or indeterminate sex, was assigned to animals with gonads lacking gametes, usually with small transparent or brown tubules. Stage 2, growing, was assigned to animals with gonads with small to medium tubules with thick tubule walls and for ovaries with small oocytes between 40 -150 μm in diameter present. For males with stage 2 testes, sperm was present in only parts of the tubule or at a lower density throughout the tubules. Stage 3 gonads were mature and only large gonads with thin tubule walls containing densely packed large oocytes or sperm were included in this category. Stage 4 partly spawned, included those with tubules with thin walls and variable amounts of fully grown oocytes or densely packed sperm in different tubules. Stage 5 were spent gonads with long shrivelled tubules containing some relict oocytes or few pockets of residual sperm.

Body metrics of mass, gutted mass and gonad index were compared for each gametogenic stage and sex. Stages of mature and part spawned were pooled into a group nominated 'spawning' for the analysis to even the sex ratio for each reproductive stage. The indeterminate animals were excluded from the analysis.

2.2.3   Analysis of tagging methods

2.2.3.1 Individual identification tags for tank based maturation trials

Seventy two *H. scabra* with an average mass of 253 g ± 53 (sd) were allocated to 12 tanks containing a 50 mm layer of fine washed sand, aeration and flow through sand filtered seawater. The tanks were covered with green 80% shade cloth covers.

To individually identify each of the six individuals in each tank they were marked by applying a line approximately 50 mm long on the dorsal epidermis with Cyanoacrylate adhesive. The adhesive, once cured, was shed by the sea cucumbers and with it the outer layers of the dermis containing the skin pigment, leaving behind a scar (Fig. 2.3). The glue scar was located in a unique position for each of the animals (1-6) in each tank (Table 2.1).

Figure 2.3: Glue scar identification tag on *Holothuria scabra.* Arrow indicates location of glue-scar tag.

Table 2.1: Location of glue scar tags for individuals within each conditioning tank.

Individual	Tag location
1	Right posterior bivium
2	Left posterior bivium
3	Right centre bivium
4	Left centre bivium
5	Right anterior bivium
6	Left anterior bivium

The tagged animals were conditioned in the tanks for 6 months and checked weekly or biweekly for growth and the presence of the tag and re-marked as necessary. Reproductive stage was checked twice during the conditioning period after 3 and 5

months using a needle biopsy as an alternative to the surgical biopsy method described above.

Animals were anaesthetised in 2% solution of menthol/ethanol in seawater using the method previously described. To sample the gonad, a 16 G needle attached to a 1 ml syringe was inserted into the coelomic cavity of the sea cucumber on the right hand side of the animal, about one third the length of the individual from the anterior. Slight suction was applied using the syringe to extract gonad tissue and gametes. The samples were observed under a dissecting microscope to assign reproductive stage as described above. Repeat samplings were conducted where necessary to obtain sufficient sample to assign reproductive stage.

Growth of the animals in the conditioning system was assessed by analysing mean growth rate and comparing mean mass for each tank graphically. Growth rates were estimated using linear regression and expressed as g day^{-1}. Growth rates were compared to daily minimum temperature data downloaded from the Australian Government Bureau of Meteorology Climate Data online tool for the nearby weather station 01472.

2.3 Results

2.3.1 Anaesthesia of adult *H. scabra*

Anaesthetic solutions slowed and stopped altogether the righting reflex of *H. scabra.* The mean righting reflex response time of *H. scabra* was determined to be 29.15 s ± 24.36 (sd.), n=64. In the anaesthetic trial righting reflex was rapidly reduced in the 3% and 4% solutions. However, evisceration was observed in the 4% treatment, and although loss of righting reflex was observed in the 3% treatment, relaxation was variable. In some cases animals in the 3% solution tended to tense up and did not appear to become relaxed in the solution. Auto evisceration response occurred in the 4% treatment after 10 and 20 min; mean cumulative evisceration of animals in the 4% solution was 26.6% (1.33 ± 0.88 se / 5) after 10 min and 53.3% (2.66 ± 0.33 se / 5) after 20 min exposure. The 4% treatment was therefore excluded from further analysis.

Righting reflex was also inhibited in the 2% solution which was effective at anesthetising 100% (5/5) of the animals within 30 min of exposure (Fig. 2.4). No evisceration or tensing of the body wall was observed. The 1% solution was less effective

at inducing loss of righting reflex, requiring 60 min exposure to anaesthetise 93% of the animals (Fig. 2.4). No loss of righting reflex occurred in the 0% menthol/ethanol control treatment (Fig. 2.4).
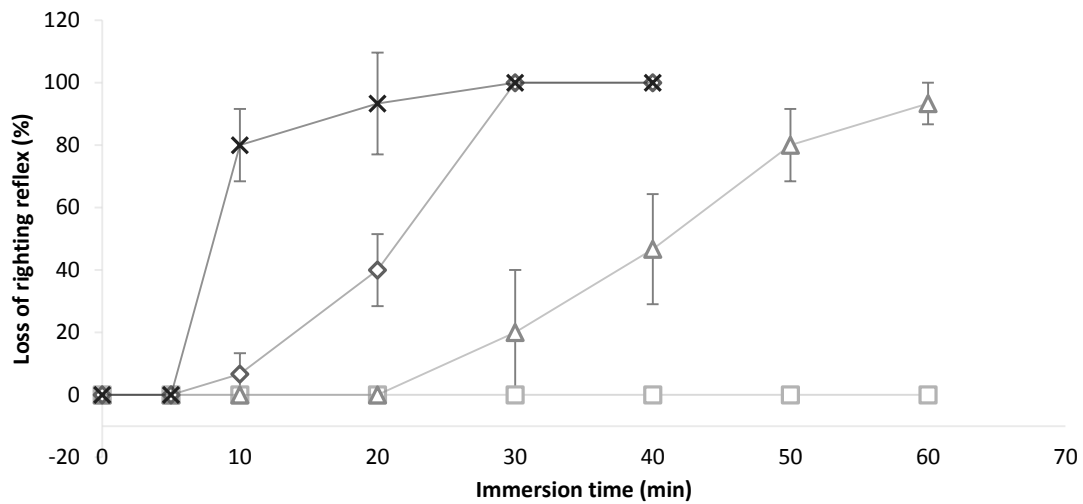
Figure 2.4: Percent *Holothuria scabra* anaesthetised in ethanol/menthol solution. 0% (squares), 1% (triangles), 2% (diamonds) and 3% (crosses) anaesthetic solution. n = 3, error bars are ± 1 s.e.

There was a significant effect of concentration for the mean time taken to anaesthetise all of the individuals in the samples at α=0.05 level ($F_{3,\ 8}$= 21.857, p=0.0003). Post-hoc pairwise comparisons with Bonferroni adjustment revealed that the 1% treatment was significantly less effective at inducing loss of righting reflex than the 2% and 3% solutions.

Change in body shape was associated with anaesthesia in the 1% and 2% anaesthetic treatments with *H. scabra* becoming elongated (relaxed) as the anaesthesia took effect. Oral features were exposed and the body of the animal became limp and soft.

2.3.1.1 Recovery of *H. scabra* post anaesthesia

Righting reflex returned in all animals in the 1% and 2% treatments after 20 and 30 min, respectively. At higher concentrations (3% and 4%), righting reflex had not returned in all individuals after the 60 min recovery period (Fig. 2.5). No mortality had occurred six weeks post-experiment and all animals from each treatment group appeared healthy.
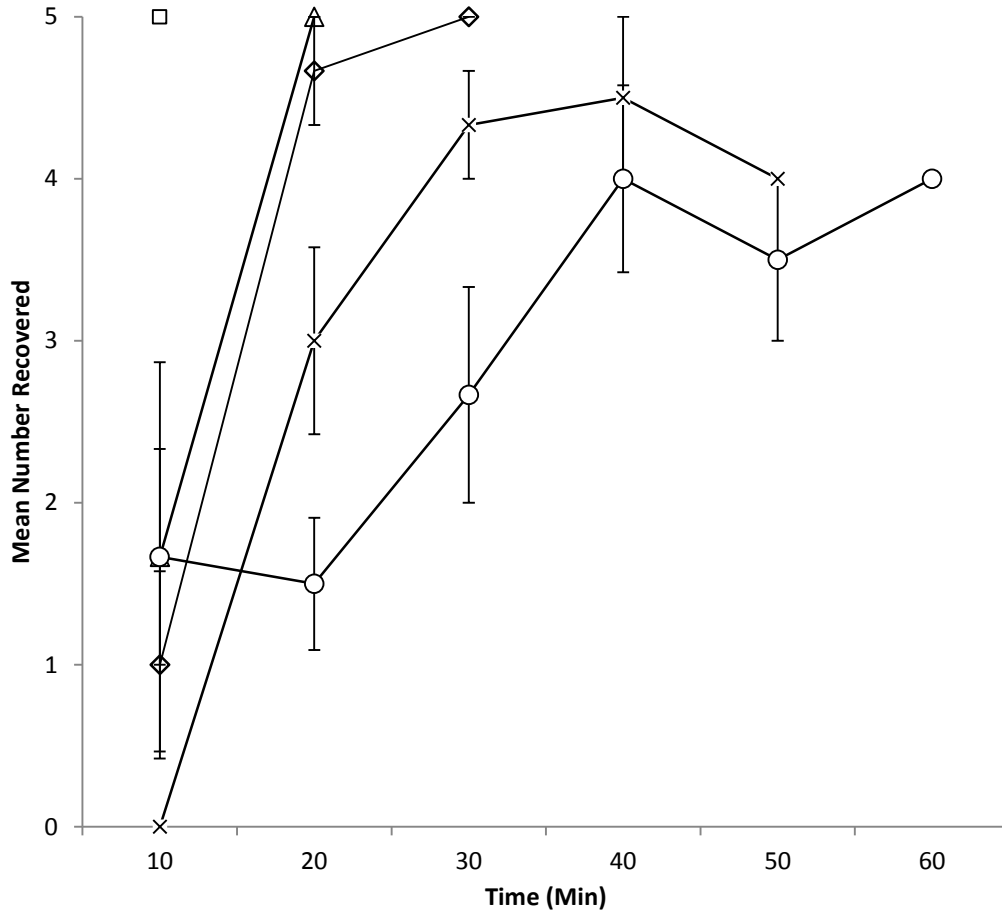
Figure 2.5: Recovery rate of *Holothuria scabra* post anaesthesia with 0% (square), 1% (triangles), 2% (diamonds), 3% (crosses) and 4% (circles) anaesthetic solution.

2.3.1.2 Effect of menthol in anaesthetic solution

The 2% menthol/ethanol solution effectively anaesthetised 100% (3/3) of the animals after 35 minutes. Ethanol alone did not induce anaesthesia in all subjects even after 40 min exposure (Fig. 2.6). However, loss of righting reflex was observed in 2/3 animals. Ethanol alone did not induce consistent loss of righting reflex. Recovery was slightly delayed in menthol/ethanol treated subjects. After 20 min all animals from both treatments were all righting in a similar time (Fig. 2.7). All animals survived and appeared healthy after one week post-treatment.
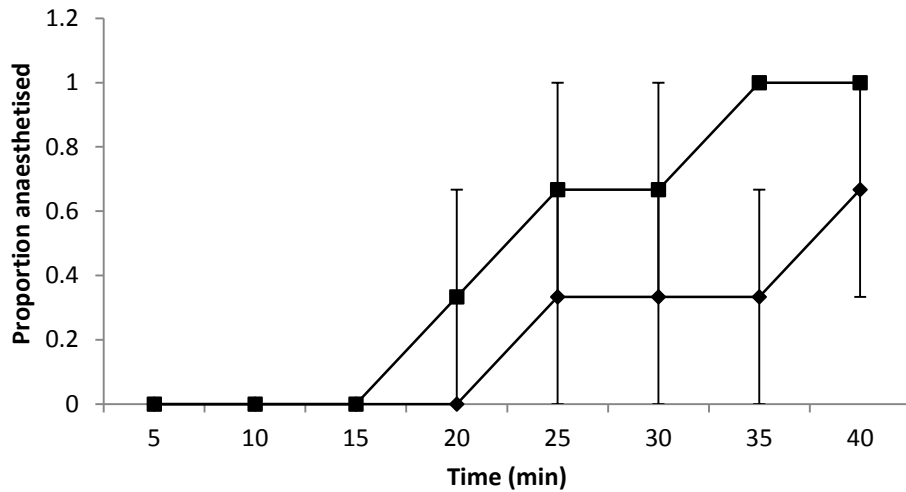
Figure 2.6: Loss of righting reflex of *Holothuria scabra* in 2% solution of menthol ethanol (squares) and ethanol (diamonds). Error bars represent ± SE.
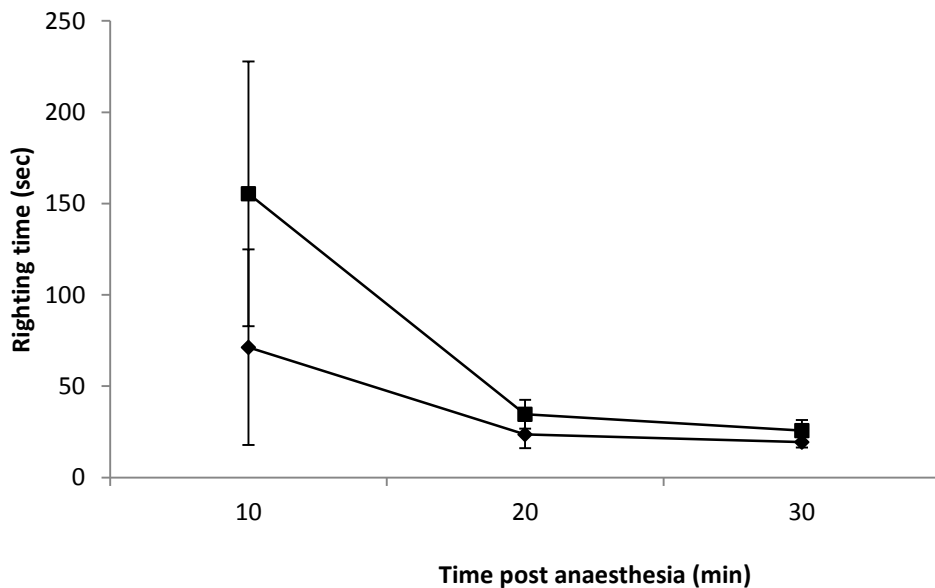
Figure 2.7: Recovery of *Holothuria scabra* post anaesthesia using 2% menthol/ethanol (squares) and 2% ethanol (diamonds) solutions. Error bars represent ± SE.

2.3.1.3 Anaesthesia as an aid in the biopsy of gonad tissue

2.3.1.3.1 Effect of anaesthesia on body metrics

At the end of the conditioning trial the animals were measured pre-anaesthesia and during anaesthesia to quantify the changes in body shape and mass resulting from anaesthesia. There was no significant difference in the mass, length or width of the animals between treatment groups so data were pooled for the analysis of changes in body shape induced by anaesthesia.

Anaesthesia caused significant changes in mass (t = -2.277, df = 26, P = 0.03), length (t = -12.477, df = 26, P <0.001) and width (t = 8.762, df = 26, P < 0.001) measurements between animals, pre and during anaesthesia. The mean change in mass was 2.5 g (99% CI: 0.5 to 5.6 g) or a mean increase post anaesthesia of 0.22%, n =27 (Fig. 2.8A). The mean width was reduced by 13.7 mm (99% CI: 9.3 to 18 mm) or a mean reduction of width of 21.1%, n=27 after an average anaesthetic exposure time of 80min (Fig. 2.8B). There was also an associated mean increase in length of 54.4 mm (99% CI: 42.3 to 66.6 mm) or a mean increase in length of 17.3%, n =27 (Fig. 2.8C).
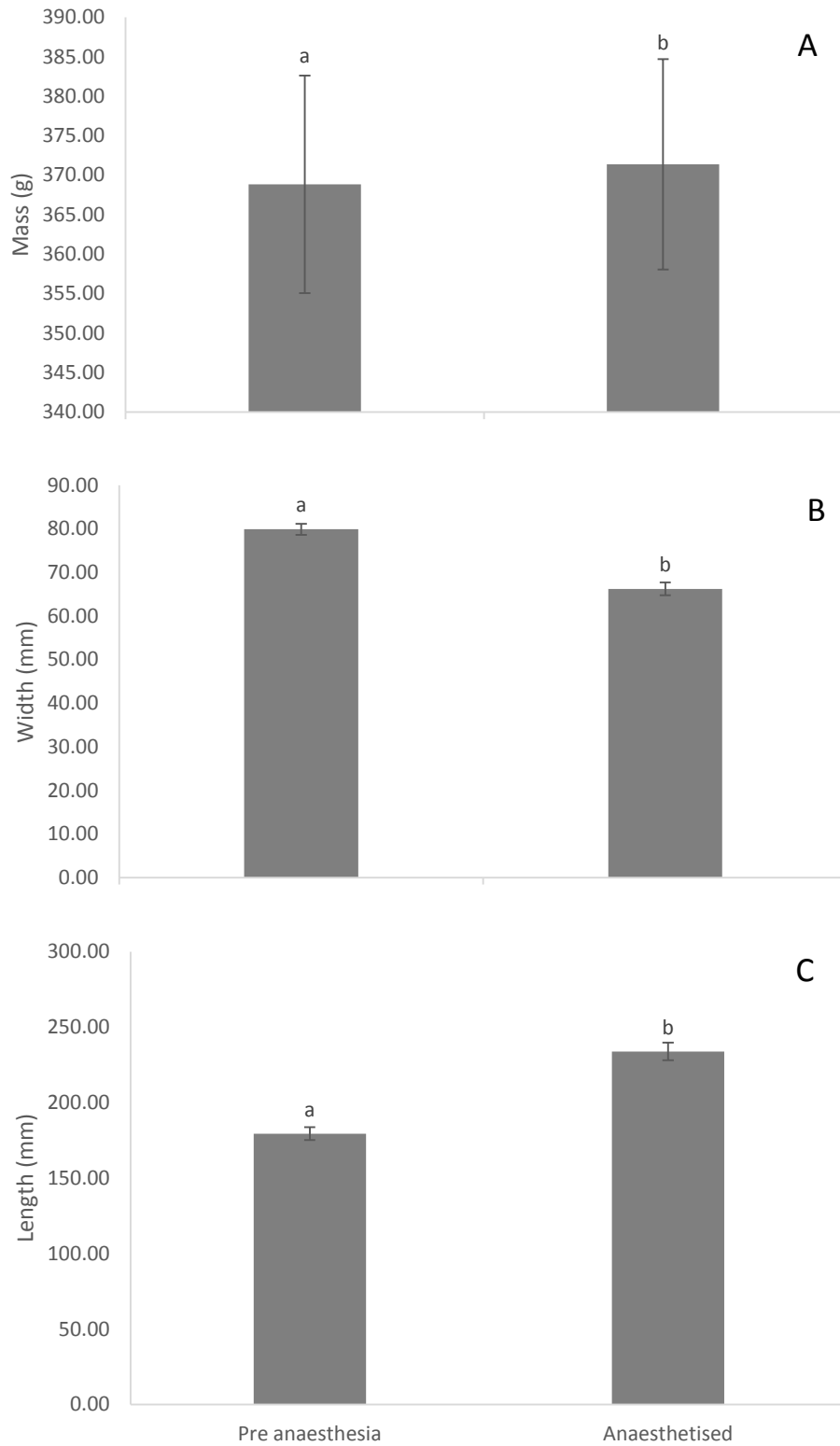
Figure 2.8: Change in body metrics associated with anaesthesia in adult *Holothuria scabra.* Anaesthesia increased mass (A), decreased width (B) and increased length (c). Error bars represent ± 1 SE. n = 27. Different superscript represent significant difference (α=0.05).

The purging process at the end of the conditioning trial resulted in a mean mass decrease in both control and anaesthetised groups. The mass reduction associated with the purging process was not significantly different between treatment groups (t= -0.530, df = 26, P=0.6). The mean mass reduction during purging for the anesthetised animals was 22.9 g ± 63.2 sd and the mass loss for the control group was 13.7 g ± 21.5 sd.

2.3.1.3.2 Effect of anaesthesia on gonad development

Eighty animals were biopsied to obtain 30 discernible females for the conditioning trial. Of those, 37 were anaesthetised and 43 not anaesthetised. Auto evisceration was caused by the biopsy procedure in some animals in the non-anaesthetised group with 13% (5/37) eviscerating during the biopsy procedure. None of the anaesthetised animals auto-eviscerated during biopsy. However, one individual eviscerated in the anaesthetic solution.

At the beginning of the trial the ovaries of the trial animals were in three distinguishable stages; growing, part spawned and spent (Fig. 2.9). There were also 18 animals sampled with indeterminate gonads in the initial biopsy. As these were not able to be sexed they were not included in the trial. The proportion of females in each reproductive stage at the beginning of the trial were not significantly different between treatment groups (FET P =0.711).

Post conditioning, a greater diversity of gametogenic stages was recorded between the treatment groups. In those not anaesthetised during the initial biopsy, each of the five gametogenic stages were recorded and in the group anesthetised during the initial biopsy; growing, mature, part spawned and spent ovaries were recorded (Fig. 2.10). Gonad stage ratios were not significantly different between anesthetised and non-anaesthetised groups at the end of the conditioning period (FET P =0.162).
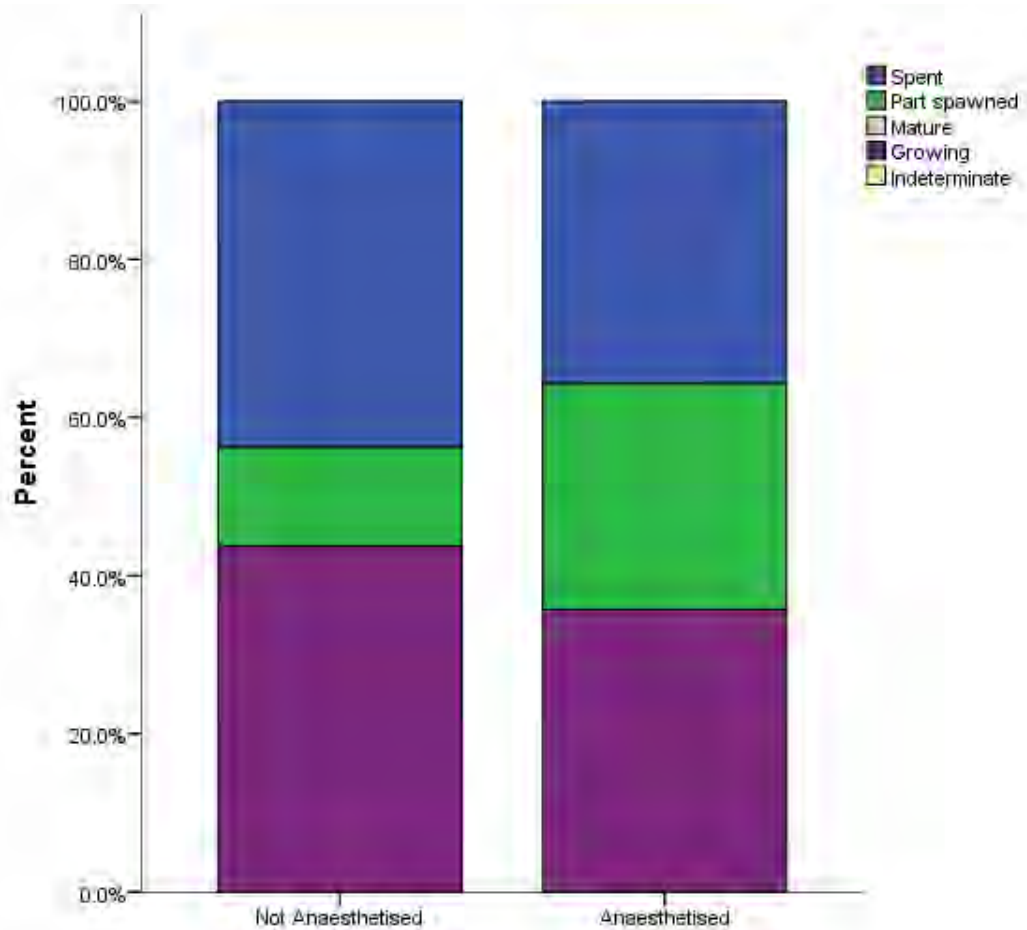
Figure 2.9: Proportions of female *Holothuria scabra* anaesthetised during gonad biopsy and not anaesthetised during gonad at each stage of ovary development pre conditioning. Ovary stages are 1 – indeterminate, 2 – growing, 3 – mature, 4 – part spawned, 5 – spent. n = 14 (anaesthetised), N=16 (non-anaesthetised).
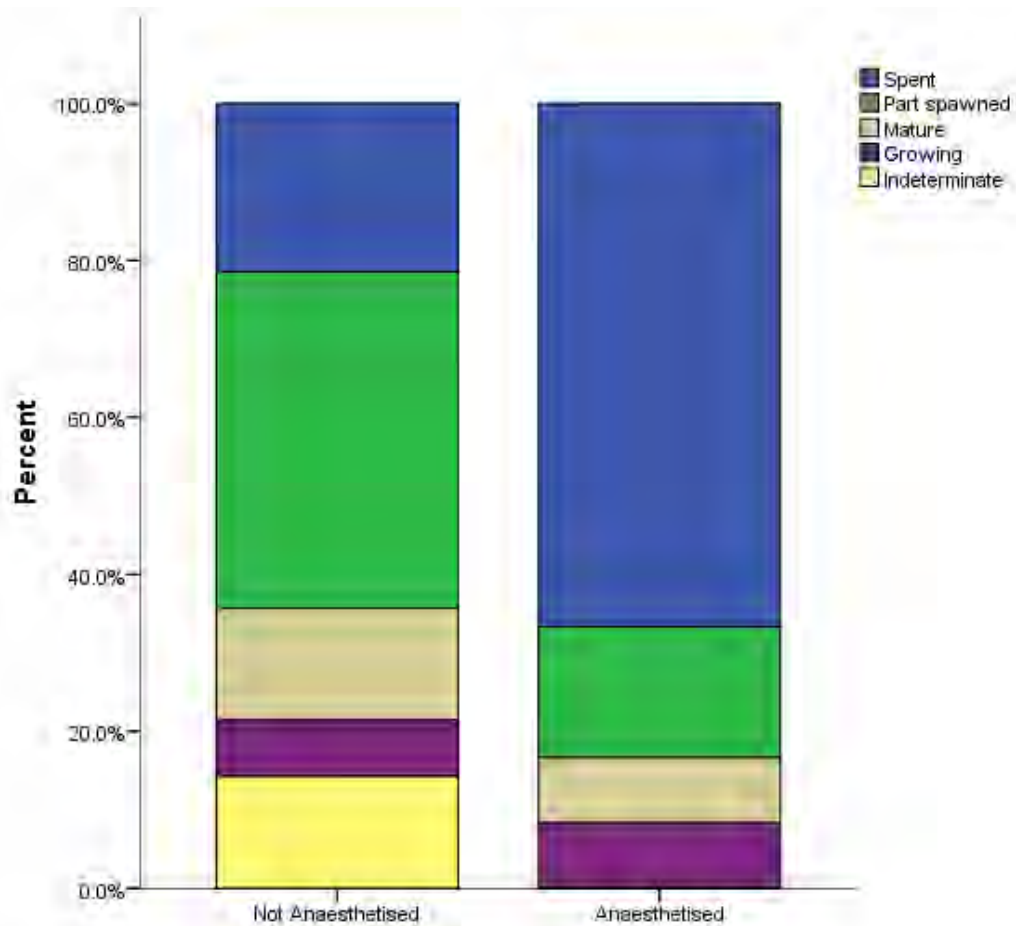
Figure 2.10: Proportions of female *Holothuria scabra* anaesthetised during gonad biopsy and not anaesthetised during gonad at each stage of ovary development after six week conditioning period. Ovary stages are 1 – indeterminate, 2 – growing, 3 – mature, 4 – part spawned, 5 – spent. n = 12 (anaesthetised), N=14 (non-anaesthetised).

Mass loss of 17% was recorded for *H. scabra* that were anasthetised before the initial biopsy (76.46 g ± 56 sd) and 16% for those not anaesthetised for the initial biopsy (72.47 g ± 47.58 sd), over the six week conditioning period. The mean mass loss observed during the conditioning period was not significantly different between the treatment groups (t_{26}=0.203, P=0.841). However, the conditioning period did result in significant mass loss within both groups; mean 443 g ± 66 sd to 370 g ± 67 sd (t_{14} =5.898, P =0.000) for the control group, mean 441 g ± 57 sd to 365 g ± 77 sd (t_{12} =4.875, P =0.000) for the anaesthetised group (Fig. 2.11).
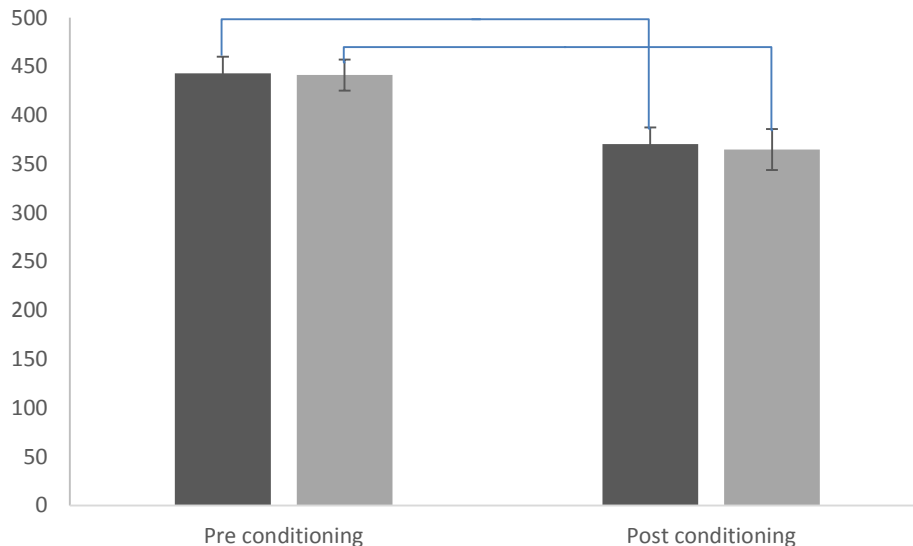
Figure 2.11: Change in mass of *Holothuria scabra* female brood stock that were either anaesthetised (light grey) or not-anaesthetised (Dark grey) for gonad biopsy prior to six week conditioning period. Error bars are ± se. Lines connect significant differences within groups (α=0.05).

There was no significant difference in the mean oocyte number (570.60 ± 639.7, 438.67 ± 596.1) (Fig. 2.12), mean oocyte size (112.61 ± 61.1, 107.19 ± 35.0 μm) (Fig. 2.13), mean tubule diameter (832.19 ± 320.0, 953.69 ± 502.4 μm) (Fig. 2.14) at the beginning of the trial between control and anaesthetised groups respectively. There was, however, a significant difference between the initial tubule length (25.19 ± 8.3, 35.95 ± 13.7 mm) of the groups (t_{26} =2.798, P =0.01) (Fig. 2.15).

After the six week conditioning trial no significant differences were detected in the ovary parameters of mean oocyte number (533.74 ± 678.9, 384.25 ± 616.3), mean oocyte size (103.50 ± 79.6, 94.54 ± 70.2 μm), mean tubule diameter (890.19 ± 453.3, 718.06 ± 391.3 μm) or tubule length (30.21 ± 18.4, 25.25 ± 9.8 mm) between the control and anaesthetised groups respectively (See figures 2.12, 2.13, and 2.15).

The conditioning period had no detectable within-group effect on any of the gonad parameters except for the decrease in mean ovary tubule diameter in the anaesthetised group which was weakly significant (t_{11} =2.280, P =0.044). This result was interpreted with caution due to the weak significance and because the Pearson correlation also showed that the decrease in ovary tubule length was not very consistent across all subjects (R =-0.112, P =0.729 n =12).
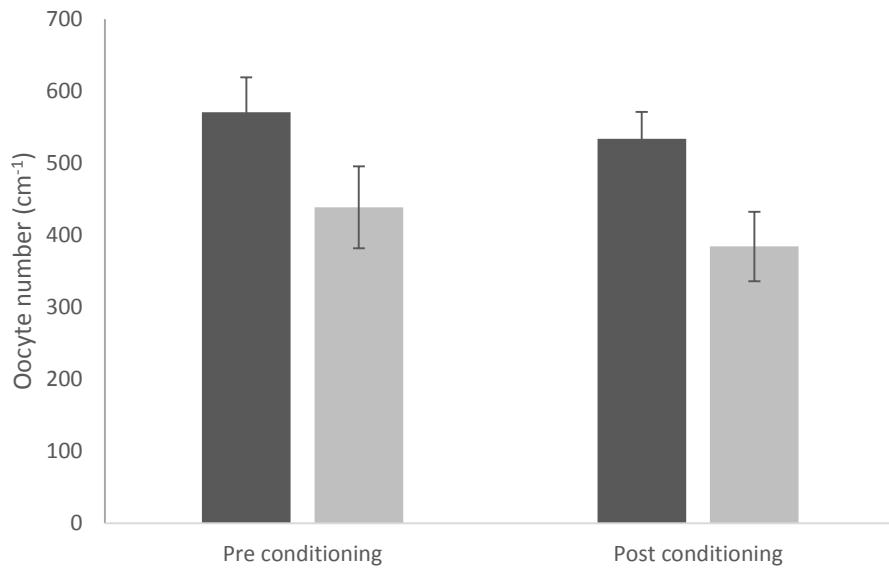
Figure 2.12: Mean number of oocytes per cm of ovary tubule before and after conditioning for anaesthetised (dark grey) and non-anaesthetised (light grey) *Holothuria scabra*. Error bars represent ± 1 SE.
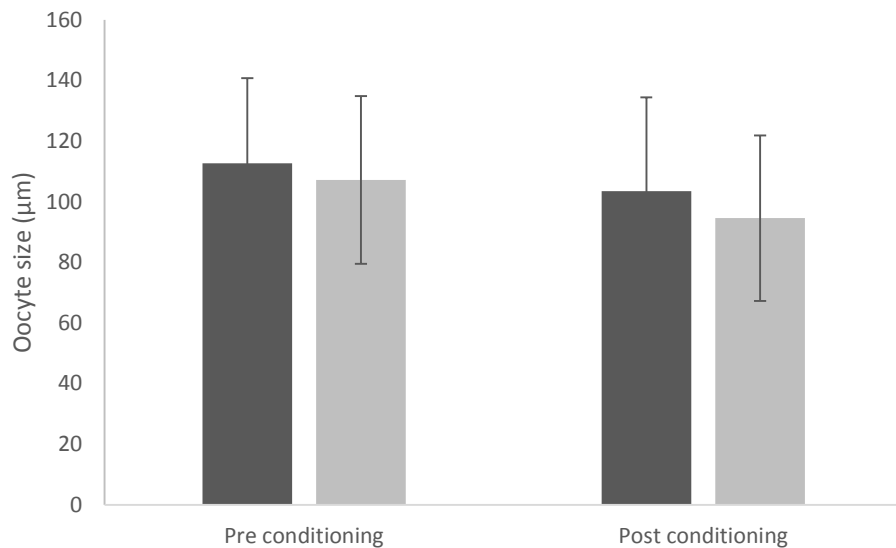
Figure 2.13: Mean oocyte size before and after six week conditioning period for *Holothuria scabra* anaesthetised (dark grey) and non-anaesthetised (light grey) during biopsy. Error bars represent ± 1 SE
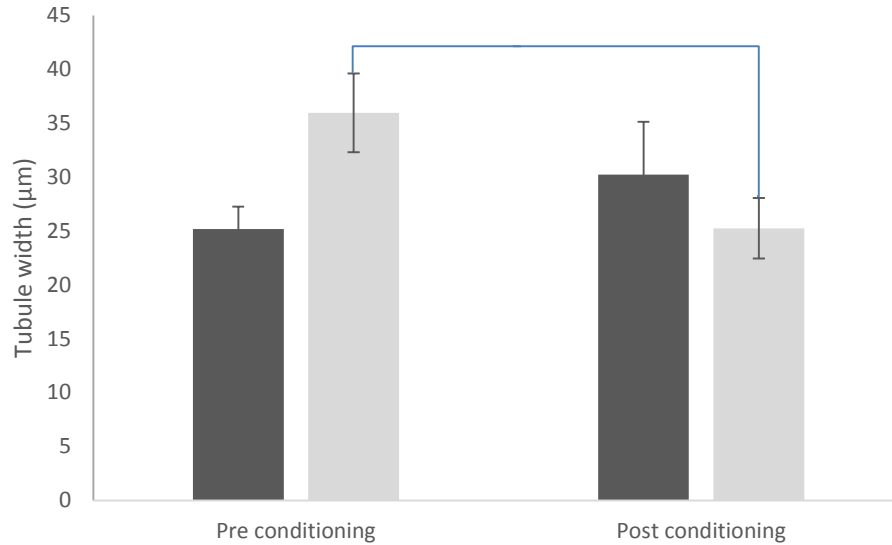
Figure 2.14: Mean tubule width before and after conditioning for anaesthetised (dark grey) and non-anaesthetised (light grey) *Holothuria scabra*. Error bars represent ± 1 SE. Lines represent significant difference (α=0.05).
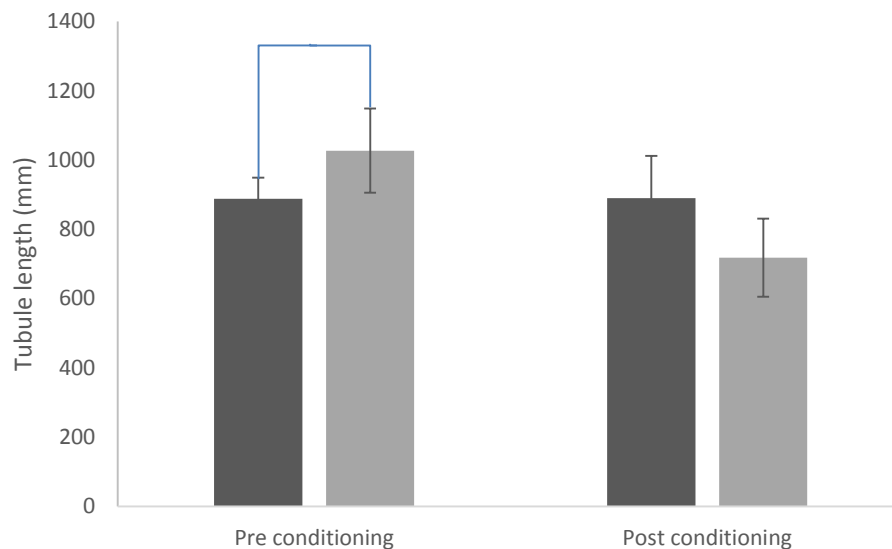
Figure 2.15: Mean ovary tubule length before and after conditioning for anaesthetised (dark grey) and non-anaesthetised (light grey) *Holothuria scabra.* Error bars represent ± 1 SE. Lines represent significant difference (α =0.05).

Oocyte size frequency of anaesthetised and non-anaesthetised females became more pronouncedly bimodal after the conditioning period. At the initial biopsy the oocyte size frequency within the ovaries of the females in both groups showed a full range of oocyte sizes from 20-250 μm with a mode of 30-40 μm and another smaller peak at 190-200 μm (Fig. 2.16 A, B). After the conditioning period the oocyte size frequency in the anaesthetised group had a mode of 10-20 μm and other smaller peaks at 150-160 μm and 190-200 μm with very few intermediate size oocytes (Fig. 2.16C). The

oocyte size frequency distribution of the control group post conditioning was similar with a mode of 190-200 µm and another smaller peak at 10-20 µm, again with few or no oocytes of intermediate size (Fig. 2.16D).
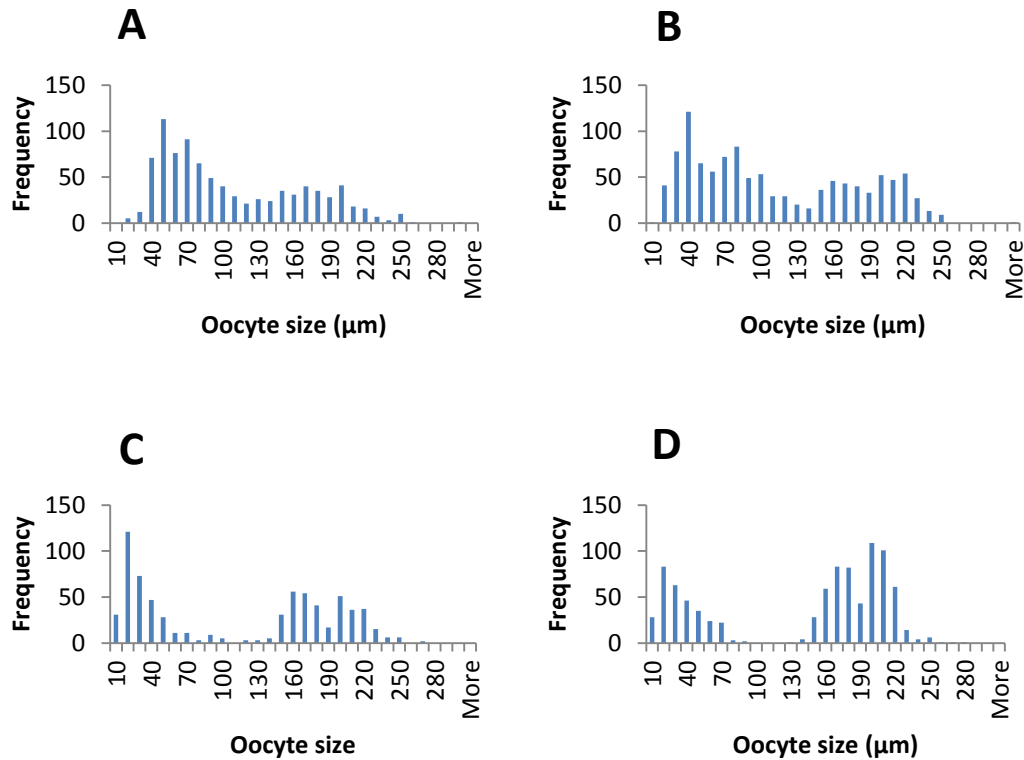
Figure 2.16: Pooled oocyte size frequency histograms for anaesthetised (A and C) and non-anaesthetised (B and D) treatments before the conditioning (Top) and after the conditioning period (bottom).

There was no difference in mortality between the two groups. During the conditioning period one animal from each treatment group died. One individual from the anaesthetised group died 14 days after the procedure and one individual from the control group died 30 days after the procedure.

2.3.2 Assessment of gonad condition of wild *H. scabra* in the Northern Territory, Australia

To assess the application of the *H. scabra* maturity scale to a Northern Territory population, one hundred adult *H. scabra* were collected. The full range of gametogenic stages were observed in both male and female *H. scabra*. Of the 100 animals sampled, four individuals were lacking a discernible gonad and therefore were not sampled and 10 individuals were of indeterminate sex. There were more males than females (7:1) in the mature stage, and because of this mature and part spawned stages were combined for further analysis. Overall

the proportion of *H. scabra* at each stage was not significantly different between females and males (FET =0.188, P =0.955) (Fig. 2.17). The female to male sex ratio of the *H. scabra* collected was not significantly different from 1:1 (Binomial test, n =85, P =0.664).
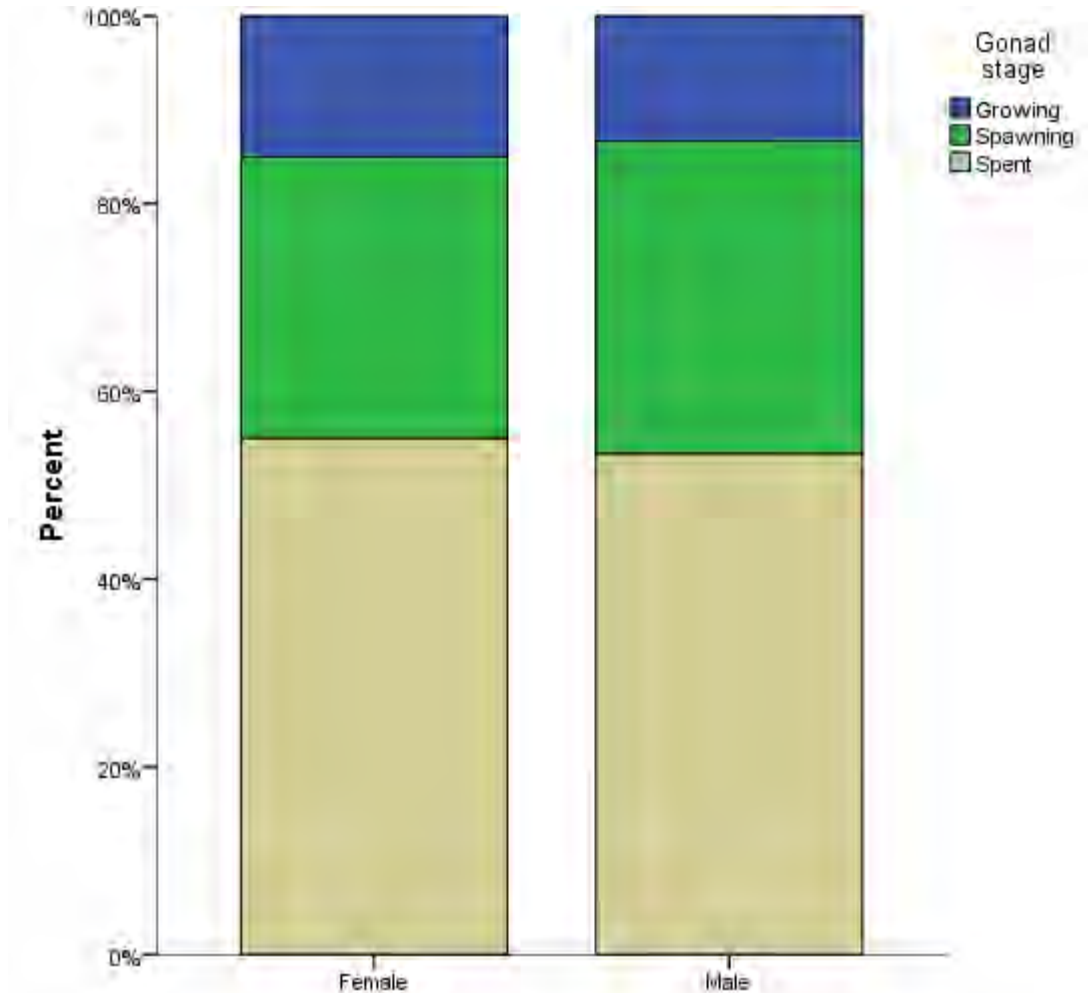
Figure 2.17: Proportion of male and female *Holothuria scabra* at each reproductive stage. n =40 Female and 45 Males.

The mean whole body mass and mean gutted mass did not significantly differ between male and female *H. scabra* (Welch F_1 =1.572, P =0.214 and Welch F_1 =1.39, P =0.289 respectively). The mean gutted weight was significantly different across reproductive stages in both males (Welch F_1 = 11.043, P =0.002) and females (Welch F_1 = 7.763, P =0.006) (Fig. 2.18). For males, Games-Howell multiple comparison revealed that the gutted mass of *H. scabra* in spawning stage was significantly higher than the gutted weight of males in the spent stage (P =0.000) but not significantly different from the mass of those in the growing stage. On the other hand the data in the female group

showed that the gutted mass of the spawning group was significantly higher than both the growing and spent stages (Fig. 2.18).
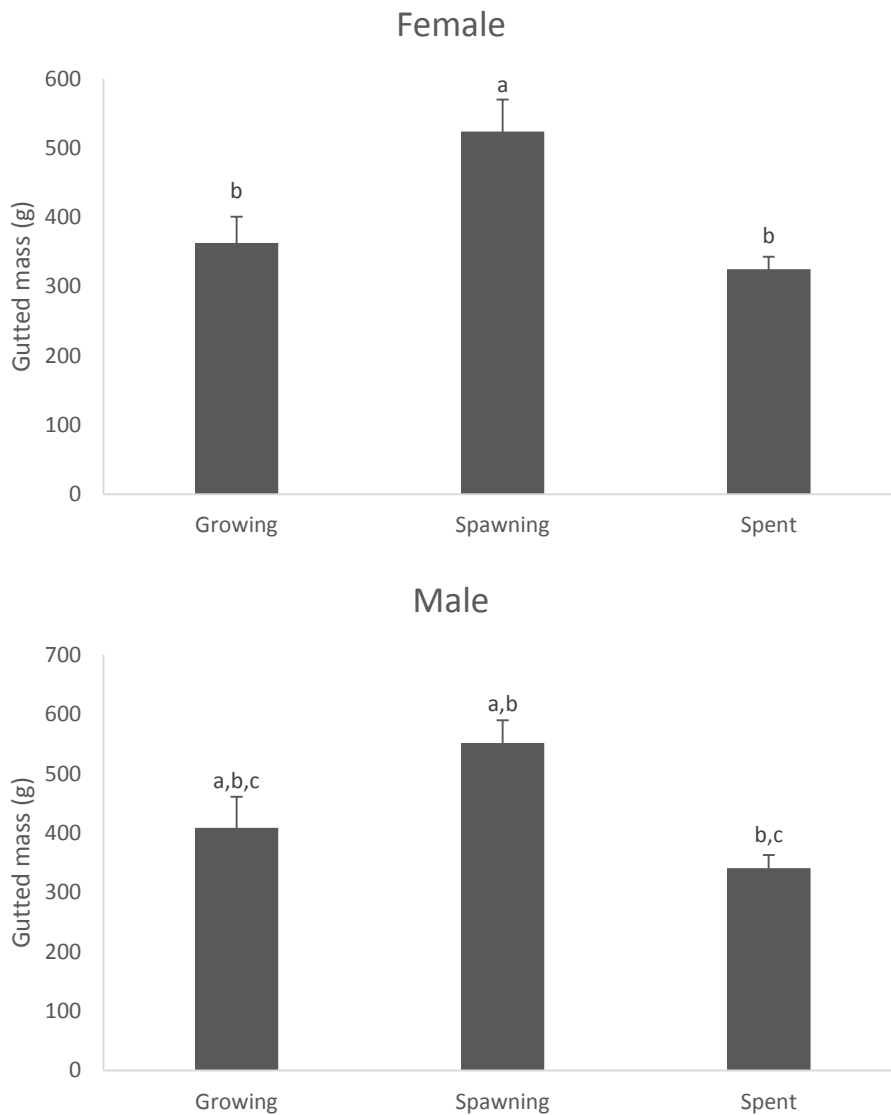
Female

Male

Figure 2.18: Gutted weight for female and male *Holothuria scabra* at each reproductive stage. Error bars are ± SE. Different superscripts denote significant difference at α = 0.05.

The mean gonad index for males was lowest for individuals in the spent stage (0.65 ± 0.70) followed by those in the growing stage (2.23 ± 0.92) and as expected highest for those in the spawning stage (5.68 ± 3.40). For females, the GI followed a similar trend to that of the males at each stage with values of 0.85 ± 0.95, 1.79 ± 0.78 and 6.99 ± 5.06 for the spent, growing and spawning stages, respectively (Fig. 2.19).
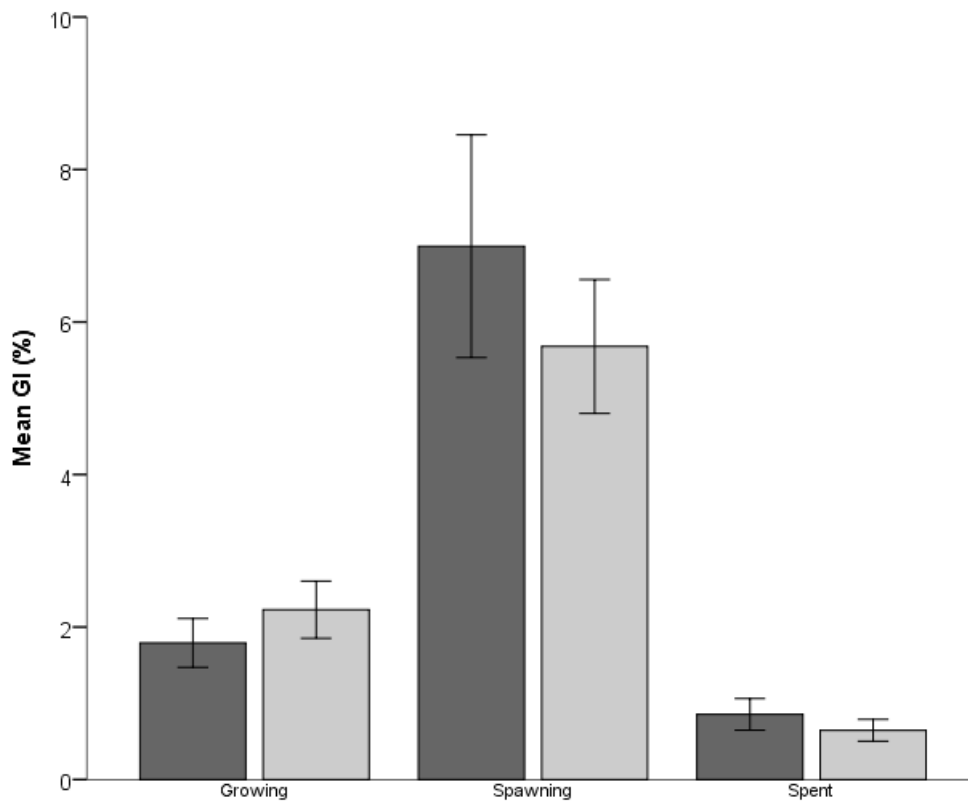
Figure 2.19: Gonad index of female (dark grey) and male (light grey) *Holothuria scabra* at each reproductive stage collected in the Northern Territory, Australia.

2.3.3 Glue-scar tagging of *H. scabra* brood stock

2.3.3.1 Growth

The mean size of the *H. scabra* was 250 ± 54 g at the beginning of the trial. The mean mass at the end of the conditioning period increased to 330 ± 79 g. Individual growth rates varied from -0.216 g day^{-1} to 1.135 g day^{-1} (mean= 0.328 ± 0.256 g day^{-1}). Whilst the growth of some individuals was negative, the mean growth rates of each tank and overall were positive.

There were no significant differences in the mean mass between each tank at the initiation of the trial ($F_{11, 60}$ =0.757, P =0.68). However, at the end of the trial there was a significant difference in the mean mass between tanks ($F_{11, 60}$ =4.418, P =<0.001). Two of the tanks had distinctively low growth rates; *H. scabra* in tank 4 and tank 9 did not gain mass over the conditioning period as well as the other tanks (Fig. 2.20). Mean growth rates over the conditioning period were significantly different between tanks ($F_{11, 60}$ =2.378, P =0.016).
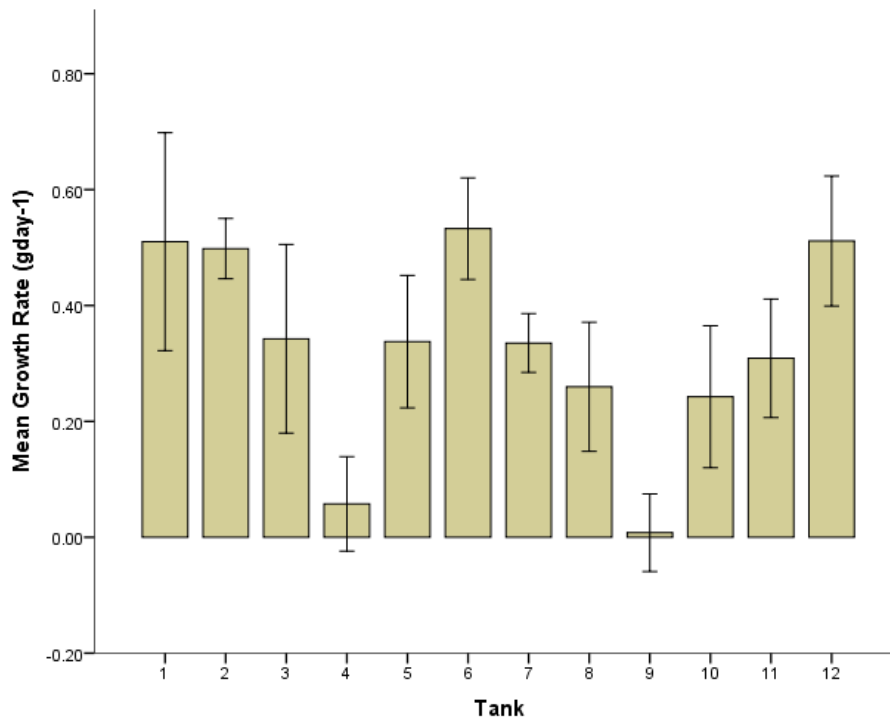
Figure 2.20: Mean growth rate of *Holothuria scabra* cultured in 12 conditioning tanks for 7 months (n =6).

There was positive growth early in the conditioning period from April to June followed by a period of negative growth from June to July followed by strong positive growth again from August through to the end of the trial in November (Fig. 2.21). The decline in mass was associated with the cooler months of the year and growth once again became positive when daily minimum temperatures rose.
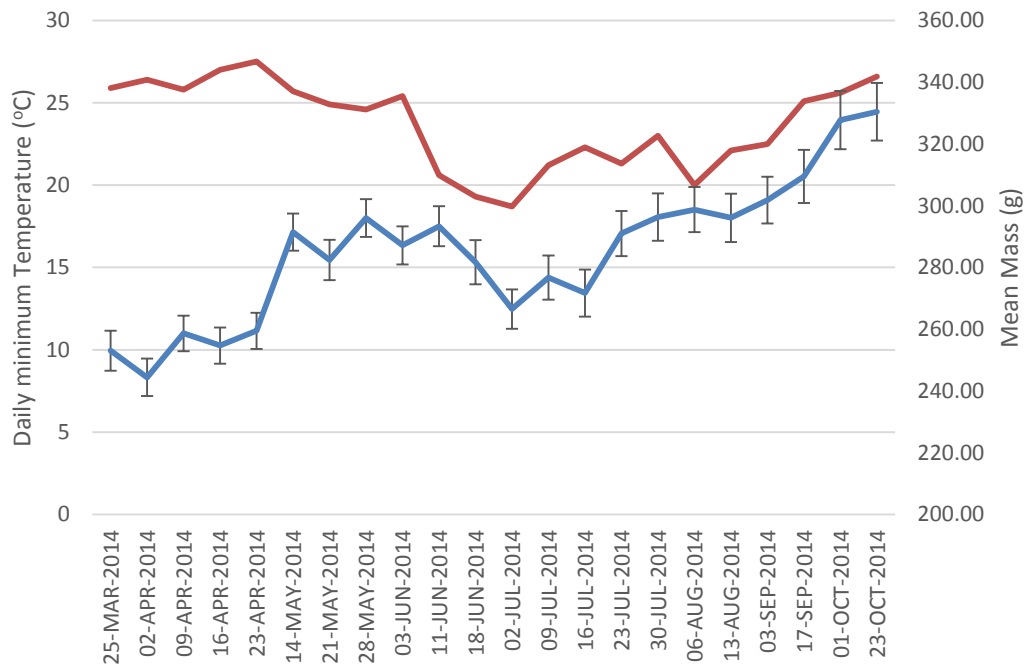
Figure 2.21: Growth rate of *Holothuria scabra* conditioned in tanks between March and October 2014 under ambient conditions (Blue line). Error bars represent ± SE. n =12. Red line shows ambient daily minimum temperatures for the conditioning period.

2.3.3.2 Needle Biopsy

The needle biopsy method was used to sample all of the 72 *H. scabra* at the first maturity check in June 2014. Two months later, 71 of the animals were able to be sexed and staged using this method. A third sampling was conducted three months after the initial check and 65 of the 72 animals were able to be sexed and staged with the needle biopsy method.

The frequency of each gametogenic stage within the population did not differ significantly with time at α =0.05 level (FET). However, there were 10 more mature *H. scabra* in the second sampling than there were in the first and third maturity checks (Fig. 2.22). Although ratios of stages stayed relatively constant over the conditioning period in the entire population, individuals often changed between stages between each sampling and the stages were not consistent between tanks (Fig. 2.23).
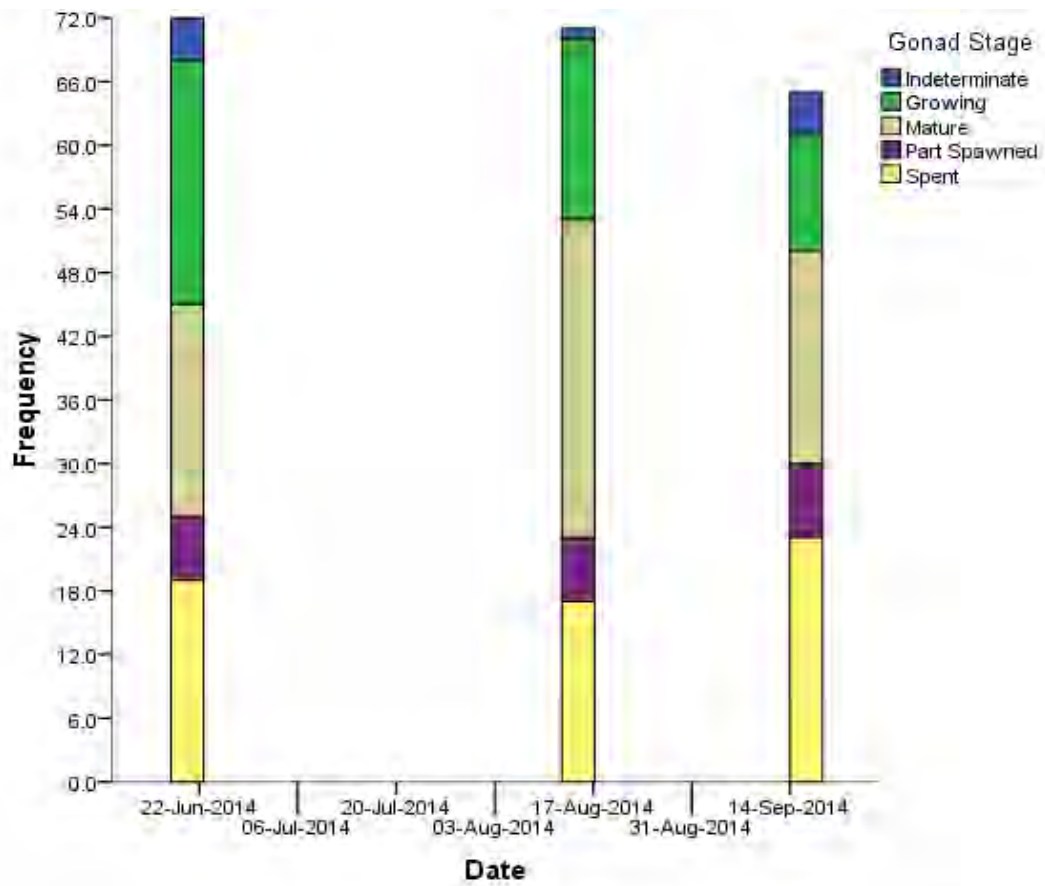
Figure 2.22: Ratios of female *Holothuria scabra* at each gametogenic stage at three time points during 7 month conditioning under ambient conditions. n =72.
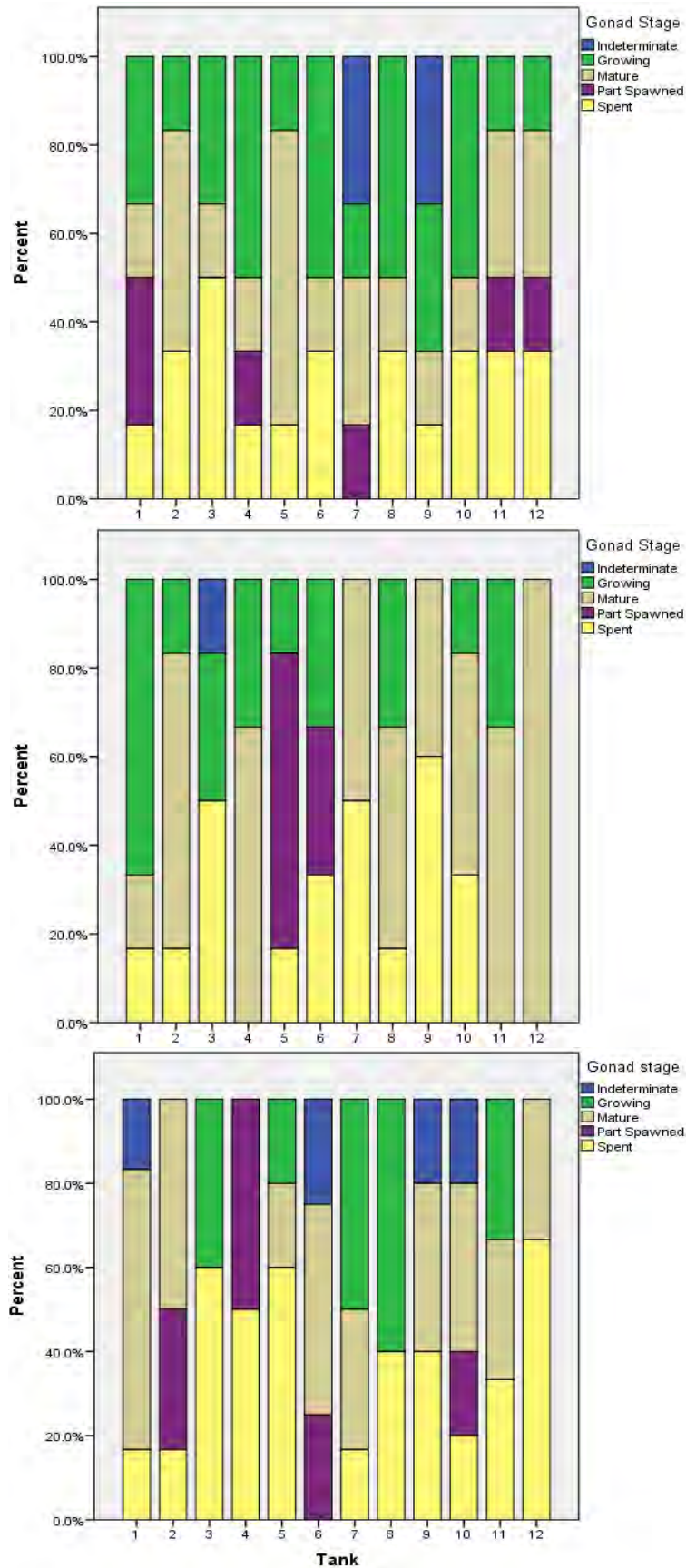
Figure: 2.23: Proportion of *Holothuria scabra* at each gametogenic stage across 12 conditioning tanks in June (top), August (centre) and September (bottom) 2014 after 3, 5 and 7 months conditioning respectively. n =6.

2.3.3.3 Marking

In general, re-marking was required in a large proportion of the animals after approximately three months of conditioning with smaller numbers of animals re-tagged at more regular intervals (Fig. 2.24). The tag retention varied with individuals; 29/72 were marked twice during the conditioning period, 34/72 were marked 3 times, 3/72 were marked 4 times and 1 was marked 5 times. Some animals did not require re-marking at all over the course of the trial (4/72).
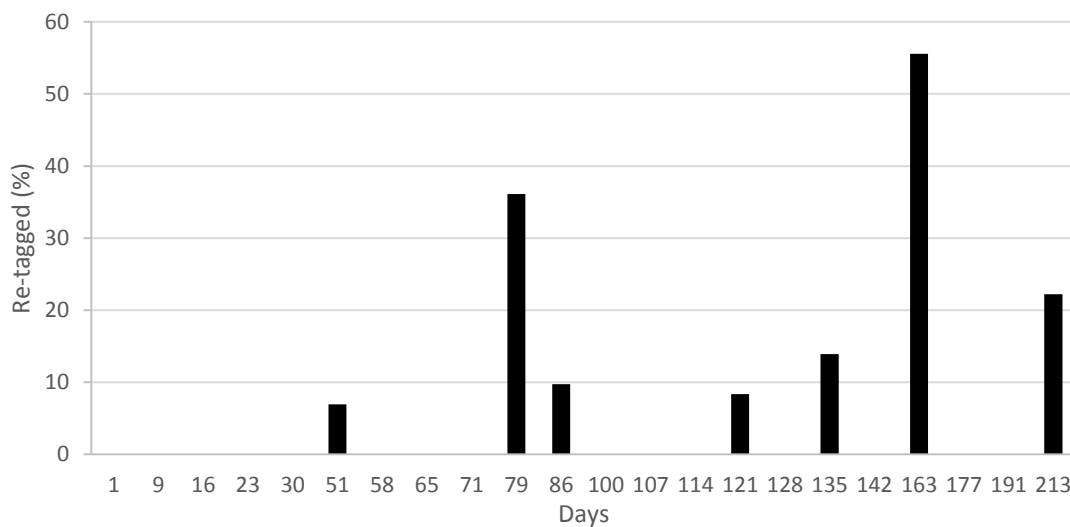
Figure 2.24: Number of *Holothuria scabra* brood stock re-tagged during 7 month conditioning. Bars represent percent *H. scabra* re-tagged during the 7 month conditioning period. n =72.

2.4 Discussion

The series of experiments in this chapter address some of the questions relating to husbandry of brood stock *H. scabra* and the monitoring of their reproductive development. The use of anaesthetics has benefited aquaculturists working with other species, and given sea cucumbers evisceration response, it should be a valuable tool for sea cucumber culture. The ability to discern the maturity of brood stock will provide better hatchery performance and efficiency. The development of methods for the assessment of the reproductive stage of individual brood stock will further the field of reproductive biology, enabling detailed studies of gonad development. A simple tagging method is also described. Holothurians are notoriously difficult to mark but can be monitored with a high degree of accuracy as long as they are checked regularly and kept in relatively small numbers.

2.4.1 Optimisation of dosage for anaesthesia and recovery in adult *H. scabra*

The most appropriate dosage of menthol/ethanol tested for anaesthesia of adult *H. scabra* was determined to be 2% menthol/ethanol solution. This is the same dosage recommended for juvenile *H. scabra* (Watanabe *et al.*, 2012)*.* The 2% menthol/ethanol solution induced the loss of righting reflex within 30-35 min at 30°C in two separate trials, slightly longer than the time reported for the anaesthesia of juveniles at 27°C in 20 min (Watanabe *et al.*, 2012). Yamana *et al.* (2005) found the menthol/ethanol anaesthetic to be more effective at higher temperatures so the longer exposure time required to induce anaesthesia in this trial may be explained by physiological differences between adult and juvenile *H. scabra*. The anaesthetic also induced muscle relaxation demonstrated by the significant elongation of the body in anaesthetised individuals. At higher concentrations the tested animals showed adverse reactions to the anaesthetic such as tensing up and auto-evisceration. Therefore, the 3% and 4% solutions were deemed to be inappropriate for large-scale use. This result, particularly the auto-evisceration response of animals in 4% is surprising given that a 4% solution is recommended for adult *A. japonicus* and was found to be effective for use with juvenile *H. scabra* (Yamana and Hamano, 2006, Watanabe *et al.*, 2012)*.* Additionally, in this experiment the 2% solution induced anaesthesia twice as fast as the 1% solution at 30°C. As rapid anaesthesia is more amenable to hatchery use, the 1% solution was not as effective as the 2% solution.

The effect of anaesthesia could not be induced completely with ethanol alone. The addition of menthol in solution resulted in more consistent anaesthesia and the results of this were observed in the slight lag in recovery of the menthol/ethanol treated animals compared to those treated with ethanol alone. This indicates the effect of menthol in the anaesthetic solution. Menthol inhibits the effect of neuropeptides which cause hardening and softening of body wall tissue in the sea cucumbers *A. japonicus* and *H. leucospilota* (Birenheide *et al.*, 1998). Some effect of ethanol cannot be discounted from these results but it seems that the primary support for its inclusion is to enable dissolution of the main active ingredient of menthol. Following these results, we recommend that possible improvements could be made to the anaesthesia protocol by decreasing the amount of ethanol used to dissolve the menthol, thus reducing the cost

of the anaesthetic. As menthol can be dissolved at higher concentrations in ethanol to make a more concentrated stock solution and then diluted in sea water at lower concentrations to be used as an anaesthetic (Pereira-da-Silva *et al.*, 2014) it is possible to obtain equivalent concentrations of menthol in the working solutions with the use of less ethanol. Additionally, the direct injection of menthol solution may be used as has been reported for the anaesthesia of mice (Galeotti *et al.*, 2002) could reduce the required volume of anaesthetic reagents significantly. This was not assessed in this trial as the aim was to derive a method of rapidly anaesthetising large numbers of brood stock where individual injections would likely be too cumbersome.

Recovery of animal's post-anaesthesia was complete for all of the concentrations tested, with no mortality recorded. Full recovery from menthol/ethanol anaesthesia is also reported for juvenile *H. scabra* and *A. japonicus* (Watanabe *et al.*, 2012, Yamana and Hamano, 2006). This supports the use of the anaesthetic for brood stock husbandry. The return of righting reflex was slower in the higher concentrations of 3% and 4% and comparable in the 1% and 2% treatments with full recovery in 20-30 min. All animals appeared healthy and displayed normal behaviour after a brief recovery.

Anaesthesia-induced relaxation of *H. scabra* caused significant changes in the mass, length and width of the animal. The overall change in mass was small (0.22%) but consistent. The changes in width (-21%) and length (17%) were more obvious and were a good visual indication of the anaesthetic taking effect. These morphological responses to the anaesthetic were larger but similar to those reported for juvenile *H. scabra* which had changes in length and width of 18% and -10% respectively (Watanabe *et al.*, 2012). The relaxation and elongation effect of the anaesthetic may be due to longitudinal muscle relaxation due to inhibition of the neurochemical signalling pathways, blocking neuropeptide/neurotransmitter action or by inhibition of calcium ion channel pathways, as hypothesised by (Wright *et al.*, 1997) regarding the ability of menthol to cause relaxation of bronchial smooth muscle in guinea pigs.

Anaesthesia aided in the surgical biopsy method by reducing the auto-evisceration response caused by the biopsy procedure. The relaxation effect of the anaesthetic also made the body wall easier to penetrate. Anaesthesia during biopsy did not have a significant effect on ovary development during the conditioning period, but there were some differences in the proportion of ovaries in each gametogenic stages

between the treatment groups at the end of the conditioning trial. There were more indeterminate and part spawned ovaries in the control group and more spent ovaries in the anaesthetised group. This could be represent a response to the anaesthetic where more spawning in the tanks was taking place in the anaesthetised group explaining the higher proportion of ovaries in the spent stage. At the same time the higher proportion of animals in the part spawned stage in the control group could represent an inhibition of spawning from the non-anaesthetised biopsy procedure. Similarly the incidence of indeterminate ovaries in the control group could be a resorption of ovary triggered by the biopsy procedure without anaesthetic. However, these differences were not significant and no effect of anaesthesia during biopsy could be determined. Further investigation is required to uncover any subtle effects of anaesthesia and biopsy on gonad development but these results suggest that the method is not likely to create problems with gonad development in cultured brood stock. Other researches have used similar biopsy methods on other sea cucumber species that have also had little effect on gametogenesis or the general health of the animals. Hamel and Mercier (2007) noted a small incision made in the body wall to sample gametes of *H. leucospilota* healed well and caused no mortality or auto-evisceration. Fujiwara *et al.* (2010) and Kato *et al.* (Kato *et al.*, 2009) described a small incision made to collect ovary material from *A. japonicus,* and did not note any short term effects. Kato *et al*. proceeded to use the same animals 4 h later in spawning trials. Hamel and Mercier (1996) also used a similar method to study the gonad of *Cucumaria frondosa*, making a 0.5 cm incision in the body wall and provide the most detailed description of the effects of the procedure. In their study, at the end of a year of biopsies the experimental subjects showed the same gametogenic stage as a control group of animals. The lesion made in the body wall of *C. frondosa* to sample the gonad healed within 3 weeks (Hamel and Mercier, 1996). In this study, a larger incision was made to enable the collection of gonad tissue from both anaesthetised and non-anesthetised animals. This larger incision did not affect gonad development either, but may have contributed to the observed mortality.

The ovary tubule parameters of length, width, oocyte size and oocyte number did not differ between groups at the end of the conditioning trial, supporting the gonad stage evidence that suggests no significant effect of anaesthesia during biopsy on gonad development. Gametogenic development was observed in both groups over the

conditioning period with the ratios of females in each gametogenic stage changing over the course of the experiment. Gonad development of *H. scabra* has been previously reported where animals held in tanks matured and were able to be induced to spawn (Battaglene, 1999, Morgan, 1999, Morgan, 2000a). Separation of the oocyte size frequency classes observed in both groups after the conditioning period could support the hypothesis that the conditions in the culture system were insufficient for growth to occur. Some of the oocytes in the ovary were apparently able to grow and increase the size with little or no growth of smaller oocytes into the middle range. This could indicate a critical size where by gametogenesis can continue in the absence of adequate environment or nutritional resource.

Mass loss was significant for both groups over the conditioning trial with a 17% loss in body mass over the month-long conditioning trial. The loss of body mass may be related to recovery from the surgical injury or suboptimal conditions in the culture system. However, mass loss in captive *H. scabra* is common for brood stock collected from the wild and conditioned in tanks. For example, previous researchers have described mass loss of 20%/month in conditioning systems for short term brood stock conditioning which is comparable to the results in this study (Morgan, 1999, Pitt and Duy, 2005, Morgan, 2000c). In Vietnam, brood stock maintained in bare tanks lost mass while those kept with sand were able to maintain constant weight (Pitt and Duy, 2005). Similarly, in Australia, adult *H. scabra* held in a large tank with sand and supplied with various feed supplements lost weight and eventually perished but were able to be used for induced spawning (Morgan, 1999). Sea cucumbers are known to be rapid regenerators (Mary Bai, 1994, Garcia-Arraras *et al.*, 1999, García-arrarás and Greenberg, 2001) and similar biopsy procedures have been used on other species without mass loss or mortality (Hamel and Mercier, 1999, Hamel and Mercier, 2007, Kato *et al.*, 2009). Both the mass loss and the mortalities observed in the conditioning trials were possibly caused by a combination of surgical injury and inadequate rearing conditions which prevented rapid healing and somatic growth. Morgan (1999) also reported animals maintained in conditions conducive to mass loss were susceptible to infection leading to death. Holothurians are thought to utilise somatic nutrient to support gametogeninc development which could explain the gonad development coinciding with significant mass loss observed in this study and others (Morgan, 2000a). Battaglene (1999) stated

225 g m^{-2} as a tank biomass at which density inhibited the growth of juveniles. Watanabe (2014) on the other hand reported positive growth at a much greater 900 g m^{-2}. Excluding social factors the biomass in this trial was much higher in each tank so insufficient rearing space for each individual may explain the loss of mass in this study. Data on stocking density for adult *H. scabra* are scarce but have been stated at 10-50 animals in 1-12 t tanks which would probably also equate to higher density than recommended by Battaglene or Watanabe (see chapter 1; Table 1).

Anaesthesia using 2% menthol/ethanol solution is deemed to have negligible effect on gonad condition and is acceptable for use for *H. scabra* brood stock management.

2.4.2 Application of a *H. scabra* maturity scale to gonad condition of wild stocks in the Northern Territory Australia; An insight into the local reproductive cycle

The maturity scale of Ramofafia *et al.* (2003) based on a *H. scabra* population in the Solomon Islands was applicable in the survey of the wild population of the Northern Territory north west coastline. Male and female gonads, where present, were able to be assigned to each of the gametogenic stages upon visual and microscopic inspection. The sex ratio was not different from 1:1 and there was no detectable difference in the mean mass between male and female *H. scabra,* in accordance with similar and more extensive surveys of other populations (Ramofafia *et al.*, 2003, Krishnaswamy and Krishnan, 1967, Che and Gomez, 1985, Tuwo, 1999, Muthiga *et al.*, 2009). There were similar proportions of each sex in each of the gametogenic stage with the exception of the mature stage. There was only one mature female found, given the timing of the sampling, may reflect that the population was outside the peak spawning periods. This would be as predicted from previous reviews which found generally that a population at that latitude (11°S) to have annual or biannual spawning peak with asynchronous or continuous spawning to a lesser extent throughout the year (Hamel *et al.*, 2001, Purwati, 2006). The peak spawning period(s) of the population in this study would probably be biannual from March-August and from October-January based on reproductive studies on populations from Indonesia, Africa, and the South Pacific between 3°S and 20°S (Hamel *et al.*, 2001, Conand, 1993, Tuwo, 1999, Kithakeni and Ndaro, 2002, Ramofafia *et al.*, 2003, Purwati, 2006, Muthiga *et al.*, 2009). Additionally, there were numerous

part-spawned animals which could represent small continuous releases of gametes through the year. Gonad stage of an individual was related to the gutted weight, with animals in spawning condition having a larger gutted weight than those at the other stages. Therefore, larger *H. scabra* should be better brood stock than smaller ones and those with thicker body wall may also have greater reproductive potential. This is supported by previous studies that found higher fecundity in larger *H. scabra* both in New Caledonia and in Egypt (Conand, 1993, Hasan, 2005). Gonad index was also higher in spawning stages than for the other stages as expected. As is commonly recommended, the collection of larger animals for brood stock over smaller ones, where possible, could improve success of induced spawning in hatcheries.

2.4.3   Simple, effective tagging method for the identification of individual adult *H. scabra*

Tagging for individual identification is an ongoing issue for holothurian research. Here, a modification of a simple marking technique of scarring, previously used in the field for juvenile *H. scabra* and *H. whitmaei* (Mercier *et al.*, 2000, Shiell, 2006), was used to identify individual adult *H. scabra* over a six month conditioning period. The glue-scar tagging method was effective at identifying all six individuals in each of the 12 tanks, tag retention was acceptable, with majority of individuals requiring re-marking after three months. However, the variability in tag retention requires continuous monitoring at the biweekly-monthly frequencies to ensure maintenance of individual identification. Identification using this method was sometimes more difficult based on colour (e.g. identification marks on light or very dark coloured individuals were more difficult to make out). The number of very dark/black coloured *H. scabra* in this trial was low (3/72), as it commonly is in wild populations in the Northern Territory. Even with two black coloured individuals in the same tank, the sampling programme was frequent enough to maintain identities.

In comparison to other tagging methods where tags are imbedded in the body wall of holothurians, the scarring and glue-scar methods enable the tagged animal to heal quickly and may have less effect on behaviour after the procedure (Shiell, 2006). Wounds created from tagging holothurians with T-bar type tags for example, may not heal around the tag, creating possible sites for infection (Kirshenbaum *et al.*, 2006,

Cieciel *et al.*, 2009). Cieciel *et al.* (2009) used T-bar tags to mark *Parastichopus californicus* for a short term movement study and noted handling and tagging increased locomotor activity for up to 24h. The T-bar tag retention for *P. californicus* was comparable to the glue-scar tag retention in this study, with retention of 70% after 4 months. The T-bar tags have the benefit of having no limit to the number of individuals that can be tagged. The glue scar technique is limited by the number of different marks and mark locations that can be used to maintain identity and therefore is better suited to individuals in tanks as opposed to large scale mark recapture. For large scale mark and recapture studies, genetic marking or fluorochrome staining methods may be more appropriate, and T-bar tags where identification needs to be made directly in the field. Sheill (2006) marked *H. whitmaei* using a scarring method and also found activity was higher in marked individuals for up to 3 days. The scarring lasted for up to 3 weeks in the field and the author suggests that the scarring method is less likely to affect the animal's physiology than penetrating tags such as T-bar types.

The glue scar method described here could be used in brood stock management to identify individuals in conditioning systems. Combined with biopsy procedures this would enable identification of the sex, and potentially the reproductive condition, of an individual at a glance, which would be of great advantage in reducing the number of failed spawning induction attempts in hatcheries. The method may also be useful in field studies of *H. scabra* where repeated identification of a modest number of individuals is required.

Tagging of the animals in the conditioning system used in this trial did not appear to affect the general health of the animal. Over the course of the trial one individual did develop lesions in the dermal layers but then recovered. It is not known if the lesions were related to the tagging method but the sloughing of the epidermal layers may create an opening for the ingress of pathogens. Disease of the tegument of cultured sea cucumber is reported and causes death and often high mortality in juveniles. A number of microbes have been associated with the disease and pathogenesis may be opportunistic (Becker *et al.*, 2004, Deng *et al.*, 2009). In contrast to the animals in the anaesthetic biopsy trial, the brood stock in this system gained mass. Suggesting that the tagging in this case did not impede growth. Growth was not consistent over the six month trial and even became negative for some tanks. Mass fluctuations were related

to temperature with the negative growth rates during the coolest part of the year and growth rates becoming positive as the temperature increased. There are little data reported for the growth rates of adult *H. scabra* and the factors affecting it. Negative growth, shrinking, has been reported for adult *H. scabra* kept at high densities (Morgan, 2000a, Pitt, 2001).

The needle biopsy method was able to provide a sufficient gonad sample to give a good indication of the reproductive state of the majority of individuals in most cases. Similar method was used by Morgan who stated that when the method was refined no evisceration was caused by sampling. In this study samples were not always able to be collected using the needle and mature animals were easier to sample. Repeated biopsies were sometimes required to get a result. The gonad stage ratios of the brood stock as a whole, did not change much over the three sampling points. There were slightly more mature and less spent individuals in the August samples than there were in the June and September samples. Although the gonad stages of the group did not change over time as a whole, the majority of individuals changed between stages at each time point, suggesting gametogenic stages progress quickly in captive brood stock. The needle biopsy is the recommended method for identifying mature brood stock for use in spawning trials because it is least invasive and can sample mature gonads readily. Where the sampling of gonad at all stages of maturity is required surgical biopsy should be used to ensure accurate sampling of small and reduced gonads.

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3 Effects of sediment nutrient level, water filtration and light level on brood stock culture and maturation of *Holothuria scabra*

3.1 Introduction

Sexually mature sea cucumber brood stock, which are used to supply gametes for aquaculture production, are generally collected from wild populations at, or just prior to, peak spawning periods. This limits productivity to seasonal patterns of larval availability, preventing year round hatchery production. Collection of sexually mature brood stock is becoming difficult in areas where over harvesting has occurred. Brood stock collection just prior to the natural spawning period takes place for the culture of the Japanese sea cucumber *Apostichopus japonicus* in China and Japan. In China, *A. japonicus* brood stock collected from the wild are held in tanks and conditioned for spawning just prior to the natural spawning period by gradually increasing the water temperature and supplying the animals with a brown alga food supplement (Liu *et al.*, 2005). Similarly, in Japan *A. japonicus* have been conditioned in ambient water temperature and supplied brown alga as food source prior to spawning (Ito and Kitamura, 1998). Methods for the conditioning of brood stock of tropical species of holothurians are less developed.

The reproductive cycle of the high value tropical sea cucumber *Holothuria scabra*, and therefore the availability of mature stock is dependent on the geographic location of the population (Hamel *et al.*, 2001). In tropical zones, the reproductive cycle is generally asynchronous which lends itself to a year round supply of mature brood stock for aquaculture production. For example, in the Solomon Islands, changes in gonad were used to determine that *H. scabra* spawn throughout the year in relatively low numbers and have a period of enhanced spawning from September to December (Ramofafia *et al.*, 2003). Whilst the population may indeed be spawning throughout the year, the success rates of induced spawning of collected brood stock for hatchery production can be very low. This is probably due to the lower proportion of reproductively mature individuals within the population at any one time. Low numbers

of released eggs from low numbers of spawning females are common at these times. For example, during a year-long study in the Solomon Islands, fertilised gametes were collected in only 46% of the spawning attempts. Additionally, at best, 35% of the induced brood stock in each attempt released gametes; an average of 1.9 females spawning per trial (Battaglene *et al.*, 2002). So although the gonad indicators of the wild population indicated prolonged spawning through the year, reliable aquaculture production was only achieved at peak periods which varied from year to year in the Solomon Islands (Ramofafia *et al.*, 2003, Battaglene *et al.*, 2002). The ability to reliably condition brood stock under controlled tank conditions is fundamental to the advancement of tropical sea cucumber aquaculture.

The development of the techniques to spawn wild brood stock and culture *H. scabra* larvae have led to the species being cultured in many countries (Purcell *et al.*, 2012). *H. scabra* grow-out production most commonly occurs in sea bed pends or open sea ranches where maturing stock will most likely reproduce and contribute to spawning biomass in wild populations (Uthicke and Purcell, 2004, Purcell *et al.*, 2012, Juinio-Meñez *et al.*, 2013). Sea cucumber stock enhancement and sea ranching initiatives must consider the genetic implications of releasing hatchery-bred stock into wild populations (Bell *et al.*, 2008). Improving the control over reproduction by conditioning brood stock is one way to mitigate potential genetic issues through increasing the number of contributing parent stock, thereby increasing the diversity of hatchery bred progeny to be released for stock enhancement, restocking or sea ranching. Brood stock conditioning may also improve the low rates of success of induced spawning of *H. scabra* and therefore improve hatchery efficiency.

There are only a few studies on sea cucumber aquaculture and very few related to *H. scabra* brood stock culture (Morgan, 2000, Battaglene *et al.*, 2002). However, there are reports, mostly anecdotal, of the difficulties with maintaining adult *H. scabra* in enclosed systems. Most suggest that density and lack of appropriate diets are the main causes for the poor performance of brood stock observed in captivity. A lack of knowledge of the physiology of the species makes it difficult to make assumptions on the best ways of housing the animals, meaning every part of the culture system may be a factor influencing the results of experimental trials. Thus, trials in this study were conducted to assess the effects of fertilisation, temperature and light levels on brood

stock conditioning, and also to enable the observation of animals held in different experimental systems to gain knowledge to inform husbandry practices for *H. scabra* brood stock.

Anecdotal evidence suggests that the addition of organic fertiliser into culture tanks may be important for tank conditioning of *H. scabra* brood stock into spawning condition. *H. scabra* at lower latitudes may reproduce opportunistically in response to increased nutrient levels in the sediment and enhanced photosynthetic productivity which could be a necessary nutritional resource for gametogenesis (Ramofafia *et al.*, 2003). The addition of organic fertiliser is therefore hypothesised to provide a nutritional benefit enabling enhanced growth and gametogenic development. An agricultural organic fertiliser (dynamic lifter) added to tanks as a nutritional supplement enhanced the growth and survival of juvenile *H. scabra* in a rearing experiment conducted at the Darwin Aquaculture Centre (Knauer, *unpublished data*). The organic fertiliser was second only to a commercially available high-protein powdered algae product, indicating it may be an effective low-cost nutritional supplement for *H. scabra*. The mechanism by which the fertiliser improved growth is unknown but it may be acting as a direct food source or promoting the production of microorganisms such as algae which are then consumed by the juvenile *H. scabra.*

Previous attempts to carry out controlled experiments on *H. scabra* brood stock maturation in small, highly replicated systems, have been unsuccessful (Chapter 2). In such systems, adult *H. scabra* typically lose weight and even die when cultured in tank based maturation systems for long periods. In trials conducted using a highly replicated indoor system described in Chapter 2, water was filtered down to 1 μm and UV sterilised, potentially removing most of the organic nutrients in the water which may be nutritionally important for the sea cucumber. More commonly used outdoor conditioning tanks rely on water that is minimally filtered i.e. sand filtered only. Due to the large silt loads of local waters around Darwin, a large amount of silt, algae, detritus and other organisms are introduced to the tanks. This is particularly evident during spring tides. The silt that enters the tanks could be providing elements or nutrients that enable the brood stock to grow and mature. Additionally, the benthic invertebrates which pass through the filtration system and into the tanks may also have significant effects on nutrient cycling in the tanks that is beneficial to brood stock. Associations

between organisms within sediments are known to improve benthic habitat productivity (van der Heide *et al.*, 2012).

Tank-based brood stock maturation systems typically consist of tanks with flow-through seawater or static seawater with daily water exchange. These systems generally house 15-30 animals in 1000l tanks with sand or mud substrates. Whilst conducting trials on *H. scabra* brood stock, Morgan (2000b) observed that captive animals lost up to 40% body mass over the period of captivity despite provision of food supplements. The study also found a decline in fecundity of female brood stock and in hatch rate after five weeks conditioning. The animals were held in tanks with a sand substrate and fed supplements including powdered kelp, lucerne pellets and prawn pellets. Moreover, after five weeks into captivity the animals showed signs of disease and did not recover (Morgan, 2000a). Such difficulties with holding *H. scabra* brood stock long-term make conditioning experiments difficult and impede aquaculture breeding advancements. Other food supplements reported for *H. scabra* include chicken manure, shrimp starter feed, ground algae, rice bran, soybean meal, prawn head waste, blue crab diet, abalone diet and seagrass powder (See table 1.3). Duy (2012) reported the conditioning of brood stock *H. scabra* in tanks at low densities in Vietnam. Although the methods enabled year round induced spawning, loss of body mass was still observed in the brood stock suggesting that the conditions in tanks would not allow for longer term culture of brood stock or may eventually result in declines in gamete viability or mortalities. It is clear that there is a need to develop suitable husbandry practices and systems that enable sufficient numbers of adult *H. scabra* to be conditioned for spawning to meet the demands of a commercial hatchery. Additionally, the development of controlled brood stock conditioning will enable researchers to experimentally test hypotheses regarding the factors effecting reproduction and gonad condition. The aim of this study is to develop effective conditioning procedures for the consistent tank conditioning of brood stock *H. scabra* for aquaculture production.

The following experiments aim to:

1) Determine the effect of added organic matter to sand substrate on the growth and conditioning of brood stock in commercial low density conditioning systems; and

2) Quantify the effect of light level and water filtration on the growth of brood stock *H. scabra* in a small experimental sized system.

3.2   Methods

3.2.1   Experiment 1. Effect of fertilisation of substrate with organic matter on gonad development and body mass of *H. scabra* brood stock.

A large-scale brood stock conditioning system was constructed consisting of three round 6000 L tanks each containing a ≈50 mm layer of fine washed sand substrate which was equivalent to 400 L of sand. The tanks were supplied with flow-through 1 μm filtered sea water (FSW) with a flow rate of 24 L min^{-1} and constant aeration. The tanks were located outdoors under ambient temperature and light with partial shade covers constructed from black 50% shade cloth. Three fertiliser application rates were tested in the trial; 0 g week^{-1} (control), 500 g week^{-1}, and 2000 g week^{-1}. The experiment was repeated 3 times consecutively with the sand substrate, animals and treatment position were changed between each replicate. The tanks were set up and pre-conditioned for 1 week prior to the addition of animals.

Adult *H. scabra* collected from a brood stock holding pond facility near Darwin, NT, Australia were used in the trial (n=54). In each tank male (n=3) and female (n=3) brood stock were conditioned together and cultured for 28 days. The animals were purged for 48 h to eliminate most of the gut contents before being added to the tanks. Each animal was given an individual ID using the glue-scar method described in chapter 2. Females were tagged on the left side of the body and males were tagged on the right with individuals tagged either at anterior, centre or posterior positions to differentiate individuals of the same sex.

To assess the effects of each of the fertilisation treatments, growth and gonad development were monitored during the conditioning period. To assess growth, each animal was weighed at the beginning of the conditioning period and once weekly thereafter. The animals were rested out of the water for 2 min to normalise the water content within the respiratory organs before whole wet weight was recorded to the nearest 0.1 g. Mean growth rate was calculated for each treatment group at the end of the trial (g day^{-1}). Gonad development was assessed by examining samples of gonad tissue before and after the conditioning period. After purging, animals were anaesthetised and biopsied using the surgical biopsy method described in chapter 2. Gonad tubule length and width was recorded for each animal, as well as oocyte size and

number for females. Gonad tubule length was measured from where the tubule was detached from the gonad basis during sampling to the tip of the most distal tubule branch, to the nearest millimetre. Tubule diameter was measured at the thickest part of the tubule using an ocular graticule fitted to a compound microscope under 40 x magnification. Oocyte size was estimated from the mean of 10 oocytes measured *in situ* from the sampled ovary tissue from each female. Oocytes were measured along the longest axis at 100 x magnification using an ocular graticule fitted to a compound microscope (Olympus). Gonad stages were assigned as described in chapter 2, based on the gonad stages defined by Ramofafia *et al.* (2003).

The tanks were checked daily for signs of spawning by taking samples from egg collectors placed in each tank. Egg collectors consisted of a floating ring with air-lift into a 63 µm mesh screen. Screens were checked daily each morning for the presence or absence of eggs, embryos or larvae. Noted spawning events were compared to the lunar cycle. Water temperature in the tanks was monitored using data loggers (TinyTag P/L) throughout the trial. Dissolved oxygen, pH and salinity were checked daily using a multi-parameter water meter (YSI, USA).

Fisher's Exact Test (FET) was used to assess differences in the gonad stage ratios between the different fertilisation rates at the beginning and at the end of the conditioning period. Split-plot ANOVA was used to test for an interaction effect of fertilisation rate and gonad parameters such as oocyte size, oocyte density, tubule length and tubule width over the conditioning period.

3.2.2 Experiment 2: Light level and water filtration on Gonad-somatic index and body mass of *H. scabra*

This experiment was set up to determine the effect of water filtration and light levels on growth and gonad condition of brood stock *H. scabra* when housed in a small scale system. Twenty 850 mm x 850 mm x 200 mm tanks containing 35 L sand as substrate were set up under 2 different light levels and 2 different water filtration levels. Ten tanks were set up indoors under artificial light (12 h light: 12 h dark) with five of those tanks receiving 1 µm filtered and UV sterilised seawater (FSW) and five receiving unfiltered raw seawater (RSW). Ten tanks were set up outside receiving direct sunlight for the majority of the day (11.5 h light: 12.5 h dark). Five of those tanks each were delivered

either FSW or RSW. Water was supplied in constant flow-through with and exchange rate of 200% day^{-1}. Each tank was supplied with constant aeration. Tanks were filled, and fertilised with 40 g dynamic lifter ground to a grain size of <2 mm, 10 days prior to stocking. During the conditioning period, the tanks were fertilised at a rate of 10% of the biomass of the individual in each tank per week.

Adult *H. scabra* used for the experiment were collected from a pond at a brood stock holding facility near Darwin and purged for 48 h in a clean tank with no substrate. Each animal was weighed and measured before stocking into a randomly assigned tank. The sex of the animals used in this trial was unknown. Animals were weighed and measured again on day 1 and every 2 days thereafter during the conditioning period. Mass data was used to compare growth rates for each treatment group. After a conditioning period of thirty days brood stock were sacrificed and gonad weight and drained body wall weight were recorded. Post-conditioning gonad index was used to compare reproductive condition of brood stock cultured under different light and filtration levels. Gonad index was calculated as gonad mass (g) / drained body wall mass (g) x 100.

Split-plot ANOVA was used to analyse within subject effects of the conditioning period and main effect of culture treatment groups. Mass data from days 1, 9, 17, 25 were used in the Split-plot ANOVA in order to meet the requirements of Box's Test of Equality of Covariance Matrices, i.e. the covariance of all mass data was too similar to calculate covariance matrices, so mass estimates from 8 day intervals were used. Where Mauchly's test of sphericity was violated, Greenhouse-Geisser adjustment of degrees of freedom was used. Chi Squared test was used to assess statistical significance of the differences in the gonad index of *H. scabra* between treatment groups.

3.3   Results

3.3.1   Effects of the addition of organic matter on broods stock conditioning of *H. scabra.*

There was no difference in the proportion of *H. scabra* individuals in each of the gametogenic stages between treatment groups at the end of the conditioning period (FET, P =0.541) (Fig. 3.1). However, there were more part-spawned males (4/10) and more part spawned females (3/8) after the conditioning period than there were in the

initial maturity check and less individuals in the growing stage after the conditioning period (males 7/2, females 11/2). Gonad development had progressed in the growing individuals to either mature or part spawned during the conditioning period (Fig. 3.1).
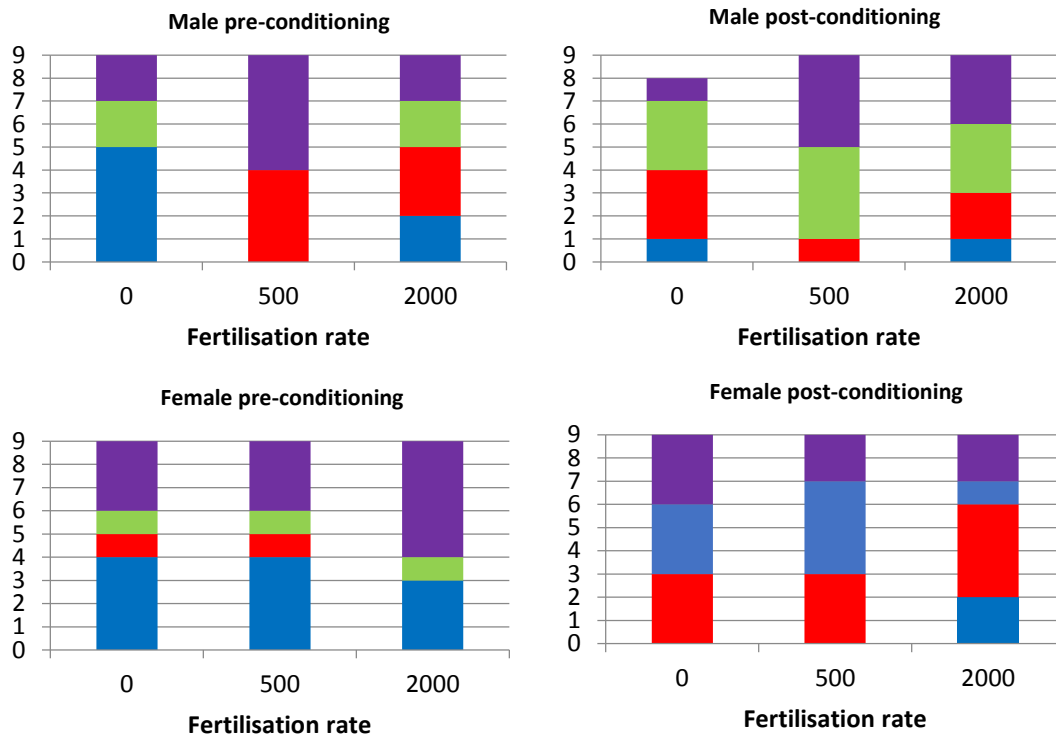
Figure 3.1: Gonad development stage of male and female *Holothuria scabra* before and after conditioning with different rates of organic matter. Gonad stages are represented by the colours purple = spent, green = part spawned, red = mature and blue = growing.

The mean daily salinity during the trial was 35.86 ppt ± 1.0, the mean daily dissolved oxygen in the conditioning tanks was 6.11 mg ml^{-1} ± 0.2 and the mean daily pH was 8.21 ± 0.0. Temperature was more variable as the trial ran over four months. Mean daily minimum and maximum water temperatures were 26.0^0C ± 1.9 to 28.6^0C ± 1.3.

Oocyte size was not significantly affected by the conditioning period ($F_{1,6}$ =0.828, P =0.398). Similarly there was no interaction effect of the different fertiliser application rates on oocyte size change over the conditioning period ($F_{2,6}$ =0.28, P =0.765). The mean oocyte size was more similar between the different fertilisation-rate groups post-conditioning, with oocyte sizes of 116.1 µm ± 31.9, 110.9 µm ± 25.0, 111.1 µm ± 53.6 for the 0 g (control), 500 g $week^{-1}$ and 2000 g $week^{-1}$ treatments, respectively. Compared to the pre-conditioning oocyte sizes of 103.1 µm ± 29.5, 110.3 µm ± 6.5 and 82.0 µm ± 18.8, respectively (Fig. 3.2).
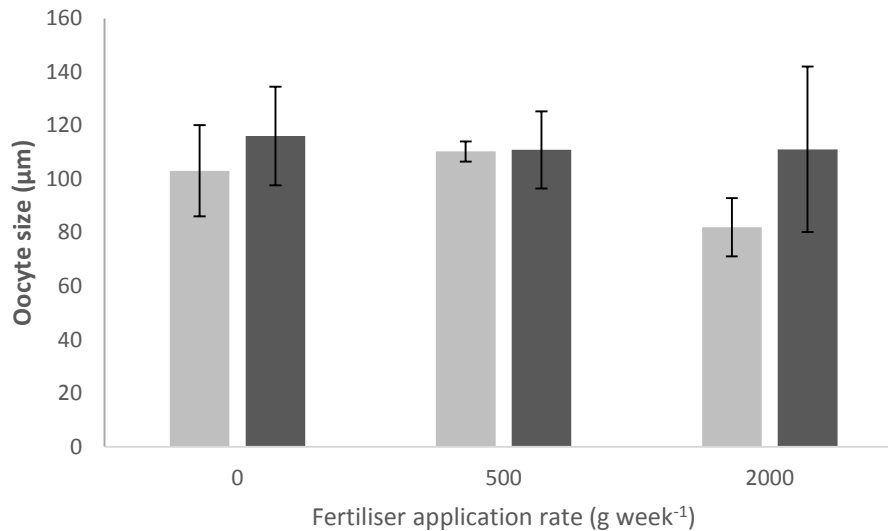
Figure 3.2: Oocyte size in *Holothuria scabra* ovary tubules before (light grey) and after four weeks conditioning (dark grey) on substrates with different fertiliser inclusion rates. n=3. Error bars represent ± 1 SE.

The conditioning period had no significant effect on the oocyte density in the ovary tubules of the *H. scabra* brood stock ($F_{1, 6}$ =0.428, P =0.537). There was also no interaction between the different fertilisation rates and the change in oocyte density over time ($F_{2, 6}$ =0.469, P =0.647). However, fertilised treatments showed a slight increase in oocyte density post-conditioning from 1120.9 cm^{-1} ± 705.2 to 1413.6 cm^{-1} ± 796.9 for 500 g week^{-1} and from 712.7 cm^{-1} ± 579.5 to 1038.1 cm^{-1} ± 721.0, for those cultured in tanks with 2000 g week^{-1}. Whereas, the control group showed a slight decrease from 1047.3 cm^{-1} ± 681.9 to 882.6 cm^{-1} ± 173.8 over the conditioning period (Fig. 3.3).
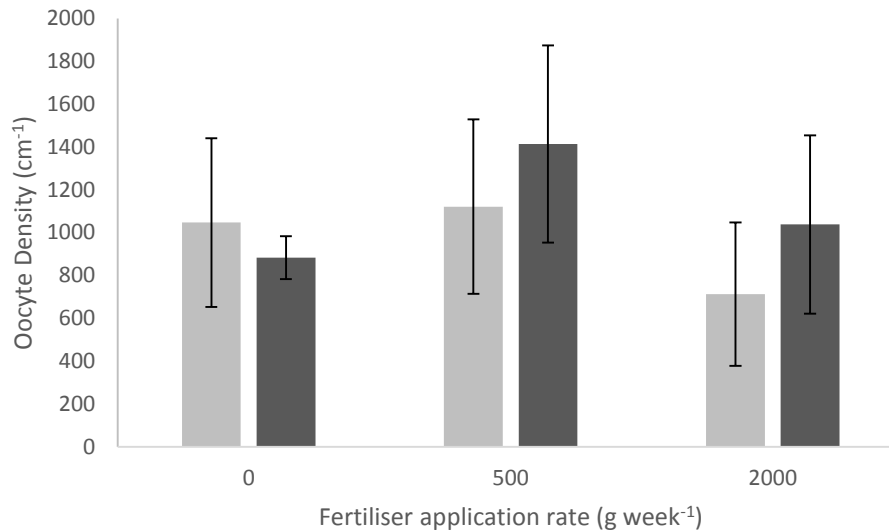
Figure 3.3: Oocyte density in *Holothuria scabra* ovary tubules before (light grey) and after four weeks conditioning (dark grey) on substrates with different fertilizer inclusion rates. n=3. Error bars represent ± 1 SE

Mean ovary tubule length was higher in brood stock that were cultured in tanks fertilised with 500 g week^{-1} (120.33 mm ± 21.2) than it was for those cultured in non-fertilised tanks (83.11 mm ± 3.0), and tanks fertilised with 2000 g week^{-1} (85.33 mm ± 15.1). For males, those cultured in the 500 g week^{-1} (96.78 ± 16.8) treatment had longer gonad tubules than did those cultured in the 0 g week^{-1} (56.00 mm ± 11.1) treatment (α =0.05) (Fig. 3.4). However, there was no significant change in gonad tubule length over the conditioning period for either males ($F_{1, 6}$ =0.219, P =0.657) or females ($F_{1, 6}$ =2.052, P =0.202). No interaction between the change in tubule length and the different substrate fertilisation rates was detected for males ($F_{2, 6}$ =0.282, P =0.764) or females ($F_{2, 6}$ =0.252, P =0.16).
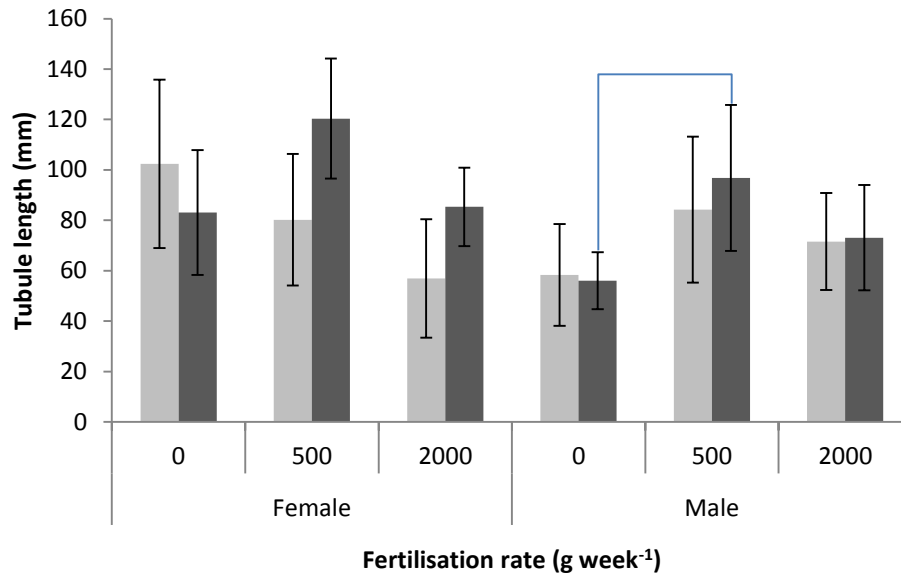
Figure 3.4: Length of *Holothuria scabra* gonad tubules before (light grey) and after (dark grey) conditioning on substrate with different fertiliser inclusion rates for four weeks. n=3. Error bars represent ± 1 SE. Connecting lines represent significant difference P <0.05.

Tubule diameter was not significantly different for male or female brood stock between the fertilisation levels before or after the conditioning period. There was less difference between the mean tubule diameter values of the different fertilisation rate treatment groups post-conditioning than there was at the beginning of the trial. In male *H. scabra,* the mean tubule diameter pre-trial was 880.0 µm ± 223.7, 1183.3 µm ± 298.3, 1188.9 µm ± 105.5 for the 0 g (control), 500 g week^{-1} and 2000 g week^{-1} groups, respectively. For females, the mean tubule diameter pre-trial was 1405.6 µm ± 197.6, 1411.1 µm ± 237.8, 1094.4 µm ± 231.6, respectively. Compared to after the conditioning trial where the mean testes tubule diameter was 1183.3 µm ± 184.3, 1125.0 µm ± 216.2, 1102.8 µm ± 127.6 in the 0 g (control), 500 g week^{-1} and 2000 g week^{-1} groups, respectively. For females, the mean tubule diameter post-trial was 1400.0 µm ± 236.3, 1341.7 µm ± 225.5, 1277.8 µm ± 166.9, respectively (Fig. 3.5).
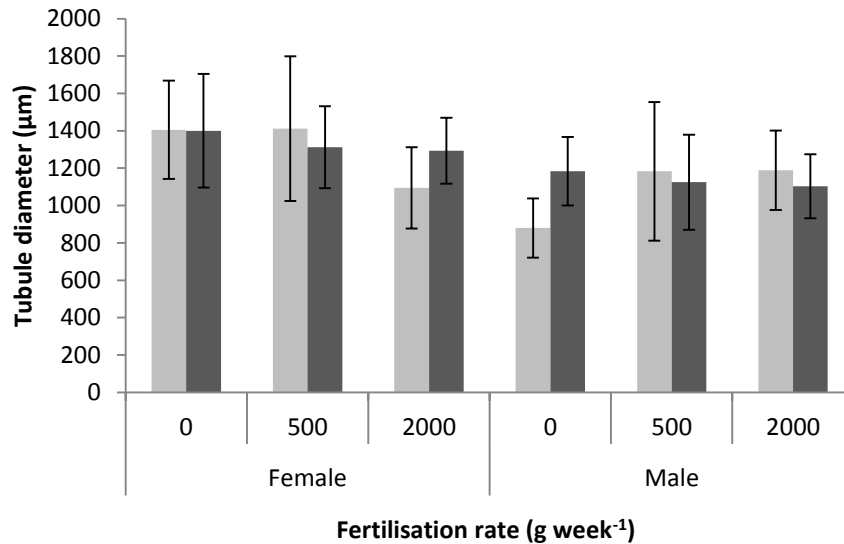
Figure 3.5: Tubule diameter of *Holothuria scabra* gonad tubules before (light grey) and after (dark grey) conditioning on substrate with different fertilizer inclusions for four weeks. n=3. Error bars represent ± 1 SE.

The mean mass of individuals in each of the tanks with different application rates of fertiliser followed very similar patterns of growth occurred and mass loss. Mass declined in the first week of conditioning then declined further, but at a slower rate, for the next two weeks before positive growth in the final week of culture (Fig. 3.6).
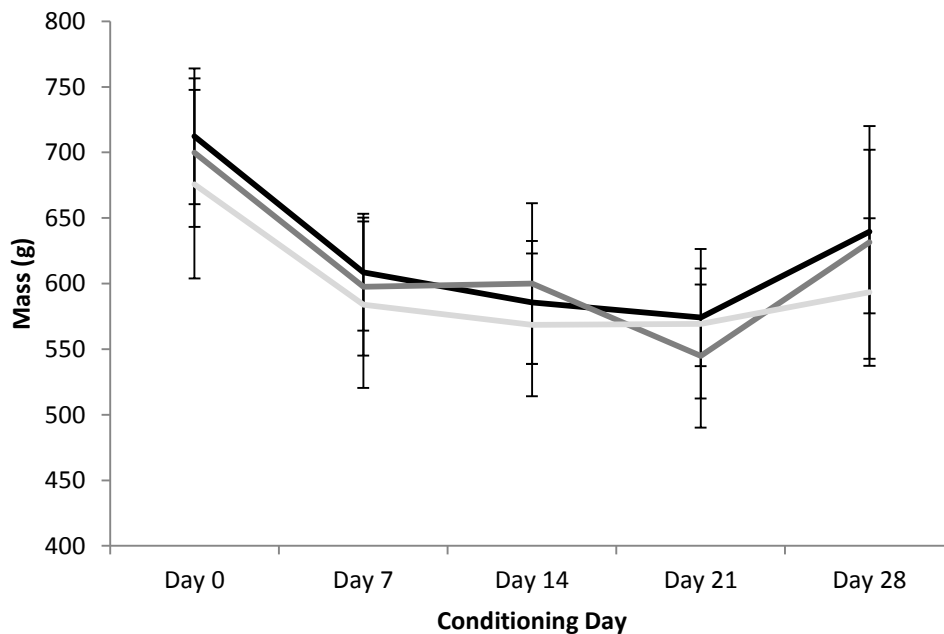
Figure 3.6: Mass of *Holothuria scabra* over conditioning period with 0 g fertilizer added (black), 500 g fertilizer added (dark grey) and 2000 g fertilizer added (light grey). n=3. Error bars represent ± 1 SE.

The mean growth rate in all treatments was overall negative. The growth rates of the treatment groups; -2.29 g day^{-1} ± 0.9 (control), -1.98 g day^{-1} ± 0.9 (500 g week^{-1}) and -2.27 g day^{-1} ± 0.3 (2000 g week^{-1}) were not significantly different (ANOVA F$_{2, 6}$ =0.048, P =0.953).

Spawning was observed in each of the conditioning tanks in each of the replicates. Spawning was often synchronised between at least two tanks and predominantly occurred from up to 6 days prior to a few days after the full and new moons from May to August (Fig. 3.7). Spawning also occurred on the first night after the tanks were stocked in each of the replicates, which may be an indication of spawning triggered by handling and transport stress. At the end of the third conditioning trial replicate, the brood stock from each treatment tank spawned in the purging tanks prior to biopsy. This would have affected the gonad data obtained from this replicate.

Several of the spawning events resulted in unfertilised or immature oocytes being observed in the conditioning tanks. Immature oocytes were common in spawns during replicate 2 during June-July. Also, unfertilised ova which appeared to be mature were identified on one occasion during replicate 1 during May-June, suggesting that only females had spawned. All other spawning events observed during the conditioning tanks resulted in normal fertilised eggs/embryos.
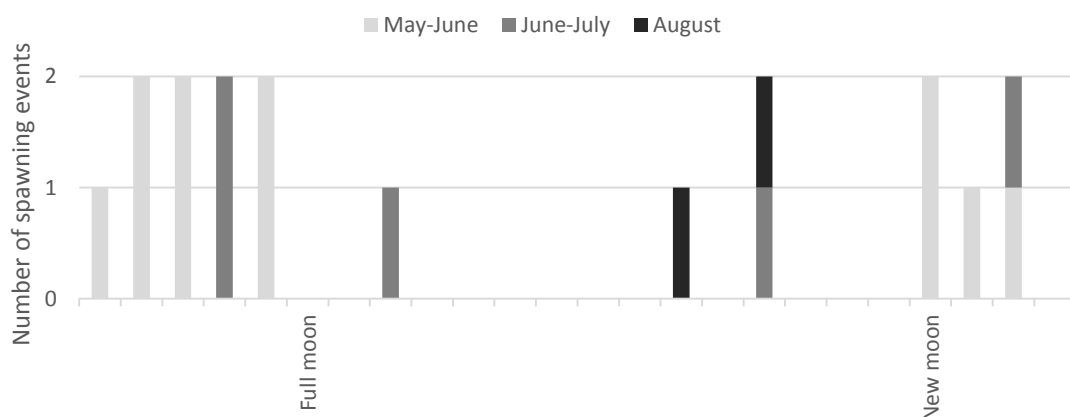
Figure 3.7: Distribution of spawning events during the conditioning experiment. Data from each treatment were pooled and spawning data from conditioning trials in May-June, June-July and August were overlaid on a single lunar scale where x axis tick marks equal one day.

3.3.2 Effects of light level and water filtration on brood stock conditioning of *H. scabra*

Gonad index at the end of the 30 day conditioning period was not significantly affected by the different combinations of light level and water filtration χ^2 =2.47, df =3, n=20, P =0.480. *H. scabra* cultured with artificial light and FSW had the highest mean gonad index of 1.2% followed by those cultured with artificial light and RSW (0.55%), those cultured with natural light and FSW (0.50%) and natural light and RSW (0.29%) (Fig. 3.8).
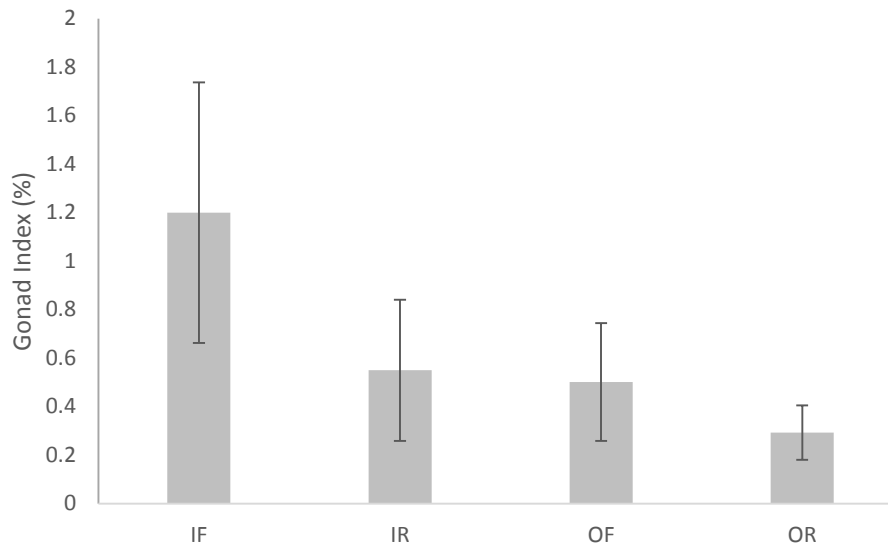
Figure 3.8: Mean gonad index for *Holothuria scabra* conditioned for 30 days under different culture conditions of light and water filtration. Artificial light with FSW (IF), artificial light with RSW (IR), natural sunlight with FSW (OF) and natural sunlight with RSW (OR). n=5.

Mass declined significantly across all treatments over the conditioning period (Fig. 3.9) ($F_{1.749, 27.99}$ =14.379, P =<0.01, part. eta squared 0.473, power 0.994). This was independent of treatment. There was no interaction within the effect on mass of the different treatments over the conditioning period ($F_{5.248, 27.992}$ =1.259, P =0.309, part eta squared 0.191, power 0.386). In general, across all treatments there was a sharp decline in mass during the first day of culture followed by a steady decline in mass for the rest of the conditioning period.
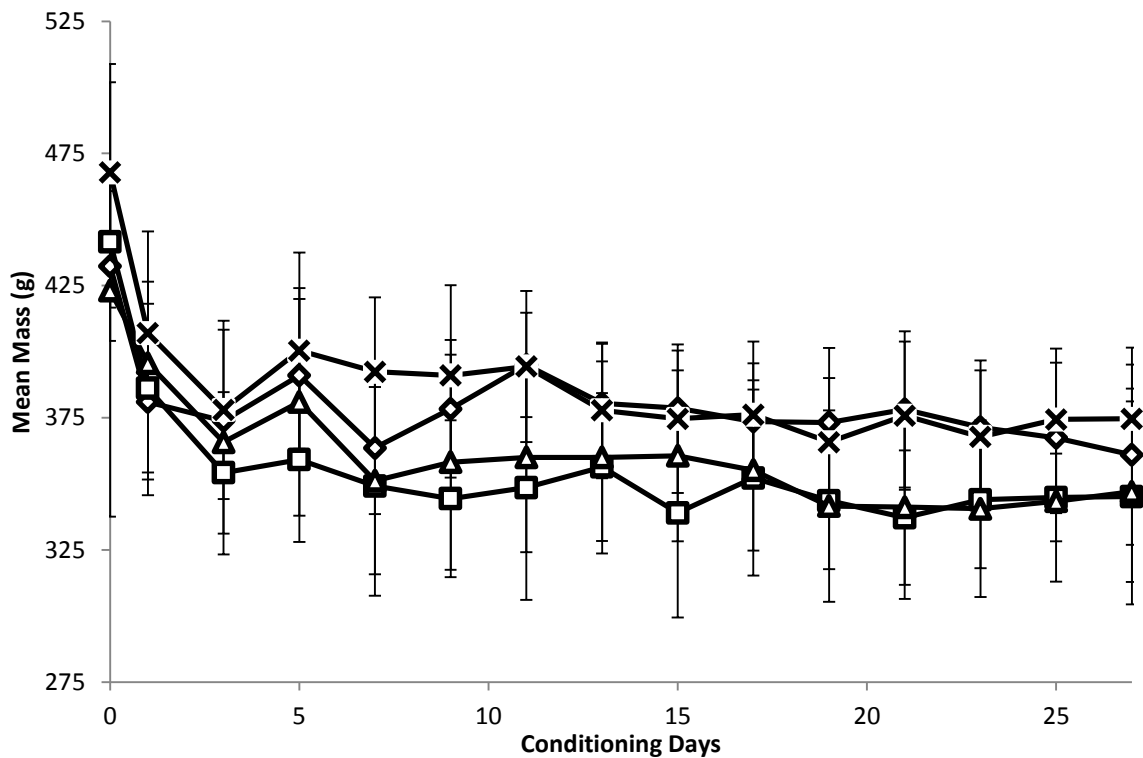
Figure 3.9: Mass of *Holothuria scabra* brood stock conditioned under different light and water filtration levels. *H. scabra* were conditioned in tanks supplied with artificial light and unfiltered seawater – triangles; natural sunlight and unfiltered sea water – crosses; artificial light and filtered seawater – diamonds or natural sunlight and filtered seawater – squares. Error bars = ± 1 sd. n =5.

3.4   Discussion

3.4.1   Fertilisation of substrate

Enrichment of sand substrate in brood stock conditioning tanks with additional organic matter did not improve the gonad condition or growth of *H. scabra*. It was expected that fertilisation of the substrate would enhance the growth and reproductive condition of the brood stock *H. scabra* because it has previously been effective for promoting growth in juveniles and anecdotally used for promoting growth of *H. scabra* in ponds (Knauer, *unpublished data*). Whilst some gonad development was observed during this conditioning experiment, as indicated by spawning, it was independent of the level of added organic matter in the substrate. Fertilisation of the culture substrate may still have an effect on long-term conditioning. Sea cucumbers are efficient at extracting nutrients from sediments (Uthicke, 2001, Michio *et al.*, 2003). High density culture of *H.*

scabra may deplete the benthic nutrients in enclosed and semi-enclosed systems, resulting in reduced growth rates and productivity for subsequent crops. By alternating crops of shrimp and sea cucumber in Vietnam, it has proven to be effective at both preventing nutrient build up (sea cucumbers) and restoring nutrients in the sediment (shrimp) (Duy, 2012). However, in the current study, addition of organic matter did not alter growth rates or gametogenesis and was seemingly unnecessary for conditioning brood stock. These results suggest that productivity in the sediment and stored energy in the tissues of the brood stock were sufficient for gonad development to progress. *A. japonicus* are also efficient at extracting and resuspending nutrients from pond sediment, with the sea cucumbers resuspending almost all the deposited nutrients which settle on the bottom of aquaculture ponds (Ren *et al.*, 2010). These results from the studies of *H. scabra* and *A. japonicus* in ponds suggest that some addition of nutrients could be required for the long-term culture of *H. scabra* but seemingly from the results of this PhD study, not necessary for short-term conditioning periods of up to one month. The uptake of benthic nutrients depends on the type of organic matter and the particular organisms involved (Gooday *et al.*, 1990). It may be in this case, that the month long trial was not sufficient to enable the primary production within the substrate to convert the nutrients to a suitable food source for the sea cucumbers. Alternatively, the productivity in the unfed treatments and nutrients entering the tank from the ambient water may have been sufficient for the sustenance of the brood stock and added nutrients were in excess to what could be utilised by the animals.

Brood stock in this study generally lost mass over the culture period. Mass loss in captivity doesn't cease gametogenesis but can eventually affect quality and survival of the offspring (Morgan, 1999). In this study *H. scabra* rapidly changed from one gametogenic stage to another and spawned repeatedly. The consequences of this for hatchery production are that short-term conditioning periods may be effective regardless of weight loss and close monitoring may be required to ensure peak maturity periods of individual brood stock are not missed as spawning was observed in the conditioning tanks. For long-term brood stock management in tanks, weight loss should be managed to avoid declining gamete quality and mortality of brood stock.

In a detailed study on spawning behaviour and timing of captive brood stock *Isostichopus fuscus*, Mercier *et al.* (2007) identified a pattern of aggregation and

spawning that correlated with the lunar cycle. This was supported by the findings of this study, where spawning of *H. scabra* occurred around the new and full moons. In this trial, the animals were biopsied before they were stocked and therefore handling stress cannot be ruled out as a possible trigger for the initial spawning observed in the tanks. However, animals used in this study were also collected and weighed weekly which involved handling that did correlate to the spawning events. The Mercier *et al.* study (2007), combined with the present results, suggest a potential large loss of gametes from spontaneous spawning events of brood stock during conditioning. The development of methods to reduce unplanned gamete release could potentially enhance yields obtained from induced spawning events. In other sea cucumber species, manipulation of temperature and light is used to inhibit spawning during conditioning periods (Liu *et al.*, 2005, Ito and Kitamura, 1998). However, *H. scabra* require large amounts of space and usually outdoor systems, making temperature and light control more difficult to achieve. Other methods for modulating the reproductive cycle such as hormonal manipulation using either gonadotropic hormones or spawn-inhibiting hormones may be more efficient in large-scale low-density systems. In conclusion, nutrient addition had little effect on gonad development and mass of brood stock but the system with larger tanks did provide a more suitable conditioning environment.

3.4.2   Light and water filtration levels

In this study, mass loss was recorded for brood stock in a replicated research-scale system with filtered and unfiltered water and under artificial light and natural sunlight. The mass losses recorded during these trials were comparable to the observations of Morgan during *H. scabra* brood stock experiments (2000a). Morgan observed mass loss of 5-10% or more after four weeks culture and this was unaffected by the addition of various feed supplements such as abalone and blue crab diets or kelp meal (Morgan, 2000a). Morgan investigated the spawning induction of individual *H. scabra* cultured over consecutive seasons. A significant loss of mass was observed and in general, the number of eggs and larval quality from resultant spawning declined in animals held for more than one month.

There was a sharp decline in mass observed during the first day of conditioning (Fig. 3.9). It is unlikely that such a decline is due to actual decline in somatic mass in such

a short period (overnight), rather, it is more likely a result of the purging process. The animals in this trial were pooled in one 300 L tank at high density for purging and some animals appeared to absorb water, becoming bloated. The following day the mean mass of the animals had substantially declined. Some individuals had reduced in mass by more than 100 g. The range in mass values for *H. scabra* used in the experiment was higher immediately after the purging process than it was on day one of the conditioning trial (Fig. 3.9). Such large differences in mass were not observed in other trials where purging was conducted in individual tanks (Chapter 2). Important to sea cucumber studies that take measurements of the body, purging is unlikely to be useful in reducing variance in mass measurements unless conducted in individual tanks with sufficient space. Even so, if animals become stressed due to lack of food or handling they may take in water. This is probably a mechanism of locomotion that evolved to reduce body density and become more buoyant and drift in current to new feeding / breeding grounds. Sea cucumbers have been observed to roll with tidal currents in reef lagoons and *H. scabra* are known to migrate to deeper waters away from juvenile settlement habitat (Mercier *et al.*, 2000). This migration pattern has also been observed in ranching studies in the Northern Territory where stocked juveniles migrate away from sea grass beds when they approach maturity. Similar migration patterns have been observed for *Cucumaria frondosa* populations in Canada (Hamel and Mercier, 1996). Margolin (1976) observed *P. californicus* response to interactions with sea stars involved increased locomotion and contact also associated with an increase in size and body contortion. When measuring mass of brood stock *H. scabra,* it is therefore recommended to measure immediately after collection or anaesthetise immediately after collection prior to measurement. If purging is required it should be conducted in individual tanks or at low density to avoid stress.

In this study, light and filtration levels had no effect on growth and final gonad condition of *H. scabra*. The growth of juvenile *H. scabra* in tanks is highly dependent on light, and thought to be related to the production of benthic and epiphytic algae in the tanks which form the main food source for juveniles (Battaglene *et al.*, 1999). In this trial however, there was no detectable difference in mass or gonad development between adult *H. scabra* cultured under different light levels. The small size of the individual tanks used in this trial (≈ 0.8 m^2) may be confounding or masking the effects of the abiotic

treatments by restricting the growth of the brood stock. The productivity within the small tanks may be insufficient to support the individual adults and therefore resulted in the declining growth rates.

There are no reports of experiments with water quality and adult *H. scabra* culture. However, *H. scabra* habitats in the NT, Australia are in turbid waters. Populations are often associated with river mouths, which discharge sediments and nutrients into the habitat (Vail, 1989). This nutrient influx may be an important energy source for sea cucumbers as it settles on the sea bed. The filtration of water used to culture sea cucumber in land based systems removes the majority of this potential food source and may necessitate the addition of nutrients and sediment to provide optimal conditions for growth. It is likely that the energy demands of the adult sea cucumbers are larger than what the productivity of the sediment substrate in the tanks could support.

The gonads of the brood stock in each treatment group were in a reduced state after the conditioning period. The gonad index ranged from 0.3% to 1.2%. The mean GI of *H. scabra* in wild populations can be up to 8-12% before peak spawning periods and 2-6% at other times (Ramofafia *et al.*, 2003). Comparison of the GI between the local wild populations (Chapter 2), those reported in the study by Ramofafia *et al* (2003) and the brood stock in the present study shows the gonads of the animals in each of the experimental treatments were in poor condition. Slightly different methods for calculating GI were used by Ramofafia *et al.* (2003) where they included viscera in the calculation of body weight. However, such calculations would have lowered the GI value in their study relative to the GI used in this study with viscera removed for the calculation of GI. In Chapter 2, the mean GI of *H. scabra* in the spent condition (<1%) was equivalent to the values from the brood stock in each treatment in this study.

In conclusion, these experiments were set up to determine the effects of culture conditions on brood stock growth and gonad development. Growth and development of the brood stock *H. scabra* conditioned in the small tanks was restricted across all treatments, taken together with the poor survival and condition of animals cultured in a similar system in chapter 2, this suggests that the tank design was not ideal and may be masking the effects of the treatments. To address this, further trials were moved to larger systems where replication was less but the condition of the brood stock improved

(experiment 1, chapter 2). Large tank systems are recommended for use when conditioning brood stock *H. scabra* and close monitoring of the gonad condition may be required to prevent missing the maturity and the loss of gametes to *in situ* spawning in the culture tanks.

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4 Radial nerve transcriptome reveals putative sea cucumber neuropeptides, including those regulating reproduction

4.1 Introduction

Neuropeptides are important signalling molecules that regulate biological processes in animals with a nervous system (Jékely, 2013). Some of these neuropeptides have been utilised to enhance reproduction in animal production systems. For instance, the vertebrate gonadotropin-releasing hormone (GnRH) is used in finfish aquaculture to stimulate or enhance maturation, ovulation and spawning success (Mylonas *et al.*, 2010). Similarly, neuropeptidergic factors have been identified from the central nervous tissues (CNS) of echinoderms that also regulate reproductive processes.

Modern molecular techniques utilising transcriptome sequencing and peptide detection by mass spectrometry can be used to identify these neuropeptide genes and their protein precursors (Rowe *et al.*, 2014, Adamson *et al.*, 2015, Stewart *et al.*, 2014, Evans *et al.*, 2012). Therefore, it may be possible to apply these methods to isolate and characterise the gonad stimulating neuropeptide components present in echinoderm neural tissues. Characterisation of such neuropeptides could have potential to be applied in sea cucumber aquaculture to stimulate reproduction.

The spawning induction techniques currently used in sea cucumber aquaculture are non-specific and involve thermal induction methods, which can be inefficient with low success rates (Battaglene *et al.*, 2002). Thermal spawning induction involves raising or lowering the ambient water temperature that triggers the animals to spawn (Morgan, 2000, Battaglene *et al.*, 2002). Using thermal induction methods, males usually spawn with a higher success rate and so research on improving spawning methods should focus on females. Typical spawning inductions for female *Holothuria scabra* result in 10% or less successful gamete release (Battaglene *et al.*, 2002). To help efficiency, extracts from echinoderm CNS have been used to induce spawning in a number of echinoderm species, including the asteroidea, echinoidea and holothuroidea. This suggests that a molecular investigation of the CNS to elucidate the active substance could be used for improved spawning induction techniques to be applied in aquaculture.

The holothurian CNS consists of an anterior circumoral nerve ring with five connecting radial nerves extending posteriorly along the ambulacra (Mashanov *et al.*,

2006). The radial nerves consist of ectoneural and hyponeural parts that form a complex with the ring canal and longitudinal muscles (Fig. 4.1). Both the nerve ring and radial nerves contain gonadotropic factors which can induce final oocyte maturation (Kato *et al.*, 2009, Maruyama, 1985), as determined by germinal vesicle breakdown (GVBD) (Fig. 4.1).
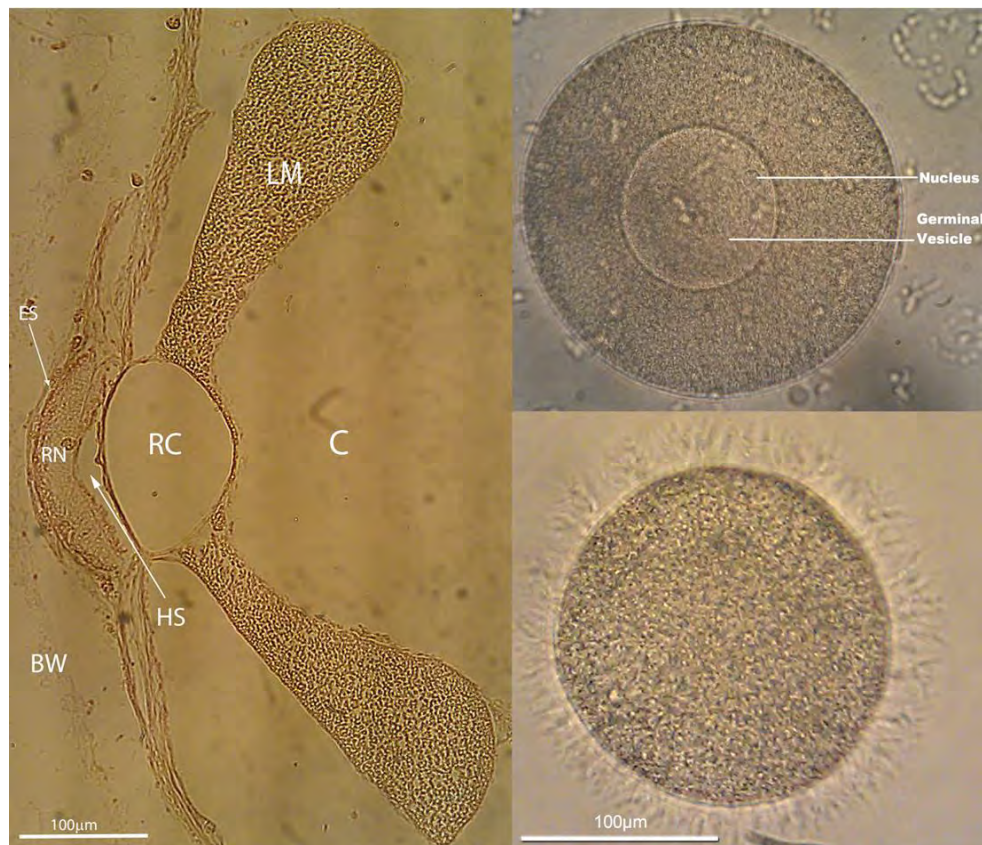
Figure 4.1: *Holothuria scabra* radial nerve and oocyte germinal vesicle breakdown (GVBD). Left - Orientation of a juvenile *H. scabra* radial nerve (RN)–longitudinal muscle band (LM) complex in relation to the coelom (C), body wall (BW), ring canal (RC), hyperneural sinus (HS) and epineural sinus (ES) in a transverse histological section. Top right - Immature oocyte with clearly visible germinal vesicle and nucleus. Bottom right - A mature ova post-GVBD and ovulation). (Photos by Luke Turner).

For most sea stars and sea cucumbers alike, final oocyte maturation occurs immediately prior to spawning, after stimulation of the gonad by a peptide substance secreted from the CNS. Without gonad stimulating substance (GSS) treatment or radial nerve factor (RNF), excised oocytes rarely spontaneously mature and become fertilisable (Maruyama, 1980). The endogenous control of oocyte maturation and spawning is most well understood in sea stars. In that echinoderm group, the process of

endogenous signalling involves secretion of GSS from the radial nerve, which in turn stimulates the gonad follicle cells to produce a maturation inducing factor (MIF). MIF binds to the oocyte, triggering the endogenous release of maturation promoting factors for final oocyte maturation. One-methyladenine (1MA) has been identified as the MIF produced in the ovary follicle cells in response to G protein coupled receptor mediated stimulation from a GSS (Mita et al., 2009, Mita et al., 2011). Sea star GSS has characteristics that place it within the relaxin-like protein family (Mita et al., 2009, Mita, 2013).

The process of oocyte maturation in sea cucumbers is likely to be similar, but much less is known. Final oocyte maturation in aspidochirote sea cucumbers involves ovulation of the oocyte from the follicle, migration of the germinal vesicle to the oocyte protuberance and GVBD (Hamel and Mercier, 2007). The germinal vesicle (GV) is centrally located and clearly visible using light microscopy in sea cucumber oocytes (Fig. 4.1). Upon resumption of meiosis the GV migrates to the oocyte protuberance and undergoes breakdown either before or after ovulation (Smiley, 1990, Hamel and Mercier, 2007). GVBD is easily observed as an indicator of oocyte maturation using light microscopy in sea star and holothurian oocytes, thus *in vitro* assays of GVBD are valuable tools for identification of active gonadotropic substances.

The sea cucumber MIF remains unidentified although is probably a molecule similar to, yet distinct from 1MA (1MA does not trigger GVBD in sea cucumber) (Kishimoto and Kanatani, 1980, Smiley, 1990). Attempts to identify the sea cucumber GSS have led to the characterisation of several neuropeptides with oocyte maturation activity *in vitro* and spawn-inducing activity *in vivo,* but the sea cucumber GSS remains unknown. Cross-reactivity of radial nerve extracts between different sea cucumbers and between sea stars and sea cucumbers with varying levels of activity suggest the GSS of holothurians and sea stars are structurally-related peptides (Maruyama, 1985, Strathmann and Sato, 1969). Sea cucumber GSS activity may be transmitted from the radial nerve to the gonad via the ceolomic fluid (CF). Mercier and Hamel (2002) experimented with transferring CF from pre-spawning *Bohadschia spp.* and *Holothuria spp*. The authors found that transfer of the aqueous fraction of CF could induce spawning in both conspecifics and between species. The GSS factor in the CF was also not sex-specific. However, the CF of a species of sea star and a species of sea urchin

collected whilst spawning was not able to induce spawning in female *Bohadschia argus* (Mercier and Hamel, 2002*)*. The active component of the CF may be an intermediary compound which is related between sea cucumbers but different in sea stars and sea urchins, or the GSS may be transmitted in the CF and activity is dependent on the level of divergence between the species.

The peptides termed cubifrin (also known as NGIWYamide) and a novel heptapeptide (QGLFSGVamide) were identified in nerve ring extracts from *Aposthichopus japonicus* (Kato *et al.*, 2009). Synthetic derivatives of both peptides induced oocyte maturation. However, the NGIWYamide and a derivative sequence NGLWYamide were more potent inducers of oocyte maturation, thus further research has since focused on these peptides. Using similar methods, a peptide termed gonad stimulating substance-like peptide (GSSLP) was identified from *A. japonicus* radial nerve extracts (Katow *et al.*, 2009). Its amino acid sequence is AEIDDLAGNIDY, which represents a novel peptide. Rapid amplification of cDNA ends methodology was used to elucidate the full-length gene sequence which shows that the peptide may be part of a larger 43 residue protein sequence – VLSKQAHHHHHEGWSLPGVPAEIDDLAGNIDYNIFKEOREKIK – which can induce oocyte maturation in 50% of follicle enclosed oocytes. Interestingly, although the N-terminal 21 residue sequence (VLSKQAHHHHHEGWSLPGVPA) does not contain the originally identified sequence (AEIDDLAGNIDY), it is able to induce oocyte maturation in 80% of follicle enclosed oocytes (Katow *et al.*, 2009). GSSL-peptides have complex expression patterns within the tissues of *A. japonicus* and vary seasonally (Ahmed *et al.,* 2011). The size of GSSL peptides appears to vary seasonally, with immunoblotting analysis of GSSL peptides revealing apparent polymorphic forms. The authors postulate that this might indicate complex post-translational processing or that a number of related peptides are differentially expressed at different reproductive stages (Ahmed *et al.*, 2011).

Other echinoderm neuropeptides have been identified with sequence homology to peptides and precursors of known bioactive peptides from other animal phyla. However, the functional characterisation of echinoderm neuropeptides is limited. The most well studied echinoderm neuropeptide is the SALMFamide and SALMFamide-related peptides. SALMFamides were discovered in sea stars and have subsequently been found in sea cucumbers and sea urchins (Elphick *et al.*, 1991, Elphick and

Thorndyke, 2005, Rowe *et al.*, 2014). These peptides contain a C-terminal motif SxL/FxFamide and have been attributed to regulatory roles in relaxation of tissues and feeding (Melarange *et al.*, 1999, Elphick and Melarange, 2001, Mita *et al.*, 2004). There is also preliminary evidence that they have a regulatory role in sea star reproduction. For example, the SALMFamide S1 may inhibit GSS secretion in the sea star *Asterina pectinifera* (Mita *et al.*, 2004). Other echinoderm neuropeptides have been identified in sea cucumbers by *in silico* sequence similarity searches using known neuropeptide sequences as queries in BLAST algorithm-based searches of echinoderm sequence databases. Twenty precursor sequences containing putative sea urchin neuropeptides were identified in the purple sea urchin genome and 17 putative sea cucumber precursors were found using a publically available sea cucumber transcriptome (Rowe and Elphick, 2012, Rowe *et al.*, 2014). Some of the echinoderm sequences share sequence homology to known reproductive hormones of other animals. These include a GnRH, a vasotocin-like peptide (echinotocin), glycoprotein hormone precursors, and a relaxin. NGIWYamides are also reported as having bioactivity in sea cucumber tissues involved in muscle contraction (Birenheide *et al.*, 1998, Inoue *et al.*, 1999). Similarly sea urchin NGFFFa and sea star NGFFYa peptides have also been attributed a contractile function in echinoderm tissues (Elphick and Rowe, 2009, Semmens *et al.*, 2013).

Transcriptomics offers a tool that can be used to enhance the power of studies to identify putative peptides and precursors by supporting molecular characterisation techniques. In this study, transcriptomic analysis of *H. scabra* radial nerve was used as a tool to identify putative secreted neuropeptides and specifically those that may be involved in reproduction. Some peptides identified in this *in silico* analysis were evaluated for use in aquaculture to enhance sea cucumber breeding methods.

4.2 Methods

4.2.1 Tissue collection

Adult *H. scabra* were dissected by making a longitudinal incision along the centre of the bivium. The digestive organs, gonad and respiratory trees were discarded before the radial nerve-longitudinal muscle band complex was excised using a sterile scalpel and forceps. Muscle bands and excess muscle tissue were separated from the radial nerve

over ice and under a stereoscopic dissecting microscope (Leica). Separated radial nerves were then either fixed in RNAlater (Ambion) at 4°C overnight and then stored at -20°C, immediately frozen on dry ice then stored at -80°C for peptide analysis, or used fresh for crude radial nerve peptide extraction.

A sample of excised radial nerve was also collected as described above and fixed in 10% formalin in FSW overnight at 4°C. For histological confirmation of neural tissue, the fixed radial nerve tissue was prepared, sectioned and stained with haematoxylin and eosin (H&E) following the procedures of Cummins *et al.* (2011) and viewed under a compound light microscope at 100 x magnification.

4.2.2 RNA extraction, transcriptome assembly and protein prediction

Radial nerve tissue (300 mg) obtained from mature male and female *H. scabra* was homogenised in duplicate. Total RNA was extracted using TRIzol reagent (Life Technologies, Australia) following the manufacturer's procedure. The amount and purity of the resultant RNA was quantified using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, DE, USA).

Two samples containing an estimated 815.2 and 860 ng/µl RNA with A260/A280 ratio of 1.68 and 1.72, respectively, were dried and sent to Beijing Genomics Institute (BGI) for Illumina sequencing, *de novo* transcriptome assembly and functional annotation (See S1). *De novo* transcriptome assembly was performed using the program Trinity (Grabherr *et al.*, 2011) using the parameter settings; seqType fq, Min contig length =100, min glue =6, group pairs distance =250, path reinforcement distance =90, min kmer cov =6. Unigenes were then organised into clusters and singletons. Functional annotation was performed by BLAST and BLAST2GO programs using the assembled *H. scabra* unigenes against the databases of NT, NR, KEGG, Swiss-Prot, COG (e-value <1x10^{-5}) and GO (default settings). Relative abundance of unigenes in the library was estimated using the fragments per kilobase per million fragments (FPKM) method (Mortazavi *et al.*, 2008).

To identify putative *H. scabra* neuropeptide precursors; tBLASTn searches of previously discovered echinoderm and other animal neuropeptides and neuropeptide precursors were conducted using CLC workbench 7. To mine for novel sea cucumber neuropeptides a *H. scabra* open reading frame (ORF) library was constructed using

sequences that did not have a significant BLAST match in any annotation databases. ORF of novel *H. scabra* sequences found by ESTscan were predicted using CLC workbench. Following translation, the ORFs were submitted to the online signal peptide prediction tool PrediSi (Hiller *et al.*, 2004) (http://www.predisi.de). Translated unigenes with predicted signal sequences with a PrediSi score of >0.5 were analysed manually and using NeuroPred (http://neuroproteomics.scs.illinois.edu/cgi-bin/neuropred.py) for prediction of putative basic and dibasic cleavage sites as well as putative sites for common neuropeptide post-translational modifications (PTM) such as of C-terminal amidation and N-terminal pyroglutamation. Sequences with possible transmembrane domains were screened using the TMHMM tool provided by the Centre for Biological Sequence Analysis, University of Denmark (http://www.cbs.dtu.dk/services/TMHMM/). Identified putative *H. scabra* neuropeptide precursors were compared with homologous sequences found using tBLASTn searches in available holothurian and sea urchin transcriptomes. At the time of this analysis, three transcriptomes were publically available from *A. japonicus* (Du *et al.*, 2012, Zhou *et al.*, 2014, Zhou *et al.*, 2013) and *H. glaberrima* (Mashanov *et al.*, 2014)*. The sea urchin sequences were found using the *S. purpuratus* genome database (echinobase.org). Putative holothurian neuropeptide precursor sequence phylogenetic relationships with other known sequences were analysed using MEGA software version 5 and 6 (Tamura K, 2013) and presented using GeneDoc software (Nicholas and Nicholas Jr, 1997). Figures of the structures of neuropeptide precursors were constructed using DOG software (Ren *et al.*, 2009).

4.2.3   Radial nerve extract used as the oocyte maturation inducing factor

4.2.3.1 Preparation of radial nerve extract

Radial nerve tissue was collected from adult *H. scabra* as described previously, and ground in a mortar and pestle. Ground tissue was then transferred to a 50 ml centrifuge tube containing 30 ml filtered sea water (FSW). The FSW/radial nerve homogenate was centrifuged at 2000 x g for 5 min. Supernatant radial nerve extract (RNE) was collected and stored at -20°C until required.

4.2.3.2 *In vitro* assay of oocyte maturation induced by RNE

Ovarian tubules were extracted, via a small incision in the dorsal bivium, from two mature *H. scabra* previously conditioned in 6 t culture tanks. Tubules were cut into ≈5 mm transverse sections and transferred to a 6 well microplate containing 3 ml of 1 μm FSW in each well. One 5 mm section from each donor female was placed into each well. One ml was removed from three alternate wells and replaced with 1 ml of thawed RNE and 1 ml was removed from the remaining wells and replaced with FSW (control). The ovarian tissue was incubated at 24°C for 3 h and continuously monitored for signs of oocyte maturation and ovulation.

After the 3 h incubation, oocytes were mechanically removed from the ovary tissue using forceps and a 500 μl sample was collected and placed onto a Sedgwick-rafter counting slide. For each well, a total of 30 oocytes were measured and the number of mature and immature oocytes recorded based on absence or presence of the germinal vesicle respectively. The numbers of mature oocytes were compared using Student's t-test.

4.2.3.3 Purification and characterisation of *H. scabra* RNE peptide inducing GVBD

To extract radial nerve tissue for assessment of biological activity and peptide purification, nine adult *H. scabra* were anaesthetised and then the radial nerve tissue was excised as previously described. A total of 7.9 g of radial nerve tissue was obtained from the animals and used to create the RNE.

Crude RNE purification was prepared by homogenising the nerve tissue in 15 ml 0.1% trifluoroacetic acid (TFA) with a homogeniser followed by sonication with a probe sonicator for 3 x 20 second pulses. The homogenate was then diluted with an additional 25ml 0.1%TFA and centrifuged at 4000 rpm for 15 min. The supernatant was purified by loading the sample onto a Sep-Pak C18 5 g cartridge and rinsed with two volumes of 0.1% TFA and the purified extract was eluted in 9 ml of 60% acetonitrile (ACN) / 0.1% TFA and lyophilised. The homogenate pellet was stored at -80°C for later use; as crude RNE positive control in the GVBD assay. A few grams of that frozen pellet was resuspended in 5 ml artificial seawater (AS; Instant Ocean, Australia), re-homogenised

and centrifuged at 4000 rpm for 5 min. The supernatant was collected and used in GVBD assays as a positive control RNE.

The lyophilised fraction of purified RNE was resuspended in 400 µl of 0.1%TFA and the biomolecules separated using reverse phase high performance liquid chromatography (RP-HPLC). The sample was loaded onto a Zorbax 300SB-C18, 5 µm particle size, 4.6 mm internal diameter, 250 mm length column (Agilent Technologies) and fractionated on a linear gradient of 0% - 60% ACN, 0.1% TFA over 60 min using a PerkinElmer series 200 autosampler and pump. Eluted biomolecules were detected with a Flexar PDA detector at 210 and 280 nm. Fractions were pooled every 5 min, lyophilised and stored at -20°C until use in the GVBD assay. Active fractions identified in the assay were lyophilised, resuspended and refractionated using RP-HPLC with an elution gradient of 5% lower to 10% higher than the expected elution range of the fraction over 120 min (i.e. a 20-25 min fraction was fractionated over a 15-35% ACN / 0.1% TFA over 120 min) with sub-fractions pooled every 1 or 5 min.

4.2.3.4 *In vitro* assay of purified *H. scabra* RNE with GSS activity

Mature female *H. scabra* were selected by needle biopsy from brood stock held at an aquaculture facility at Darwin, NT, Australia. Four mature females were transferred to USC and housed in aquaria with aeration under ambient conditions until use. Immediately prior to use, the ovary was removed from one female, and then large fecund tubules were selected. Ovary tubules were cut into roughly 5 mm long segments using a scalpel and a stereoscopic dissecting microscope (Motic). Approximately 60 ovary segments were transferred to a 96 well microplate before the assay solutions were added.

Twelve lyophilised 5 min fractions of the RNE RP-HPLC purification were each resuspended in 20 µl of water and 10 µl transferred to clean tubes and diluted with 1.3 ml of 35 ppt of AS. The remaining 10 µl was re-lyophilised and stored at -20°C for MS analysis, or further purification. AS and crude RNE were used as negative and positive controls, respectively. A cocktail consisting of a pooled sample of the 12 RP-HPLC fractions (100 µl of each) was used as an additional treatment. Each well of the 96 well microplate containing an ovary fragment was delivered 300 µl of one of the fifteen

treatment solutions (Fraction 1-12, cocktail, AS and RNE) with 4 replicate wells for each treatment. The plate was incubated at 28°C for 3 h to allow GVBD to occur.

To determine GSS-like activity of each treatment, oocytes were separated from ovary tissue by gently aspirating with a pipette. Oocytes in a standardised area of each well were then photographed at 10 x magnification on a Leica inverted microscope. The number of immature oocytes containing a GV and the number of mature oocytes without a GV were counted for each image. The mean number of mature oocytes determined as oocytes lacking a GV was compared graphically, then ANOVA and Tukey's HSD post-hock test was used to assess for any statistical significance detected in the results.

Sub-fractions of the active RNE fractions detected in fractions 1-12 (fraction 7 and 5) were retested for activity. These sub-fractions were resuspended in 20 μl of water and 10 μl transferred to clean tubes and diluted in 600 μl of AS, then tested in a similar GVBD assay following the procedure stated above, except the assay volume was reduced to 150 μl per well. The remaining 10 μl of the sub-fractions were lyophilised and stored at -20°C for LC-MS/MS analysis and peptide identification.

4.2.3.5 NanoHPLC-ESI-Triple TOF peptide identification

The RNE were analyzed by LC-MS/MS on a Shimadzu Prominence Nano HPLC (Japan) coupled to a Triple-ToF 5600 mass spectrometer (ABSCIEX, Canada) equipped with a nano electrospray ion source. Aliquots (6 μL) of each extract fraction or sub-fraction were injected onto a 50 mm x 300 μm C18 trap column (Agilent Technologies, Australia) at 30 μL/min. The samples were de-salted on the trap column for 5 min using solvent A [0.1% formic acid (aq)] at 30 μL/min. The trap column was then placed in-line with the analytical nano HPLC column, a 150 mm x 75 μm 300SBC18, 3.5 μm (Agilent Technologies) for mass spectrometry analysis. Peptide elution used a linear gradient of 1-40% solvent B [90:10 acetonitrile:0.1% formic acid (aq)] over 35 min at 300 nL/minute flow rate, followed by a steeper gradient from 40% to 80% solvent B over 5 min. Solvent B was then held at 80% for 5 min to wash the column and then returned to 1% solvent B for equilibration prior to the next sample injection. The ionspray voltage was set to 2400V, declustering potential (DP) 100V, curtain gas flow 25, nebuliser gas 1 (GS1) 12 and interface heater at 150°C. The mass spectrometer acquired 500 ms full scan TOF-

MS data followed by 20 by 50 ms full scan product ion data in an Information Dependent Acquisition (IDA) mode. Full scan TOFMS data was acquired over the mass range 350-1800 and for product ion ms/ms 100-1800. Ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion, ms/ms spectra of the resultant 20 most intense ions. The data was acquired and processed using Analyst TF 1.5.1 software (ABSCIEX, Canada).

Proteins were identified by database searching using PEAKS v7.0 (BSI, Canada) against the protein database built from the *H. scabra* radial nerve transcriptome. Search parameters were as follows: no enzyme was used; variable modifications included methionine oxidation, conversion of glutamine to pyroglutamic acid, deamidation of asparagine and amidation. Precursor mass error tolerance was set to 20 ppm and a fragment ion mass error tolerance was set to 0.05 Da. Maximum expectation value for accepting individual peptide ion scores [$-10*Log(p)$] was set to ≤0.01, where p is the probability that the observed match is a random event. Proteins and their supporting peptides were obtained and analysed. [1]

4.2.3.6 *In vitro* assay of synthetic peptides for oocyte maturation activity

Ovary tubules were removed by biopsy from five mature female *H. scabra*. Tubules were cut into transverse sections about 5 mm long and transferred into 340 microplate wells (across 4 x 96 well microplates) with each well receiving 1 section of ovary tissue. A total of 68 different treatments were carried out with five replicates, each using ovary tissue from a different female. Selected putative *H. scabra* neuropeptides identified from the transcriptome analysis were synthesised (ChinaPeptides Co.,Ltd.) and solutions of each made to concentrations of 10 pM, 1 nM, 10 nM, 100 nM and 10 µM using serial dilution. Peptides were dissolved at a concentration of 1 mg ml^{-1} in distilled water before being diluted into the appropriate concentrations in 0.45 µm FSW. Each of the peptides was tested at all of the concentrations; additionally peptides predicted from the same precursor were tested in combination at each concentration and one positive control

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[1] I would like to acknowledge the expert support of Dr T Wang in LC-MS/MS analysis, peptide identification and peptide modelling. Dr Wang conducted the LC-MS/MS and PEAKS database search as described in section 4.2.3.5 of this thesis as well as the peptide modeling described in section 4.2.3.8.

(crude RNE) was used (Table 4.1). Two negative controls were used, consisting of 0.45 μm FSW (Control) and 0.45 μm FSW diluted with 10% distilled water equivalent to the same dilution factor of the smallest peptide (ControlD). The ovary fragments were incubated in 300 μl of treatment solution for 4 h at 28°C.

After incubation, oocytes and tubule fractions from each well were transferred to a microtube and oocytes and ova separated from the tubule by gentle aspiration with a pipette. A homogenous sample of oocytes was pipetted onto a glass slide and photographed using a digital camera connected to a Leica dissecting microscope (60 x magnification). Oocytes and mature ova were distinguished as previously described and counted in each photograph.

Table 4.1: Sequences and masses of synthetic *Holothuria scabra* neuropeptides selected for GVBD assay screening. [p-] represents pyroglutamation.

TREATMENT	SEQUENCE	MOLECULAR WEIGHT (DA)
NP1	NGIWYamide	650.73
NP2	NGIWFamide	634.73
NP1+2		
NP3	CFVTNCLLGamide	966.18
NP4	SYSPFMFamide	877.02
NP5	SFSRLYFamide	918.06
NP6	FKSSFYLamide	890.04
NP4+5+6		
NP7	[p-]QYFTamide	539.58
NP8	[p-]QLPGGDAGDVED	1154.15
NP7+8		
NP9	FGNYPMDPLSQSLMamide	1598.86
NP10	NGGIA	430.46

4.2.3.7 *In vitro* assay of synthetic oocyte maturation associated peptide

The single peptide identified from RNE that had gonadotropic activity was synthesised (China Peptides Co., Ltd). The peptide consisted of the amino acid sequence SYHNPVDISGGYYLGSGVHRGTamide and was termed oocyte maturation associated peptide. For the assay, the peptide was solubilised in distilled water and then diluted to test concentrations of 10 µM, 10 nM, 10 pM, 1 pM and 10 fM. Ovary sections of a mature *H. scabra* were incubated in the test solutions with positive (crude RNE) and negative (FSW) controls in a 96 well microplate, as described previously. Ovary sections were incubated at 27°C for 3 h. After incubations, the number of mature oocytes in a subsample of 30 oocytes were counted for each well, as previously described.

4.2.3.8 Peptide modelling

The initial conformation of the oocyte maturation associated peptide was built/predicted using the LEAP module of AMBER version 11 (Case *et al.*, 2010). Then, molecular dynamics (MD) simulation was fully unrestrained and carried out in the canonical ensemble using the SANDER module. The ff14SB force field (Duan *et al.*, 2003) was employed. Energy minimisation with 2500 steps was first performed to remove unfavourable contacts. The AMBER structure was then heated to 325K over 50 ps to avoid being kinetically trapped in local minima, and cooled down to 300K, then subjected to unrestrained MD simulations at 300K for the purpose of peptide equilibration. The structural information was sampled every 1 ps (i.e., 400,000 structures were calculated for 400 ns MD simulation). Then a lowest energy structure can be determined, and considered as the representative of the conformations simulated over this period. Visualisation of the systems was effected via VMD software (Humphrey *et al.*, 1996).

4.2.4 Workflow of experiments

A summary of the workflow of experiments conducted to identify *H. scabra* neuropeptides and identify gonadotropic peptide hormones is given in figure 4.2.
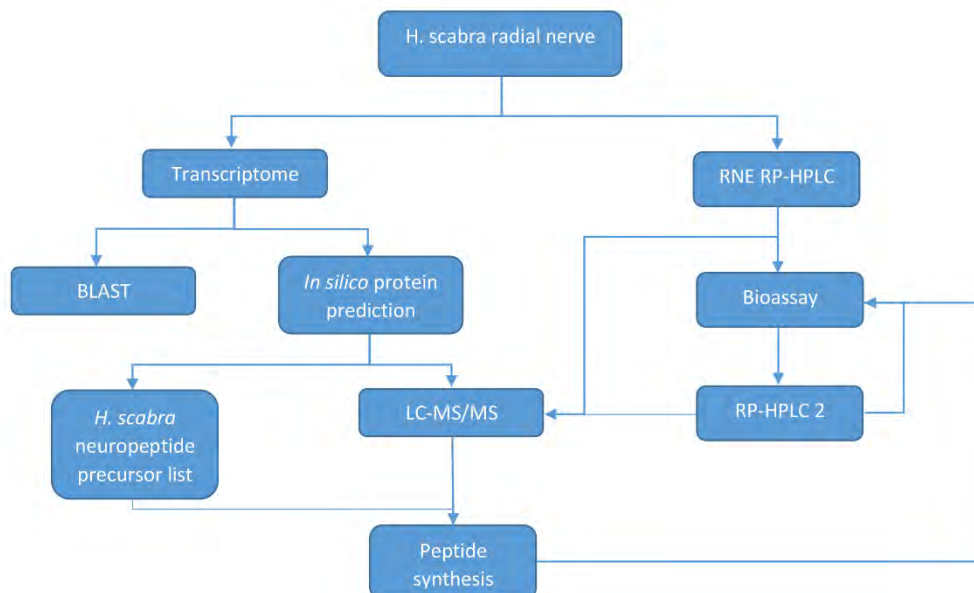
Figure 4.2: Workflow diagram of experiments conducted to identify *Holothuria scabra* neuropeptides and identify putative gonadotropic peptide hormones.

4.3   Results and Discussion

4.3.1   *H. scabra* radial nerve histology

A transverse histological sections of a sample of *H. scabra* radial nerve tissue stained with haematoxylin and eosin revealed features consistent with radial nerve sections of other holothurians (Mashanov *et al.*, 2006, Mashanov *et al.*, 2009, Inoue *et al.*, 2002). Ectoneural and hyperneural parts of the radial nerve cord, lacunae, sinus' and ring canal were clearly identified with muscle tissue from the inner body wall and longitudinal muscle surrounding the nerve components (Fig. 4.3). Once histology had confirmed the presence of neural tissue in our samples, we proceeded with Illumina sequencing. Since some excess muscle tissue was also present, we expected that our radial nerve transcriptome would also contain some muscle tissue transcripts.
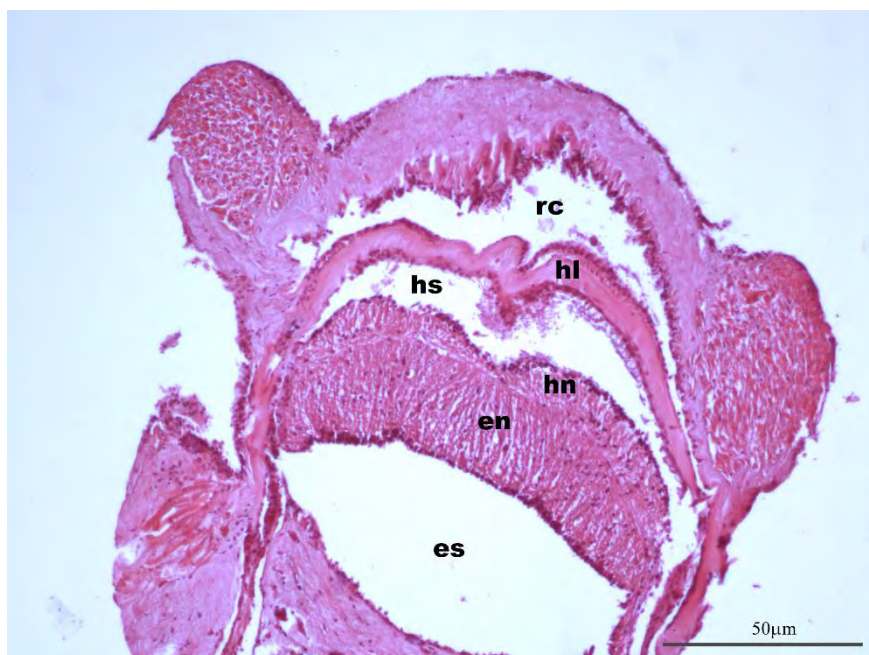
Figure 4.3: Micrograph of a transverse histological section of excised *Holothuria scabra* radial nerve and surrounding muscle tissue stained with haematoxylin and eosin. Features observed included; ring canal (rc), hyperneural lacunae (hl), hyperneural sinus (hs), hyponeural (hn) and ectoneural (en) parts of the radial nerve and the epineural sinus (es).

4.3.2 *H. scabra* radial nerve transcriptome sequencing, assembly and gene annotation

A total of 4.9 Gb of *H. scabra* radial nerve transcripts were sequenced, generating 58,264,200 total raw reads and 54,975,732 total clean reads. Sequence assembly resulted in 112,276 contigs (average length 285 nt) and 51,874 unigenes (average length 528 nt), including 6,308 clusters and 45,566 singletons. Only 10,337 (19.9%) of those unigenes produced a significant hit following annotation databases of Nt (7%), Nr (18%), KEGG (13%), Swiss-Prot (15%), COG (6%) and GO (9%), reflecting the dearth of sequences available in data banks from closely-related species, including other echinoderms. Slightly higher rates of annotation (30-40%) were reported for transcriptome annotation in the sea cucumber *A. japonicus* (Du *et al.*, 2012, Zhou *et al.*, 2014). However, our study is very similar to the rates reported for another non-model echinoderm (Delroisse *et al.*, 2015).

Analysis of the *H. scabra* radial nerve unigenes in the non-redundant sequence database (Nr) produced 9,380 hits (18.08%); of those, 52% had a significant BLAST match with sea urchin *S. purpuratus* sequences, 10.5% with acorn worm *S. kowalevskii* sequences and 37% had a match with other species, with each species having sequence similarity proportions of the BLAST matched sequences of less than 5% (Fig. 4.4). Relatively few hits in the Nr database reflect the small resource of sequences from closely related species and is to be expected for a non-model organism.
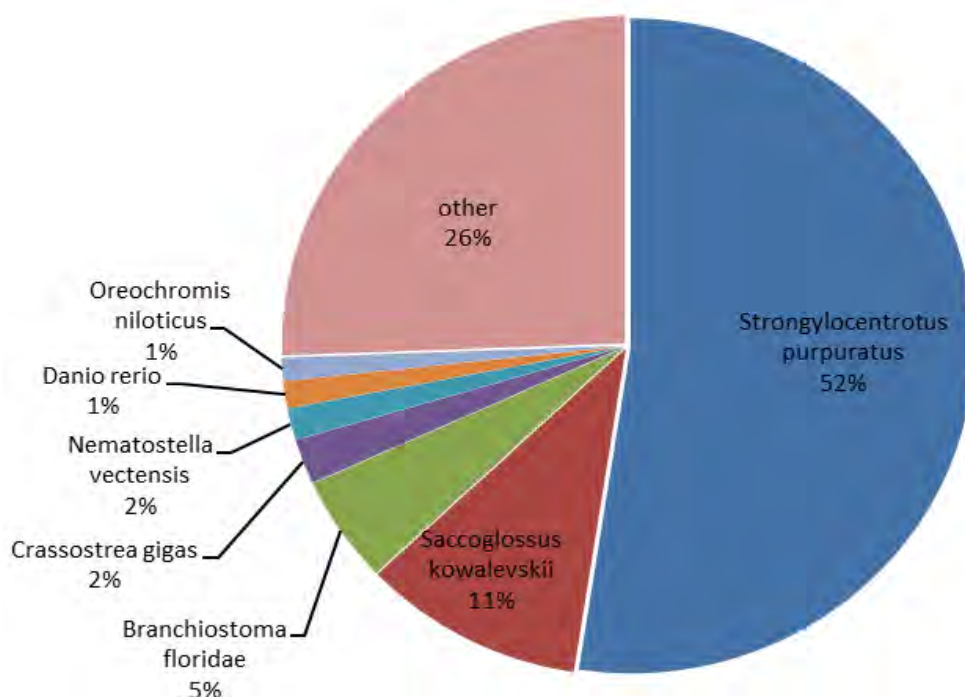
Figure 4.4: Species similarity matches to *Holothuria scabra* radial nerve unigenes (E>1x10^{-5}). BLAST match was with Nr protein sequence database.

Gene Ontology (GO) functional annotation classified 4,767 unigenes into one or more GO classifications (Fig. 4.5). Classifications with the highest number of genes in the biological process ontology were cellular process (3,102 sequences), metabolic process (2,540 sequences) and biological regulation (1,568 sequences). Approximately 1,452 sequences were classified as positive regulation of biological processes and 249 and 227 unigenes were involved in reproduction or reproductive process. Within the cellular component ontology, cell, cell part and organelle classes contained the highest number of unigenes. Binding and catalytic activity were the dominant classes represented in the molecular function ontology.
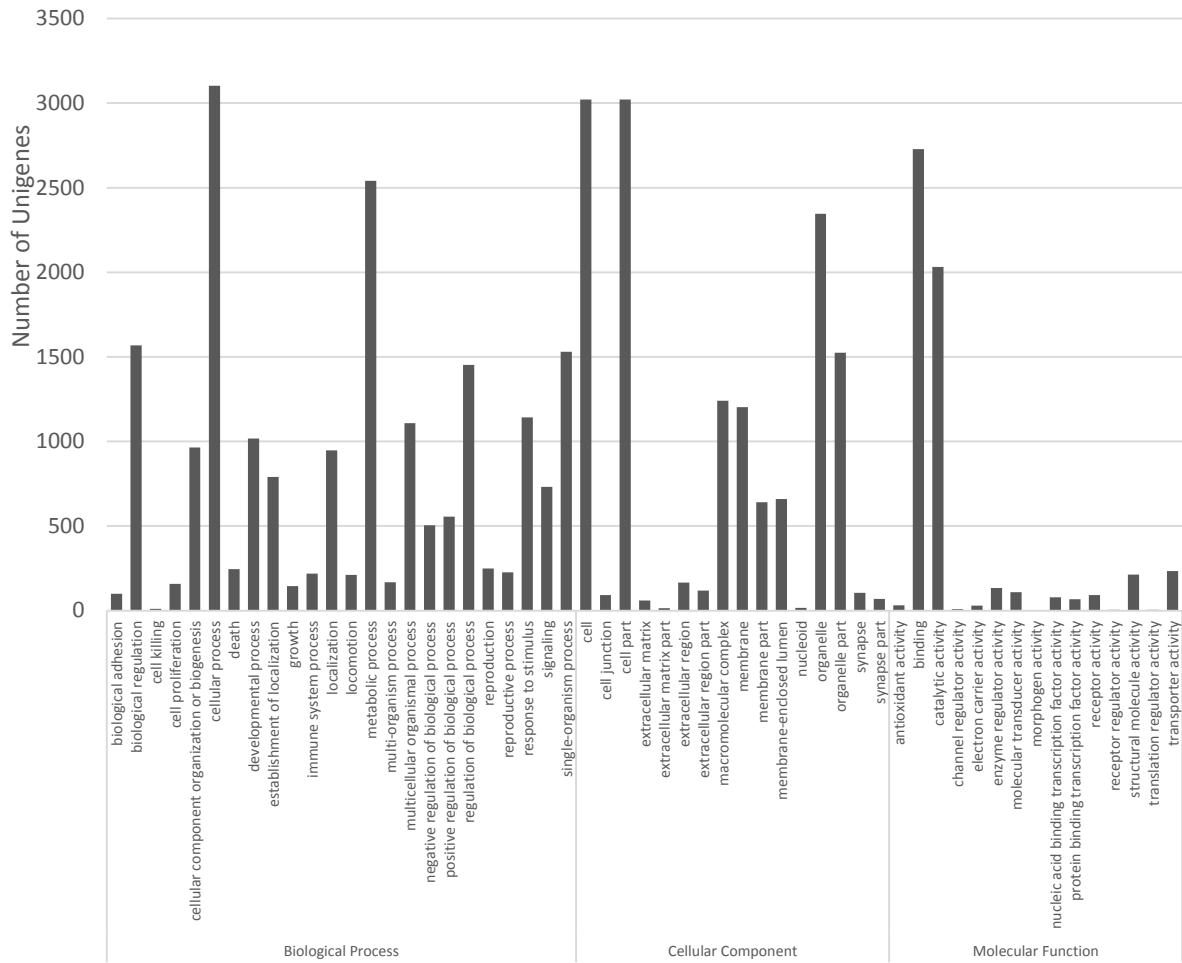
Figure 4.5: Gene Ontology functional classification of *Holothuria scabra* radial nerve unigenes.

Functional classification of *H. scabra* unigenes in the Clusters of Orthologous Groups (COG) database predicted functions for 3,125 unigenes. The majority of annotated unigenes were classed as "general function prediction only" and "translation, ribosomal structure and biogenesis" followed by "transcription" and "replication, recombination and repair" (Fig. 4.6).

COG Function Class
A: RNA processing and modification
B: Chromatin structure and dynamics
C: Energy production and conversion
D: Cell cycle control, cell division, chromosome partitioning
E: Amino acid transport and metabolism
F: Nucleotide transport and metabolism
G: Carbohydrate transport and metabolism
H: Coenzyme transport and metabolism
I: Lipid transport and metabolism
J: Translation, ribosomal structure and biogenesis
K: Transcription
L: Replication, recombination and repair
M: Cell wall/membrane/envelope biogenesis
N: Cell motility
O: Posttranslational modification, protein turnover, chaperones
P: Inorganic ion transport and metabolism
Q: Secondary metabolites biosynthesis, transport and catabolism
R: General function prediction only
S: Function unknown
T: Signal transduction mechanisms
U: Intracellular trafficking, secretion, and vesicular transport
V: Defence mechanisms
W: Extracellular structures
Z: Cytoskeleton

Figure 4.6: Abundance of *Holothuria scabra* radial nerve genes within COG functional categories.

4.3.3 Identification and characterisation of putative sea cucumber neuropeptides

Within the *H. scabra* radial nerve transcriptome, 77 putative neuropeptide precursors were identified. Similar precursor sequences were also found in publically available *H. glaberrima, A. japonicus and S. purpuratus* sequence databases. Of the 77 *H. scabra* putative precursor sequences identified, 74 (96%), 46 (60%) and 30 (39%) have partial or full matches in the available *H. glaberrima, A. japonicus and S. purpuratus* databases, respectively. A list of all identified *H. scabra* precursor proteins of known, previously reported neuropeptide precursors and neuropeptides is provided in figure 4.7. In addition, those that have a BLAST match with *H. glaberrima* (Mashanov *et al.*, 2014), *A. japonicus* (Du et al., 2012, Zhou et al., 2013) and *S. purpuratus* (http://Echinobase.org) (Cameron *et al.*, 2009) sequences are presented in Table 4.2.

>Hsc_GVBDassociatedpeptide
MKYVGELSFVWLVILALYAVVPTIVQARALDGDNYIPESDEDNTNDEAPIQDMDD
NFTVSKDDLVNIVGMVELYLANKAKSGASFLWNRPIEPLSNGFGGFYPSKRSADSL
YDARDYYARAALKRNVRNELRNALAAKRSYHNPVDISGGYYLGSGVHRGTGRR

>Hsc_Relaxin
MASKTTRVVFFAAVCVLLVLEHAASVRLCGADLSRAVYRVCSHGKRGYPMIDIEED
DFSQELDTELDEYLAQALTGFLESRSFAADIESDRYYTIPQRFRRNGGIARRCCASGC
SSSDIAKLC

>Hsc_NGIWYa(Cubifrin)
MAVEARIVFFSWVVCIWFASFVTCQTITGRPQDYEEISKAVDRFLDILIKEDTTDPD
FKLDSWESLLDEEINPKLKILAHVMRSLSSRPDVPSVREQLYIPNSYLQSFITEEDPFS
DDPSSKLPSLQSSGLDQKGKERNNYWGENDIFPGNEEKRNGIWYGKRATNTND
AAAKRNGIWYGKRNSPTLEDKRNGIWFGKRNGIWYGKRNGIWYGKRNSGYFPSV
ADEVM

>Hsc_Vasotocin
MSLKYVCGAVLFLILVVCLRESRSCFVTNCLLGGKRSASRPYRQCLPCGPRQAGRCV
GPGICCGSSFGCLINTKETITCRRENELPTPCEVIGDRCLTVSNGKCTAFGVCCNERG
CVLEENCKYSPSRIRRNEPLLMTSSNENLYDGGIGERFTDFLFEESEK

> Hsc_SALMFamide-like
MALPSSFLFIAVTVLGGFHLTASQPLDSKEVPGFQAEVPDDLETAEILFQNQKDLAH
QLALDYLYDEEADGFTNFAKHDPRNEEMDKKDTRTLYDNYAKRNIPPNFFKLNFG
KRTTDKHFNNPFVFGKREDLSDVDKRSYSPFMFGKRENDLNLDPEKRSWNSKFMF
GKRDGSDADKRAGYSVLYFGKRVPETAKRSFSRLYFGKRGRSAYNKFMFGKREEA
DKTEDKTEDKRFKSSFYLGKRNFAADNDGVQAMENV

>Hsc_SALMFamide-spnp1like
MKAYQVLVPAFLCVMAAILARTEAEEDVIPLNEELYKITEELEERLRQREMEENLLL
ADGVYKPGIKRASRPWSPLLGQTGIAFGKRSDIERTRDQTDQRARKTRTRGMFGH
SALAFGKRTEYLPQDLLELEGANNGFGDFQQVPVKKAMGFTGNTGILLGKRNADD
EQD

>Hsc_Ntermpartial_PPLNP2
MKLVVYTIGFAVAACVLAVFAEDEDAQAEDKRFGNSAMLDPLSQSLMSGKREED
KRFGNYPMDPLSQSLMGKRSQDKRFGNYPM

>Hsc_CRH-like
MQKFLVLIVSVLVVLLGLEAVTEAVALPLSGIPDDENLTDLELMEDVNDYVKRIGDN
DFAARRGQQISQIARNRALYQQRKHVLDLAGKRGGMDKRNLSQNDLSQGRSNYI
NQLLAYQKWSQLMGAAGRR

>Hsc_GN19
MIKMAEKLLLIGLVVAALILVCSSMYVKRTDDQDDLSPLLLQMKRNERMNEMEML
ADTLKRNGPIRYRNNYAALPPMPWLMNNKR

>Hsc_GPHA2
MMTRKALSQILLFAFVCLLIGAFFPQKTEADRLWQRPGCHRVGFKKLVEIPGCRSV
EILINACRGYCMTYSFPSDIHTLFLSGGNHVLTSHGSCCTIKTTHDVHFTLECENNQ
VYQDVIKSAEDCECSLCDVEY

>Hsc_GPHB5
MARRERLYLLGLTGCFIVSLLCSTVTSVDPSTTIECFVHTAMKHRAIKKGCRPYDITIR
GCWGRCDSYQVPELLPPYVYSVHPSCIQDGVTPTTIQLPDCDEGVDSTYVYNSANS
CKCQSISGVTTAYDYRPDYFLT

>Hsc_Gremlin_GBH-like
MISVFWRSVLQFVVILPALLSQVASSLDNNPVASQEQTIDTSQFPITNPMSMLRHE
TAQSRY5SGLPQELRVAPDKVLKSSSEEVIQSEANYLRDDWCKAYTLRQRIEEPGCI
SRTITNRLCYGQCNSFYIPKQSNDYNQAFQSCSFCKPQKVAYITITLRCPGQDPPIKT
RRVKRIKKCRCMAIDVS

>Hsc_Spnp12-like partial
CKAAIAILLIVIVSMACQAHNTYSMKGKYRWRAGKRSYNLRDSLEKRMPSYSNMF
QGIQQFPLQDDVPPSSKSLKSIVTDLRDYC

>Hsc_SP_MPMNPADYFSRGTVYIPTRDS-like
MQSNVTRVLSLVVCIYMCIVAVSSVPINLPEYQTGGTVFFPTRYRRNIGDSDYWVN
SGTVFLPDILDKRGLGDGRFSRYDDYYTRGTEYFPSRIKRNYVPVEPGMWDHMCV
NKPMFYCMCSSNGLVSFCENHTSSYFSRR

>Hsc_Spnp11-like
MNTFLVCLFSMILLAGVVLGAPALDFGDDATYDAILDQRMRRSYNKLNECLHDCL
FCGKYSKKFNVKSCLDECWGKTTGNEQTWAACSLFTGKR

>Hsc_Spnp15-like
MSQLTSLCLLLIVAAAAVFAYPADNQLDLIDIDDIFDAAQADKQETVPEVASNSFDR
ERRHQFYSCMKECSHCAKIHTRYSMVRCFRTCSSGRTDFGCNNRMHFV

>Hsc_CTermpartial_PPLNP2
PLSQSLMGKRSEEKRFGNFPMDPLSQSLMGKRSQDKRFGNYPMDPLSQSLMGK
RSEEKRFGNYPMDPLSQSLMGKRSEEKRFGNYPMDPLSQSLMGKRNQEKRFGNY
PMDPLSQSLMGKRSAEKRFGNFPMDPLSQSLMAGGGRR

>Hsc_TRH-like_PRQVa-like
MSTLAFLLYFIYLLQGNVASGTLEGTIADVEGEILKEIEENAQLNEISADGEEDKRQY
FAGKRQLPGGDEYEDKRQYFTGKRQLPGGDAGDVEDKRQYFAGKRQLPGGDAG
DFEDKRQYFAGKRQLPGGKDYEDKRQYFNGKRQYFAGTREFLGVGQDNTEDKRQ
YFAGKRQLPGGQEYEDKRQYFAGKRQLPGGEAGDLEDKRQYFAGKRQLPGGNEF
DHKRQYFAGKRQLPGGDVKDFADKRQYFAGKRQLPGGEAGDLEDKRQYFAGKR
QLPGGDAYEDKRQYFVGKRQLPGGEEYEDKRQYFSGKRMQTDAELNDETQLFSE
KREYEGDAEHEDKRQYFAEKRQLPGGDVEDGMMAKRQHFIGKRQLPEGWVNV
NSDGKRQYFVGKRQLPGGDFEDLADKRQYFVGKRQLPGGQTDADYIKRQFFHGK
RQLPGGQTMNTNRQYFAQKRQLPAGPWAFWE

>Hsc_Luqin
MALSRCRTTELFCWCAILLVLVALVVPLSSAQSMEASRKRKPYKFMRWGKRFYDD
TDVLEVGSSEDIGFEQQQLPTELLLPMFGDKEIICMRVSEGGLYQCSQYTARSDTLR
HK

>Hsc_CT
MKTSVVVPITLCIFCYLLATVSAASISRPSAELDLSRQYPELYEYILRQFSADQPMEYK
RSCSDRFSGCAHLKVAKALLDQARREENSRFGISGPGKRDSSDFELVKEKRRMGGC
GDFSGCASLKAGRDLVRAMLRQPSKFGSGGPGKR

>Hsc_CT2
MKTSVVVPITLCIFCYLLATVSAASISRPSAELDLSRQYPELYEYILRQFSADQPMEEK
RRMGGCGDFSGCASLKAGRDLVRAMLRQPSKFGSGGPGKR

>Hsc_ANP-like
MQFPLRLYLLASVLLLSVFPTCIHTAEEEVPTEDVGGDDWTAIEKVYAAEEKVDDKD
GDQTSGEAADEDKRGNIFSKLRGKQRFRLRTRDDDNEDGMMELEEEEEKRANHH
RMMNFRPRYRIRTRDGEEEYPDEEEGEEEEEEEEDPTEDLRGNKRNSLRGKVRYRI
RNRSGDEFYEIDDENEEEKRANKFKALRGRLGSPRFRIRTRDGEEEEEEEEEEEEEEEE
EGVDKRANMMKMMRGRPRYRIRTRSEEGDEEYYDEDEQDLEEEKRANFYFKLRF
KPRYRISARGFDEDDEGYYEEGDNLQEEKRANHFNTLRGKSRYRIRNRDDQVDAE
EYQLDGLEDKRGNHIGVLRGKGRRYHLVSRSGDPVLEQDATLQE

>Hsc_Spnp16-like
MSGKTNLVMFLFGLVVTVVAARNLDLRDFKEPQQTTNEQNFEDLVNEVPDSPRTI
NQRIRDELMANLLNLLGKVDAADPFEYIPPMDSRPEFPVSGSDRFVEGSLSKRSKG
RYHNNPWCNLVDIWKGQGSGSRRCR

>Hsc_Spnp17-like
MEFGAKIMVLATLLLLCLIVASSHGESTQNRISRRDIEGGTALINDLDTNFGYPNNG
NENVESILRRTLLLKTYWQLCTELDACPEKITKTEGVLPQRSALRGGHLFWRTGILVP
PKPGR

>Hsc_Spnp18-like
MNMSNLIYLAAFATVSAFLMHTVSSISYNTIDGTIPYKRLFWIDKRSSYDDSSLYTVP
ISSAPSIVECFLDDCTKSFATCAKSCIMTNEEIPCMTSCKQQRTLCSIRCFQRYAIKSS
DNAA

>Hsc_Spnp19-like
MSQVRVGVVLFTTVLVCWLFTTSAHTWGNEDTDAFDTNPFEQDPILEDLDDTTLR
TLIIKMFSDRIRSQLKLLNEVDLNSEPNRVYKPPEDWDYQKEMDTKEGNTDIIRSRR
GRKCIGRFVPILSKCGR

>Hsc_SpnpB-Like
MNTKTVLLALTLVLALVISTSDARNYYYAPGNRGGQGRSRNRASKPFKNPFLKRFM
EEFYGTDQGTPYFNPDDVFPDDLDVE

Figure 4.7: Predicted amino acid sequences of *Holothuria scabra* neuropeptide precursors. Precursor identity is presented in bold font with neuropeptide name or *Strongylocentrotus purpuratus* neuropeptide (spnp) homologue number from Rowe and Elphick (2012). Predicted bioactive peptides are highlighted in yellow, signal peptides in green and putative basic and dibasic cleavage sites are highlighted in red. Red font signifies dibasic residues which are not predicted to be post translational cleavage sites (NeuroPred). Conserved inter and intra molecular bonding cysteine residues are underlined.

Table 4.2: *Holothuria scabra* neuropeptide precursors containing homologues of previously-reported known and predicted neuropeptides. Presence (✓) or absence (-) of homologues in available transcriptome databases of two other sea cucumber; *Holothuria glaberrima* and *Apostichopus japonicus* and the sea urchin *Strongylocentrotus purpuratus*.

Neuropeptide	*H. glaberrima*	*A. japonicus*	*S. purpuratus*
CRH[1]	✓	✓	✓
SALMFamide[1]	✓	✓	-
GN-19[2]	✓	✓	-
GPHA2[1]	✓	✓	✓
GPHB5[4]	✓	-	✓
GPH/Gremlin-like	✓	✓	✓
GSS[3]	✓	-	-
Hscnp1[1,4]	✓	✓	✓
Hscnp11[1]	✓	-	✓
Hscnp12[1]	✓	-	✓
Hscnp15[1]	✓	-	✓
Hscnp16[1,4]	✓	✓	✓
Hscnp17[1]	✓	-	✓
Hscnp18[1]	✓	✓	✓
Hscnp19[1]	✓	✓	✓
TRH[1]	✓	✓	✓
Calcitonin[1]	✓	✓	✓
Calcitonin[4]	✓	✓	✓
AN peptide[1,4]	✓	✓	✓
PPLNP2[1,4]	✓	✓	-
PPLNP2[1,4]	✓	✓	✓
Hscnp8[4]	✓	✓	-
Luqin[1]	✓	✓	✓
Predicted precursor[5]	✓	-	✓
Nerve Growth Factor-like	✓	-	-
NGIWYamide[6,4]	✓	✓	✓
QGLFSGVamide[6]	✓	✓	-
Vasotocin[1,4]	✓	✓	✓

[1](Rowe *et al.*, 2014) [2](Elphick, 2012) [3](Mita *et al.*, 2009) [4](Rowe and Elphick, 2012) [5](Menschaert *et al.*, 2010) [6](Kato *et al.*, 2009)

4.3.3.1 Putative reproductive neuropeptides of *H. scabra*

4.3.3.1.1 Holothurian oocyte maturation associated peptide

Our RNC transcriptome did not appear to contain the previously reported oocyte maturation inducing peptide QGLFSGVamide (Kato *et al.*, 2009). However, we found that it is present within the available *A. japonicus* transcriptome dataset (Zhou *et al.*, 2013), as a full-length precursor sequence. The predicted mature peptide product from the *A. japonicus* precursor is a 23aa sequence SYHSPVDMSGGGLLGQGLFSGVamide (Fig. 4.8A). Using this precursor as a query, a homologous precursor was found in *H. scabra* and in *H. glaberrima* transcriptomes. Both *H. scabra* and *H. glaberrima* predicted oocyte maturation inducing peptides are also 23aa amidated peptides (Fig. 4.8B).

A

MKYIGELSLAWLVILAFYAVIPATVDARALDADTFISEREAEENSPAENDEANMEDNLTISEDELYTI
VEMALKTYLNERGAKGPRKTGASSLWYRPIDSLTNGFTGNYKRTLSNEVAARDYARTALKRNMRN
ELRSELAKKSYHSPVDMSGGGLLGQGLFSGVGR

B

```
                          *              20
Hsca  :  SYHNPVDISGGYYLGSGVHRGTG  :  23
Hglab :  TYHNPVDIAGGHFLGSGIFKGTG  :  23
Ajap  :  SYHSPVDMSGGGLLGQGLFSGVG  :  23
```

Figure 4.8: Identification of full length oocyte maturation inducing peptide. (A) Full length precursor of the *Apostichopus japonicus* oocyte maturation inducing peptide QGLFSGVamide (yellow) and predicted full-length sequence of the bioactive peptide (underlined) which is flanked by dibasic cleavage sites (red). Predicted signal peptide is shown in green. (B) Multiple sequence alignment of oocyte maturation associated peptide homologues from *Holothuria scabra* (Hsca), *Holothuria glaberrima* (Hglab) and *Apostichopus japonicus* (Ajap).

Synthetic QGLFSGVamide induced oocyte maturation in *A. japonicus* ovary tissue (Kato *et al.,* 2009). In the same study, the activity level of the heptapeptide was lower than that of NGIWYamide (cubifrin), presumably leading to further research focused on the NGIWYamide peptide (Yamano *et al.*, 2015, Yamano *et al.*, 2013). Comparison of the full peptide with sequences from two other holothurians shows a greater degree of conservation in the N- terminal region of the peptide which was lacking in the original

assays (Kato *et al.*, 2009). This may indicate that the component missing from the previously tested synthetic heptapeptide may be important for biological function.

The *H. scabra* precursor of the oocyte maturation associated peptide is a 164aa protein containing a 27aa N-terminal signal peptide. The remaining sequence contains 4 predicted dibasic cleavage sites, including 2 flanking the bioactive peptide (Fig. 4.9).

==MKYVGELSFVWLVILALYAVVPTIVQA==RALDGDNYIPESDEDNTNDEAPIQDMDDNFTVS
KDDLVNIVGMVELYLANKAKSGASFLWNRPIEPLSNGFGGFYPS==KR==SADSLYDARDYYAR
AAL==KR==NVRNELRNALAA==KR==SYHNPVDISGGYYLGSGVHRGTG==RR==

Figure 4.9: *Holothuria scabra* precursor for the putative oocyte maturation associated peptide SYHNPVDISGGYYLGSGVHRGTamide identified from radial nerve transcriptome.

4.3.3.1.2 NGIWYamide- cubifrin- NGFFFa

A BLAST search of the *A. japonicus* NGIWYamide precursor sequence (AB758561.1) (Kato *et al.*, 2009) revealed a homologue in the *H. scabra* RNC transcriptome. The *H. scabra* precursor contains a predicted N- terminal 24aa signal peptide, 4 copies of the NGIWYa peptide (cubifrin) and 1 copy of a novel cubifrin-like peptide NGIWFa (Fig. 4.10A). In contrast, the *A. japonicus* sequence only contains 5 copies of NGIWYa peptide and not the novel cubifrin-like peptide. Precursor sequence analysis of holothurian cubifrin precursors with Echinoid NGFFFa and Asteroid NGFFYa precursor sequences show that although the bioactive peptides are quite similar in structure (Fig. 4.10B) the echinoid and asteroid precursor sequences are quite different from those of the holothurians. As expected the phylogenetic analysis cluster the echinoid and asteroid sequences on a separate branch (Fig. 4.10C). Both the asteroid and echinoid precursors contain 2 copies each of the active peptides followed by a neurophysin domain. The neurophysin sequence is not present in the holothurian NGIWY amide precursors. A homologue was also identified in the *H. glaberrima* radial nerve complex transcriptome (Mashanov *et al.*, 2014) which, like the *A japonicus* precursor, contains 5 copies of the NGIWYa peptide (Fig. 4.10D).
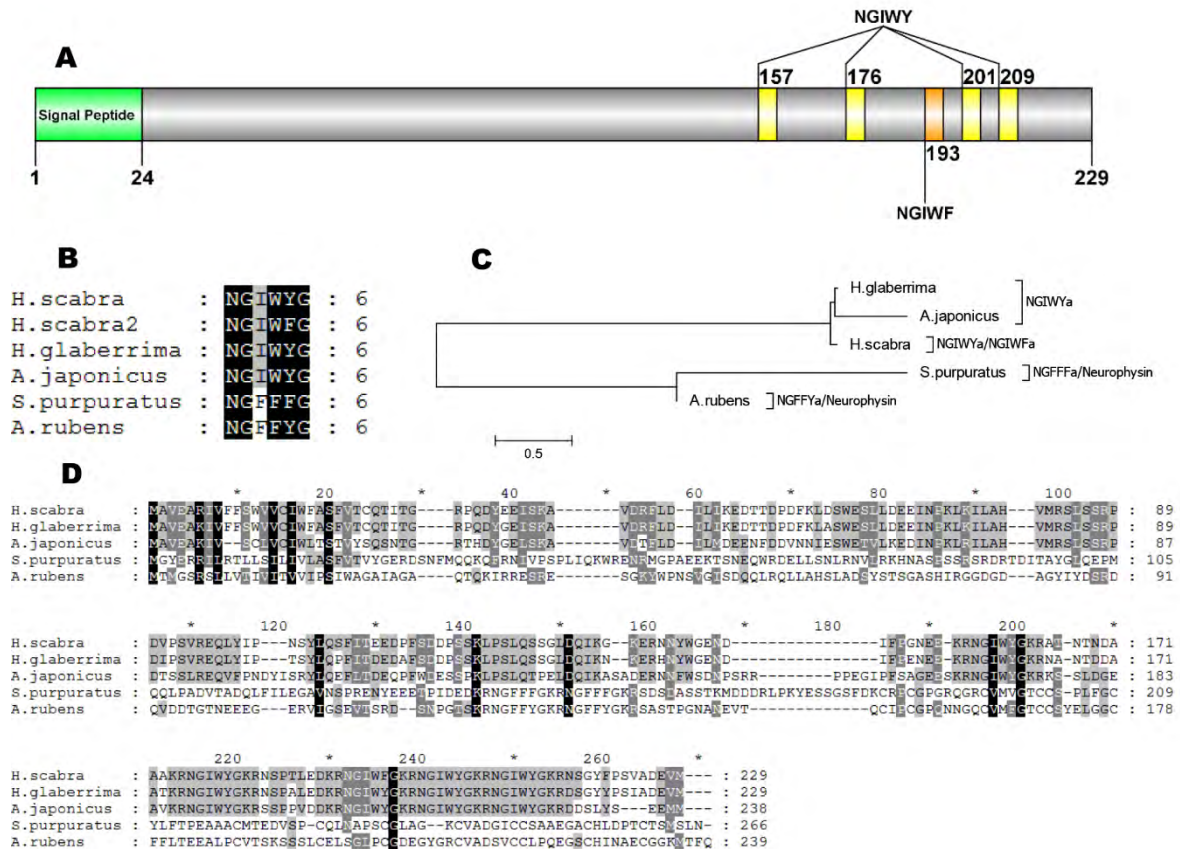
Figure 4.10: Structure of the *Holothuria scabra* NGIWYamide precursor and relationship with related echinoderm sequences. (A) *H. scabra* preprohormone structure showing positions of signal peptide (green), 4 copies of the NGIWY neuropeptide (yellow) and NGIWF neuropeptide (orange). (B) Multiple sequence alignment of structurally related neuropeptides from holothuroids (NGIWY/ NGIWF), echinoids (NGFFF) and asteroids (NGFFY). (C) Maximum likelihood tree showing phylogenetic relationships of echinoderm NG neuropeptide precursors. (D) Multiple sequence alignment of holothurian NGIWYamide precursor homologues, asteroid NGFFYamide and echinoid NGFFFamide precursor sequences. Shading represents 100% (black), 80% (dark grey), 60% (light grey) conservation in the alignment.

4.3.3.1.3 *H. scabra* relaxin/GSS-like precursor

Using the *A. pectinifera* Gonad-Stimulating Substance precursor ([BAI44654.1](#)) (Mita *et al.*, 2009) as a query to tBLASTn our *H. scabra* transcriptome, we identified a putative homolog. The *H. scabra* GSS-like precursor consists of a 25 aa signal peptide, a putative beta chain peptide from residue 25-44 with a putative C- terminal amidation site and 2 cysteine residues (Cys29 and Cys41), and a putative alpha chain peptide from residue 102-123 containing 4 cysteine residues (Cys109, Cys110, Cys114 and Cys123). The alpha and beta peptides are separated by a C peptide from position 44-102. There are 2 putative dibasic cleavage sites for post-translational cleavage to release the C peptide

from the A peptide, both are sequential arginine residues at position R100-R101 and at R107-R108 (Fig. 4.11).
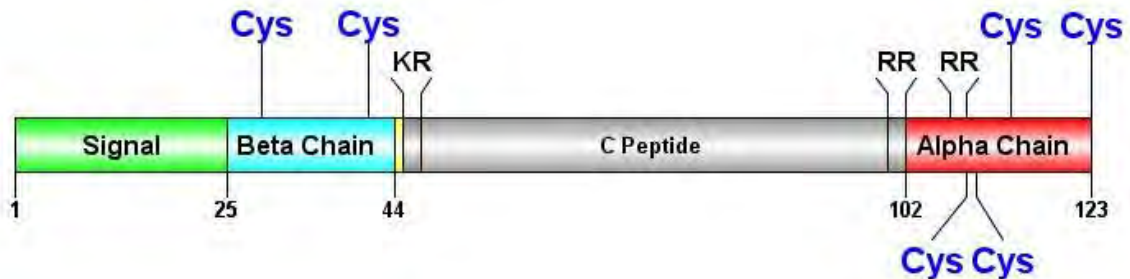
Figure 4.11: Structure of the *Holothuria scabra* GSS-like precursor with post-translational processing sites for amidation (yellow), Cleavage (KR and RR) and intramolecular and intermolecular disulfide bonding sites (Cys).

A precursor with high similarity occurs in the *H. glaberrima* radial nerve complex transcriptome and the structures of both show a high degree of conservation of structurally important prohormone features such as basic and dibasic cleavage sites and cysteine residues with the reported sea star GSS (Fig. 4.12). A unique feature of the holothurian relaxin-like sequences compared to the asteroid homologues is the C-terminal glycine residues on the predicted beta peptides. N-terminal glycine residues have been described for beta peptides of relaxins in other species and can indicate potential sites for post translational amidation (Schwabe and Büllesbach, 1994, Eipper *et al.*, 1992).
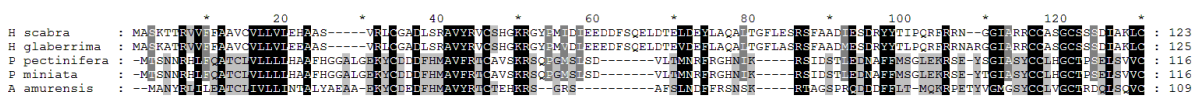
Figure 4.12: Multiple sequence alignment of newly identified *Holothuria scabra* relaxin-like neuropeptide precursors with sea star GSS precursors. Shading represents 100% (black), 80% (dark grey), 60% (light grey) conservation in the alignment.

A. japonicus and *S. purpuratus* homologues were not found in the available datasets. However, a relaxin receptor-like (Rxfp2) partial sequence encoded in the *H. scabra* CNS transcriptome does have a homologue in the *S. purpuratus* genome, which is a predicted protein with GPCR superfamily homology and human relaxin receptor (rxfp2) similarity.

Sea star GSS was the first non-mammalian relaxin-like peptide proven to have a role in reproduction and is the first identified relaxin-like gonadotropin. GSS activates

GPCRs in follicle cells to stimulate oocyte maturation and ovulation via 1MA-mediated oocyte GVBD (Mita *et al.*, 2014). Sea star GSS is predominantly expressed in the neural tissues (Haraguchi *et al.*, 2015). Our discovery of a holothurian relaxin neuropeptide in mature female *H. scabra* radial nerve transcriptome suggests this relaxin-like peptide may also act as a gonadotropin in sea cucumbers. GSS of one sea star species is not functional when administered to sea stars belonging to a different order (Mita *et al.,* 2015). For example, *Asterias amurensis* GSS does not induce oocyte maturation of *A. pectinifera* ovary fragments. However, *A. pectinifera* relaxin is able to induce oocyte maturation of *A. amurensis* ovary fragments (Mita *et al.,* 2015).

Sea cucumber GSS shows differential activity across different species (Maruyama, 1985). Differences in the relaxin primary sequence could explain the variation in effectiveness of GSS from separate species. The importance of sea star GSS and other relaxins in reproduction in vertebrates creates an intriguing question of the role that relaxin plays in sea cucumber physiology. Homologues of relaxin were not found in *S. purpuratus* or *A. japonicus* data sets but a homologue was in the *H. glaberrima* RN transcriptome. This could suggest it is a neural-specific precursor in echinoderms as it was only found in sea cucumber transcriptomes which were enriched with neural RNAs. Relaxin's complex quaternary structure makes it difficult to synthesise and therefore it was beyond the scope of this project to examine the physiological activity of the *Hsc*-relaxin.

4.3.3.1.4 Vasotocin-neurophysin precursor

A BLAST search of *S. purpuratus* echinotocin precursor revealed a vasotocin-neurophysin type precursor in the *H. scabra* dataset. The *H. scabra* vasotocin (VT) precursor contains a 24 aa signal peptide followed by the amino acid sequence CFVTNCLLGG, a putative dibasic cleavage site, followed by a neurophysin peptide (residues 37-162). The predicted mature form of *H. scabra* vasotocin-like peptide (CFVTNCLLGamide) differs from the *A. japonicus* sequence at 2 residues, from the *S. purpuratus* echinotocin at three residues while the homologue identified from *H. glaberrima* is identical to the *H. scabra* vasotocin-like peptide (Fig. 4.13).

```
                                            *
H scabra     :  CFVTNCLLGG : 10
H glaberrima :  CFVTNCLLGG : 10
A japonicus  :  CFITNCPLGG : 10
S purpuratus :  CFISNCPKGG : 10
```

Figure 4.13: Multiple sequence alignment of echinotocin neuropeptides. *Amidation site.

The holothurian vacotocin-like neurpeptide belongs to a family of nonapeptides which include vasotocin (VT), oxytocin (OT) and vasopressin (VP). The neuropeptides of this family are believed to have evolved from a common ancestor of the VT prohormone. VT, OT and VP peptides are involved in osmoregulation, reproduction and social behaviours (Insel and Young, 2000). Echinotocin causes muscular contraction in echinoid tissue preparations via an unknown mechanism (Elphick and Rowe, 2009). There is recently discovered activity of VT in the gonadotropic pathway leading to oocyte maturation and ovulation in the catfish *Heteropneustes fossilis* (Joy and Chaube, 2015). Further research is required to discern the physiological roles of vacotocin-like peptides in holothurians and what potential they have, if any, in stimulation reproduction.

4.3.3.1.5 Glycoprotein hormones

Putative glycoprotein hormone subunit precursors were found in the *H. scabra* radial nerve transcriptome. The predicted *H. scabra* GPHA2 homologue is a 134 aa protein consisting of a 30 aa signal peptide followed by a 104 aa sequence containing 9 spatially conserved cysteine residues. The *H. scabra* GPHB5 precursor has a 27 aa signal peptide followed by a 110aa peptide containing 8 conserved cysteine residues similar to GPHB5, gonadotropin subunit B2 (GTHB2). A Gremlin2-like peptide with glycoprotein hormone sequence similarity, with cysteine-knot protein homology was also found that had a total length of 181 aa with an N- terminal signal peptide of 25 aa and 9 spatially conserved cysteine residues (See figure 4.7). Gremlin-2 in vertebrates regulates the activity of bone morphogenetic protein and bone and tissue formation (Zuniga *et al.*, 2011). Homologues of GPHA2 and Gremlin-like sequences were identified in *A. japonicus*, *H. glaberrima* and *S. purpuratus,* while homologues of GPHB5 have been identified in *H. glaberrima and S. purpuratus* (See table 4.2).

The glycoprotein hormone family includes the gonadotropins chorionic gonadotropin (CG), follicle-stimulating hormone (FSH) and luteinising hormone (LH) as

well as thyroid stimulating hormone (TSH). In vertebrates, glycoprotein hormone release is regulated by other neuropeptides such as gonadotropin releasing hormone (GnRH) and thyrotropin releasing hormone (TRH) (Gopurappilly *et al.*, 2013, Mateos *et al.*, 2002). The discovery of TRH-like and GnRH-like neuropeptides in echinoderms together with the presence of glycoprotein hormone-like sequences suggests a similar signalling pathway might exist in echinoderms (Rowe and Elphick, 2012, Rowe *et al.*, 2013). The existence of a gonadotropic glycoprotein hormone–like hormone in echinoderms is an interesting prospect for aquaculture because gonadotropic glycoproteins and their regulatory neuropeptides have been useful in stimulating reproduction in fish (Black and Black, 2013). Therefore similar potential may exist for the use of similar hormones in echinoderm aquaculture if a reproductive role for glycoprotein hormone like proteins exists in the echinoderm phylum.

4.3.3.2 Echinoderm neuropeptides absent from *H. scabra* radial nerve transcriptome-

Despite the discovery of an echinoderm GnRH in the *S. purpuratus* genome (Rowe and Elphick, 2012), and in most other animals, a GnRH-like precursor was not identified in our *H. scabra* radial nerve transcriptome. Additionally, a GnRH homologue could not be found in the other available sea cucumber transcriptome datasets that contain a greater range of tissues sampled (Du *et al.*, 2012, Zhou *et al.*, 2014, Mashanov *et al.*, 2014). The absence of GnRH in sea cucumbers could reflect the timing of the sampling of the transcriptomes analysed or a lack of sequence conservation between sea urchin and sea cucumbers. The reproductive peptide GSS-LP identified by Katow *et al.* (2009) was also absent from the datasets analysed.

Stichopin is a 17 aa holothurian neuropeptide first isolated from the sea cucumber *A. japonicus* (Birenheide et al., 1998)*.* A full length precursor sequence for *A. japonicus* stichopin is available (Rowe *et al.*, 2013). Despite this, a precursor was not found in *H. scabra* radial nerve transcriptome. This particular precursor may be expressed in other tissues as it was originally isolated from connective tissue preparations (Birenheide *et al.*, 1998). Although, there is immunohistological evidence that it is also expressed in neuronal cells in *A. japonicus* (Tamori *et al.*, 2007, Elphick,

2012). Stichopin has a regulatory role in the inhibition of stiffening of the connective tissues of the body wall (Birenheide *et al.*, 1998).

4.3.3.3 In silico identification of putative neuropeptides in the *H. scabra* radial nerve

As shown in table 4.2, 28 neuropeptide precursors were identified with sequence similarity to other previously reported neuropeptide precursors. Further investigation, using algorithms that predict proteins with neuropeptide-like characteristics, indicate that there are an additional 50 putative precursor sequences encoding putative neuropeptide with features including (but not limited to) signal peptides, conserved cysteines and basic or dibasic cleavage sites (Fig. 4.14). Six of the precursors contain putative peptides with conserved C- terminal glycine residues, possibly required for C-terminal amidation which is a common post-translational modification for neuropeptides (Fig. 4.14).

Figure 4.14: Precursor sequences of novel putative *Holothuria scabra* neuropeptides predicted by *in silico* transcriptome analysis. Predicted novel bioactive peptides are highlighted in yellow, signal peptides in green and putative basic and dibasic cleavage sites are highlighted in red. Red font signifies dibasic residues which are not predicted to be post translational cleavage sites (NeuroPred). Putative inter and intra molecular bonding cysteine residues are underlined.

4.3.3.4 Relative expression of putative and known neuropeptide precursors genes in *H. scabra* radial nerve

The highest expression level belonged to a transcript (Unigene18122) encoding a unique neuropeptide precursor with no known sequence similarity or function (see figure 4.14). The second most highly expressed transcript encoded a peptide that was identified by HPLC-MS of RNE, indicated it was translated in the developmental stage used. The third most highly expressed precursor transcript was that of the precursor containing the oocyte maturation associated peptide SYHNPVDISGGYYLGSGVHRGTamide followed by the transcript for the precursors for HscNP11 (*Holothuria scabra* neuropeptide 11), HscNP15, HscNP5, HscNP18, HscNP19, another unique precursor transcript (Unigene2166), and NP16 (Fig. 4.15).
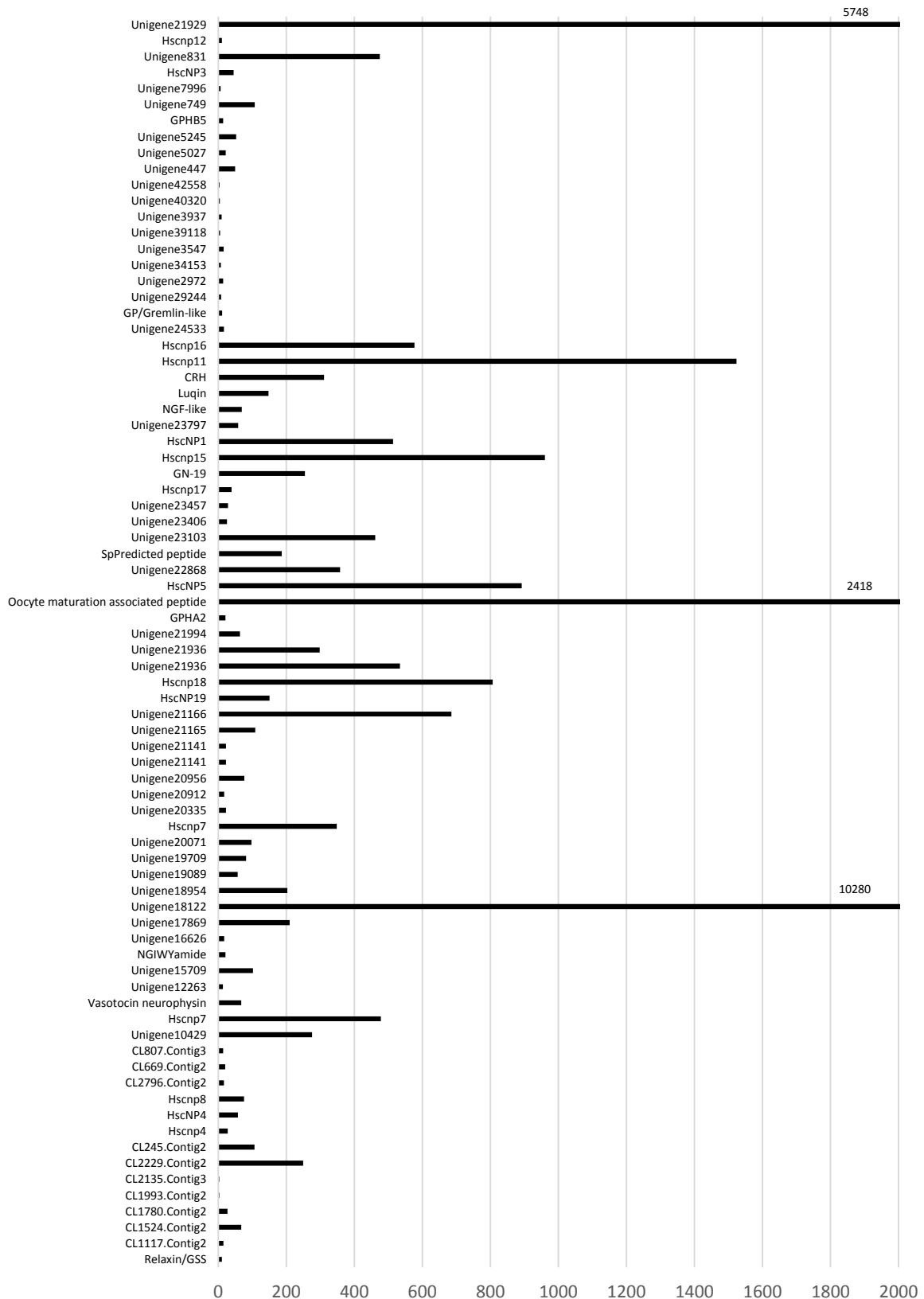
Figure 4.15: Relative expression levels of *Holothuria scabra* radial nerve transcripts encoding putative neuropeptide precursors. Expression level was estimated from sequencing data as fragments per kilobase per million fragments (FPKM).

4.3.4 Oocyte maturation substance from *H. scabra* radial nerve

4.3.4.1 Gonadotropic activity of radial nerve extract (RNE)

Oocyte maturation was observed in the RNE-treated ovary after c.a. 50 min incubation, as determined by GV migration towards the polar process and break down, in some oocytes. After 80 min, ovulation of mature ova was observed in the RNE-treated groups. Mature ova were observed free from the ovary section, at the bottom of the microplate wells (Fig. 4.16A). In the control groups immature oocytes remained in the follicles attached to the ovary segments (Fig. 4.16B). There were many more mature ova lacking a GV than there were immature oocytes with an intact GV in the RNE treated samples (Fig. 4.16C). A small proportion of oocytes in the control group did undergo spontaneous maturation (Fig. 4.16D).
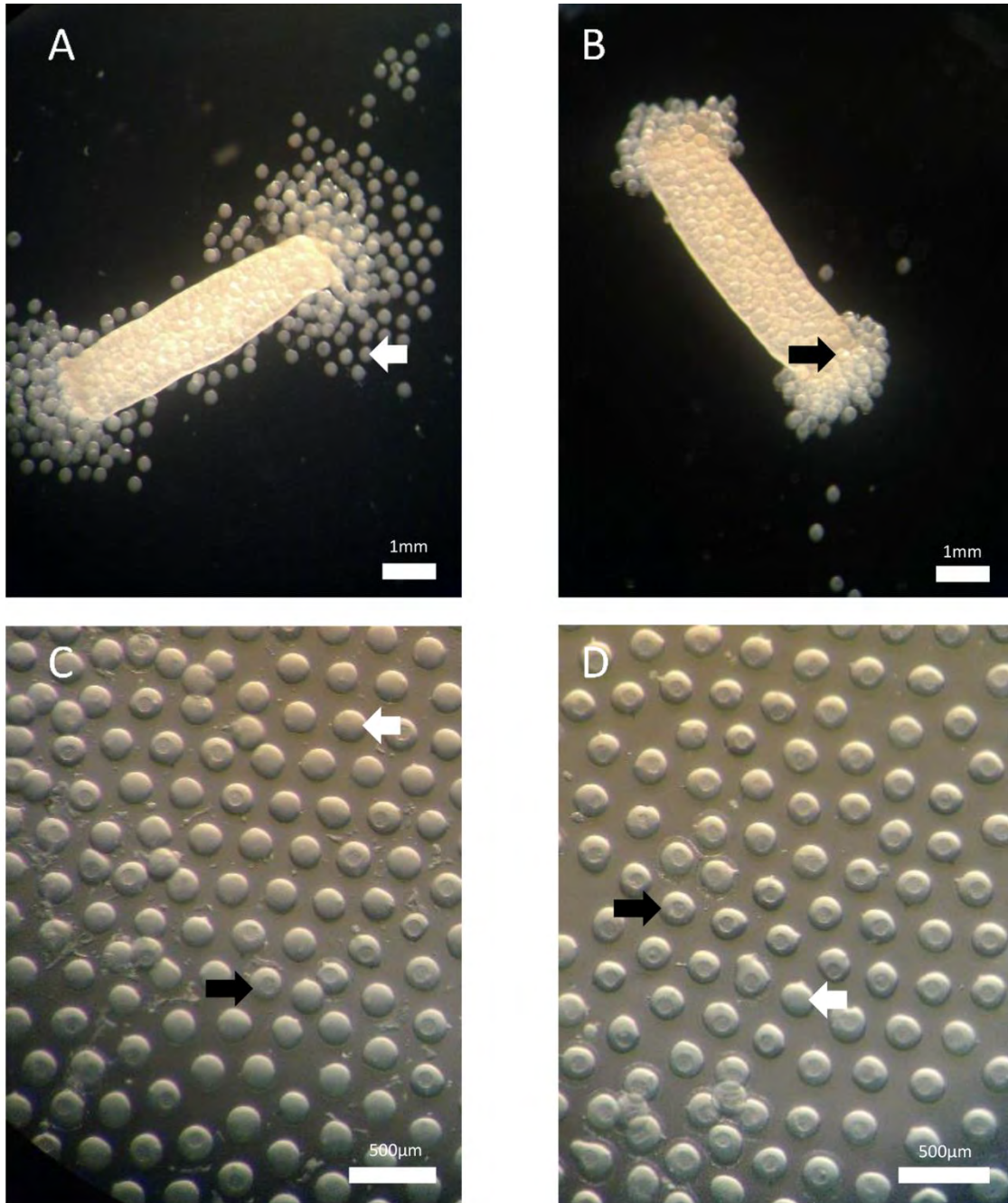
Figure 4.16: Photomicrographs of induced GVBD in *Holothuria scabra* ovarian sections Incubated with (A) RNE and (B) FSW, showing immature oocytes with intact germinal vesicle (black arrows) and mature ova (white arrows). Samples of gametes extracted from ovarian sections incubated with (C) RNE and (D) FSW and photographed on a stereo-dissecting microscope (Leica).

After 3 h incubation, the mean number of mature ova was significantly higher in the RNE-treated groups than in the FSW-treated groups (t= -3.977, d.f. = 4, n=3 P= 0.016). GVBD had occurred in 58% (17.33 ± 6.51) of oocytes in RNE-treated ovary fragments, and only 8% (2.33 ± 0.58) in the FSW-treated group (Fig. 4.17).
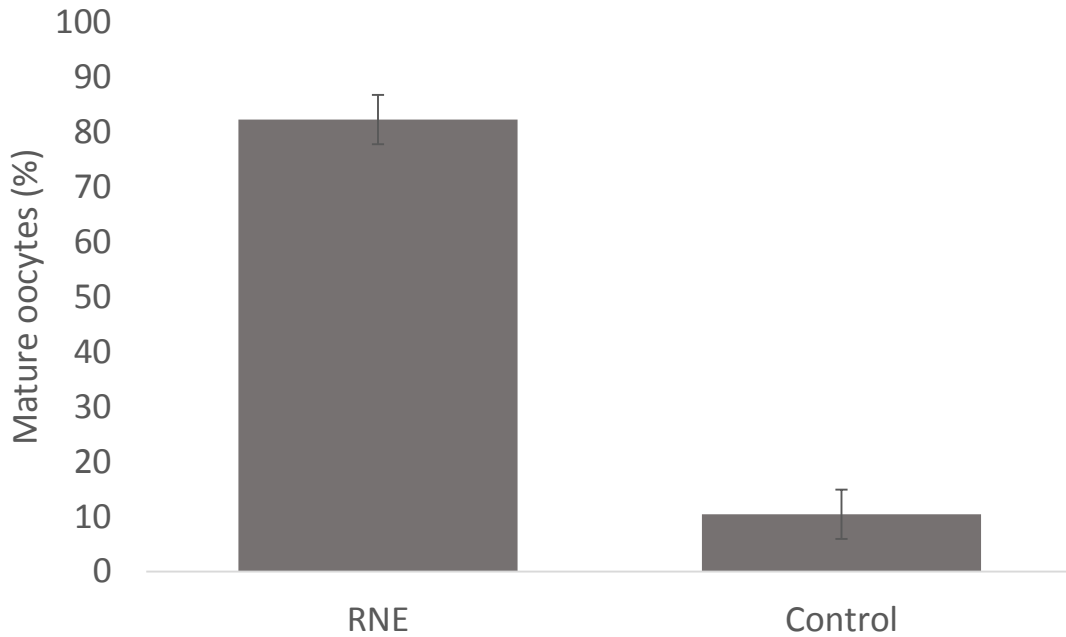
Figure 4.17: Oocyte maturation within *Holothuria scabra* ovarian tubule following incubation with radial nerve extract (RNE) and FSW (control). Samples of 30 oocytes were counted for each sample. n=3, error bars represent ± 1 SE.

4.3.4.2 Purification of RNE peptide with GSS activity

A significant difference was observed in *H. scabra* oocyte GVBD between different radial nerve extract HPLC fractions (ANOVA F =$20.59_{13,\ 4.}$ P =0.000). A cocktail containing combined fractions 1-12 induced GVBD at significantly higher levels than the negative control and most other individual fractions (Fig. 4.18). Only fraction 7 induced similar significant induction of GVBD. The proportion of mature oocytes in the RNE-treated samples was 70%, which was significantly higher than in all other treatments. Fraction 5 caused GVBD in 20.6% of oocytes but this was not at significantly higher levels than that induced by the fractions 1-4, 6, 8-12 and the negative control. However, GVBD activity in fraction 5 was also similar to the activity observed in fraction 7 (Fig. 4.18).

The oocytes obtained from ovary tissue treated with fraction 2 were degraded and did not allow direct observation of the GV.
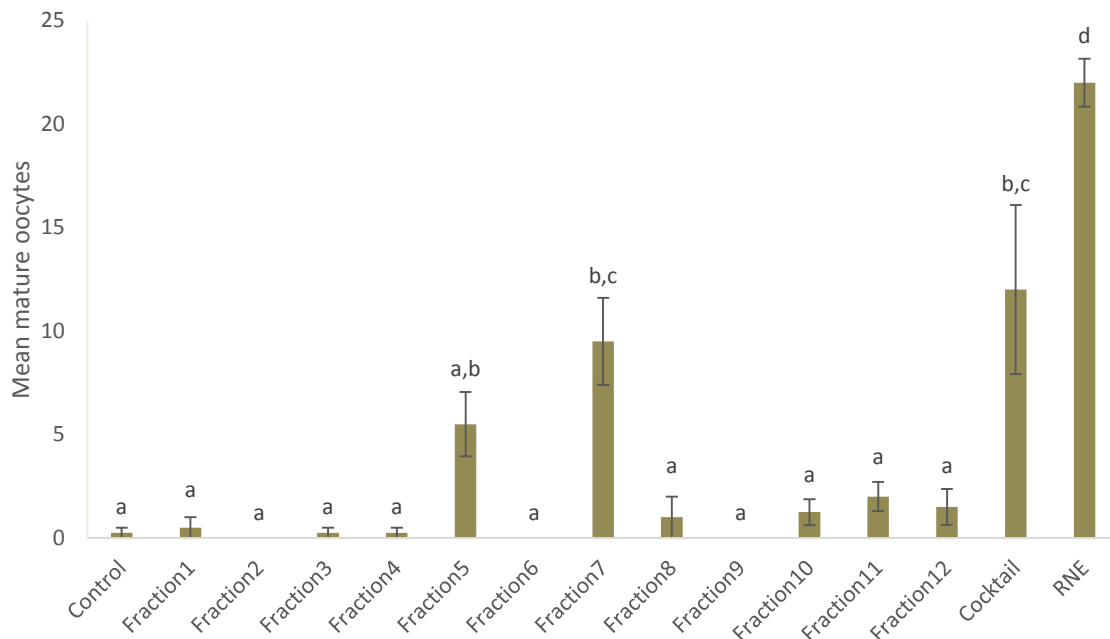
Figure 4.18: Mean number of mature oocytes per sample of *Holothuria scabra* ovary tissue treated with different fractions of purified radial nerve extract. Error bars represent ± 1 SE, n =4. Different superscripts represent different homogeneous subsets at alpha 0.05.

The RNE extract appeared to be complex based on RP-HPLC chromatograms. Active fractions (5 and 7) eluted at 21-25min and 31-35 minutes corresponding to CAN / 0.1% TFA concentrations of 21-25% and 31-35%, respectively (Fig. 4.19).
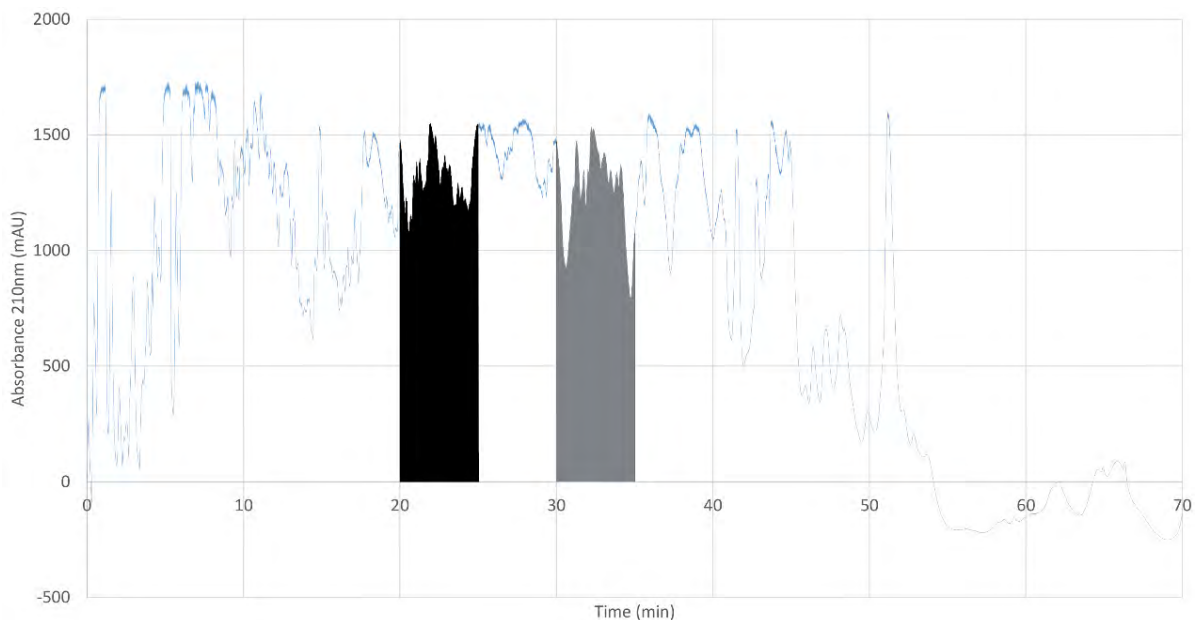
Figure 4.19: RP-HPLC chromatogram showing purification of oocyte maturation peptide from the *Holothuria scabra* radial nerve extract. Fractions 5 and 7, which contained oocyte maturation inducing bioactivity are highlighted in black and grey shading, respectively.

GVBD activity observed in fraction 7 was lost following re-purification as demonstrated by loss of activity of sub-fractions (Fig. 4.20). However, sub-fraction 24 (23-24 min) obtained from the re-purification of fraction 5 induced significant GVBD [63% ± 11 (22/34)] of fully grown oocytes in ovary tissue segments (ANOVA F $=14.898_{40, 4.}$ P $=0.000$) (Fig. 4.20). Those biomolecules detected within the active fraction were present at low absorbance (ca. 40-28 mAU; 210 nm) (Fig. 4.21). Crude radial nerve extract did not induce oocyte maturation in this assay.
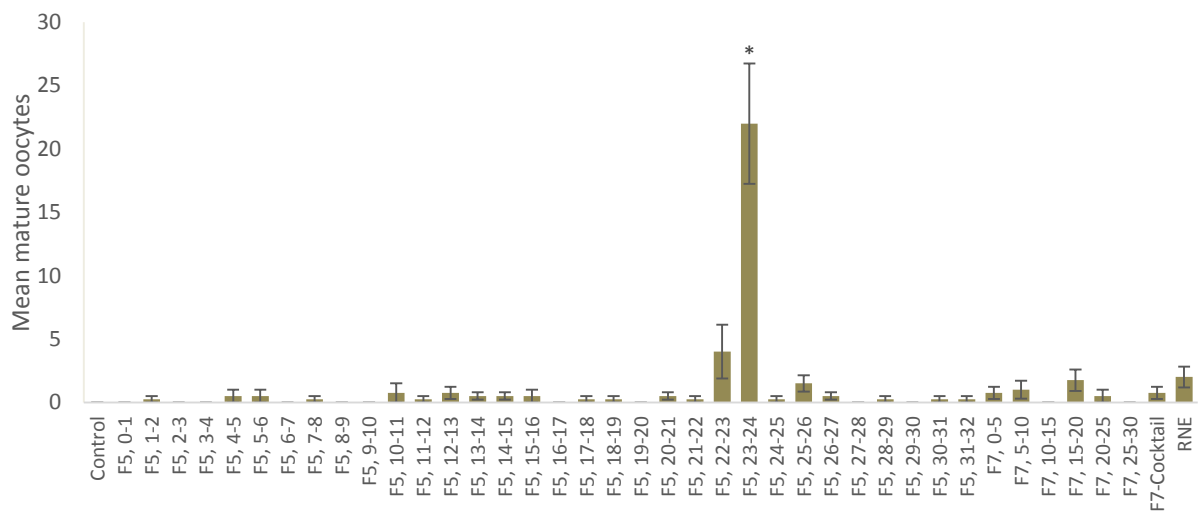
Figure 4.20: Oocyte maturation observed in *Holothuria scabra* ovary sections following incubation with sub-fractions of RNE fractions 5 (F5), 7 (F7), FSW (control), combination of all sub fractions (Cocktail) and crude radial nerve extract (RNE). Mean GVBD in oocytes per sample, error bars are ± 1 SE, n = 4.
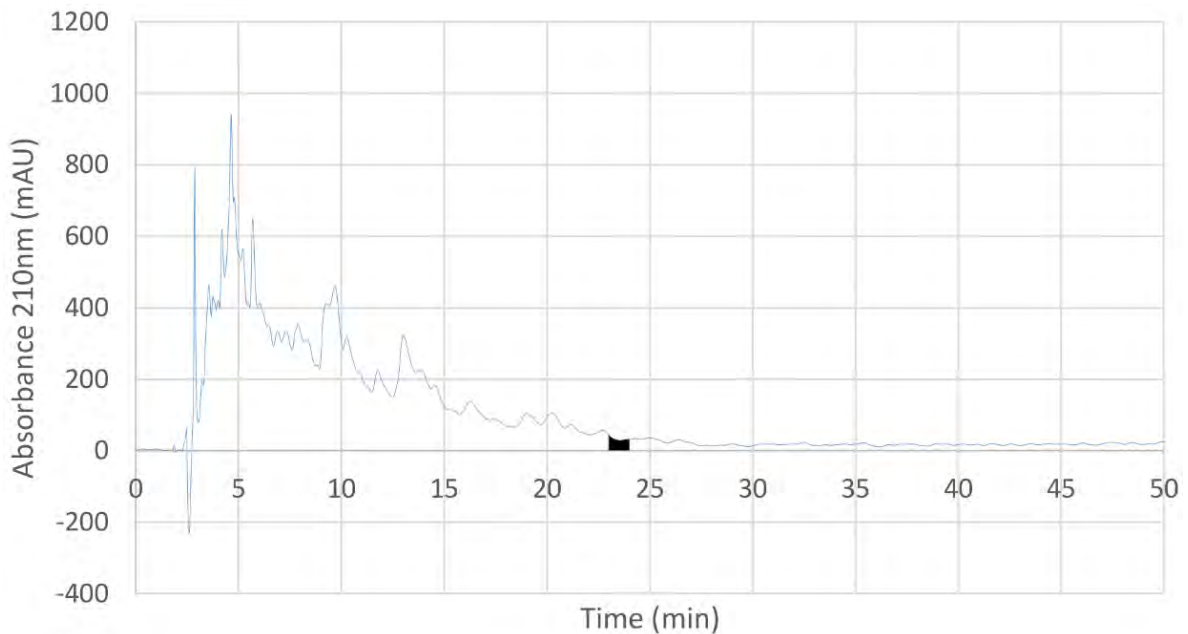
Figure 4.21: RP-HPLC chromatogram showing repurification of RNE Fraction 5. Oocyte maturation activity was found to be within sub-fraction 23-24 min (black shading).

RNE was not equally active across all assays and experiments in the study. This is further evidence to suggest varying amounts of the active components in different individuals or changes in the receptivity of the ovary to be a factor. In one instance, complete loss of activity occurred; possibly due to degradation of the active extract component when stored, or from repeat freeze-thaw cycles. Holothurian GSS is known to degrade rapidly when kept frozen and can be sensitive to protease degradation (Smiley, 1990). Despite the varying effectiveness of the RNE, the *in vitro* assay was still a powerful assay for the detection of GVBD activity.

4.3.4.2.1 Peptide detection in fraction 24

LC-MS/MS of the components present in bioactive sub-fraction 24 provided a peptide with a match to a *H. scabra* precursor protein (Fig. 4.22A). The full sequence of the *H. scabra* precursor containing the bioactive peptide with GVBD activity was found in our transcriptome (Fig. 4.22B). The precursor includes the exact sequence of the purified peptide flanked by dibasic cleavage sites and the peptide itself contains a C-terminal glycine residue which is a putative site for C-terminal amidation as was detected in the radial nerve purification. The detected peptide is a larger homologue of the QGLFSGVamide peptide identified in CNS purifications from *A. japonicus* (Kato *et al.*, 2009). This new evidence of our detection of a larger 22 aa peptide suggests that the

148

QGLFSGVamide peptide, which showed GVBD activity in *A. japonicus,* may have been a partial peptide (Kato *et al.*, 2009). The newly discovered peptide will be an interesting candidate for further analysis in GVBD assays as presented below.
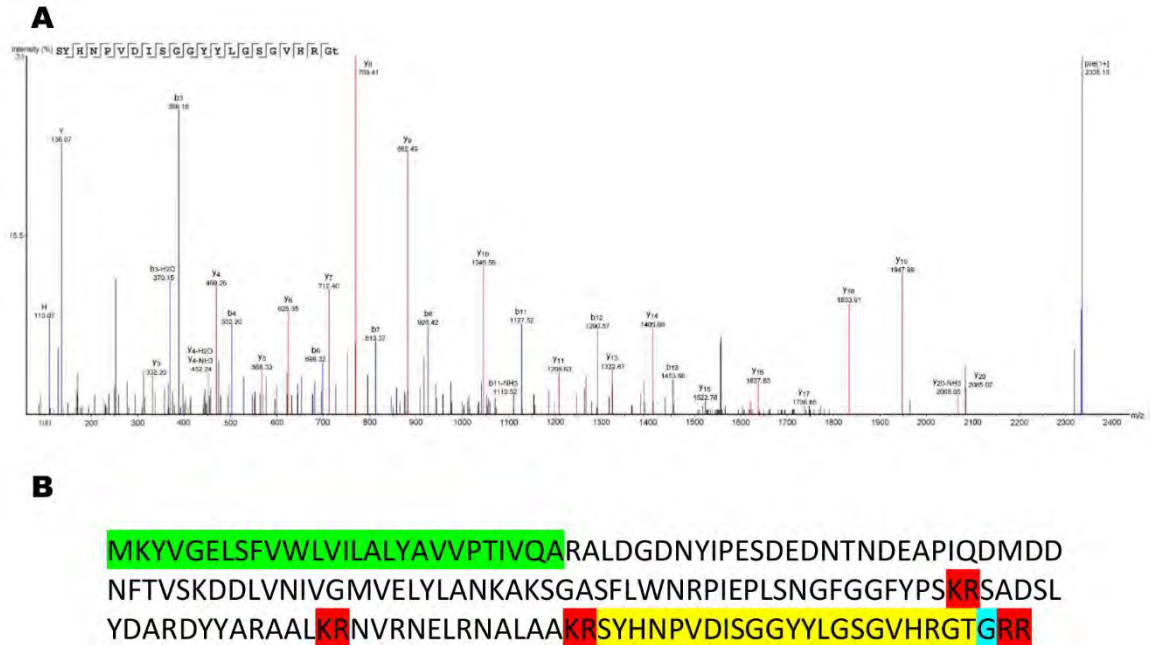
Figure 4.22: Characterisation of the neuropeptide identified from the bioactive fraction of *Holothuria scabra* radial nerve extract by MS. (A) Predicted *Holothuria scabra* precursor protein containing the peptide purified from fraction of radial nerve extract with *in vitro* GVBD activity. Identified by LC-MS/MS (yellow). The precursor has characteristic features of a neuropeptide precursor such as a signal peptide (green), dibasic post-translational cleavage (red) and an amidation (blue) site (B).

4.3.4.3 Neuropeptide detection all RNE fractions by MS

Three peptides that were detected by MS analysis of the RNE have sequence matches in our list of putative neuropeptide precursors. Peptides were identified which matched the precursors of Hscnp11, oocyte maturation associated peptide precursor and Unigene23103 (a putative neuropeptide precursor). Further, one additional putative neuropeptide precursor was identified by the MS analysis. Unigene21929 encodes a 91 aa precursor with 4 dibasic cleavage sites and a 20 aa signal peptide. One putative bioactive possibly cleaved out contains 2 conserved cystein residues (Fig. 4.23A). The identified peptide sequence detected by mass spectral analysis was EYAPLAEVLGDLY and it was detected in fraction 10 of the radial nerve purifications. The precursor has homologues in *H. glabberima* and *A. japonicus* transcriptome datasets with high sequence conservation (Fig. 4.23B). The sequence was highly expressed in the *H. scabra*

library (FPKM = 5748) as the second most represented of the putative neuropeptide precursor transcripts (see figure 4.15). No significant BLAST similarity was found for this in the NCBI Nr database.

A peptide (APALDFGDDATYDAILDQR) was detected from fraction 6 of the *H. scabra* RNE purifications. The peptide sequence had an exact match with the same precursor as the *H. scabra* homologue of the previously reported predicted neuropeptide Spnp11 (*S. purpuratus* neuropeptide 11) (Rowe and Elphick, 2012) (Fig. 4.23C). A potential homologue of this precursor was also identified in the *H. glaberrima* transcriptome database. The *H. glaberrima* precursor sequence lacked altogether the C-terminal np11 sequence and contained a partial sequence matching the peptide detected in the MS (Fig. 4.23D).

The novel peptide AESQYKEDVWNFLDNLL was identified in fraction 10 of the RNE HPLC purifications. The peptide had an exact match with part of one of the precursor sequences identified by *in silico* protein prediction in this study encoded by Unigene23103 (Fig. 4.23E). *A. japonicus* and *H. glaberrima* homologues were also identified which had high level of sequence conservation between the detected *H. scabra* peptide as well as cleavage sites and cysteine residues (Fig. 4.23F).

A

>Unigene21929_Hsc

MKGLILSVFSAVLVFCLATAAVLPDNADLAKREYAPLAEVLGDLYTNENSMDKRKGYGGCQDYMMDWISFQHC
MIKRAAAVARFRRGLQNL

B

C

>Unigene24070_Hscnp11

MNTFLVCLFSMILLAGVVLGAPALDFGDDATYDAILDQRMRRSYNKLNECLHDCLFCGKYSKKFNVKSCLDECWG
KTTGNEQTWAACSLFTGRK

D

E

>Unigene23103

MRALVISCLGFMLLTTIAAESQYKEDVWNFLDNLLNTDETNEVVGDENPDAAGTFPLAKRGSRISQAVCRRECASC
VKIMNVLFISCYRDCLSSNSGVSSSSSSWSMCRKFLVG

F

Figure 4.23: Analysis of precursor sequences for predicted neuropeptide which contain matches for peptides detected by LC-MS/MS analysis of *Holothuria scabra* radial nerve extract. Signal sequences are shaded green, putative dibasic cleavage sites in red and peptide detected by MS are shaded in yellow (A, C and E). Multiple sequence alignment of precursor homologues in other echinoderms (B, D and F). Shading represents 100% (black), 80% (dark grey), 60% (light grey) conservation in the alignment.

The oocyte associated maturation peptide (SYHNPVDISGGYYLGSGVHRGTamide), which was identified from mass spectrum analysis of active purifications of fraction 24, is most likely a homologue of the *A. japonicus* peptide QGLFSGVamide, reported by Kato *et al*. (2009) to induce GVBD and oocyte maturation. They had found that QGLFSGVamide was less effective at inducing oocyte maturation compared to NGIWYamide, thus NGIWYamide became the focus for further study (Kato *et al.* 2009). Our new information on the oocyte associated maturation peptide, which includes the identification of the full-length peptide sequence, might explain the lower activity observed in the Kato *et al.* (2009) study, where only a partial peptide was used.

Transcripts for the *H. scabra* SYHNPVDISGGYYLGSGVHRGTamide precursor were highly expressed in the radial nerve. These had been derived originally from a pooled sample containing, exclusively, radial nerve tissue from reproductively mature male and female animals. This contrasts the relative low abundance of the peptide observed during RP-HPLC purification. The radial nerve used in peptide identification was collected from animals from a range of reproductive stages, which may explain the low abundance of the mature peptide, although we cannot rule out that it is under translational control. Taken together, these results provide a preliminary indication that the peptide expression may be correlated with the reproductive cycle and is present most abundantly in radial nerve from reproductively mature *H. scabra*. Despite the low abundance in the RNE used in this study, the peptide was still a potent inducer of GVBD in our *in vitro* bioassay. Bioactivity at very low concentrations is a common feature of echinoderm gonadotropic peptides, where effective concentrations have been observed at 1 pM to 2 nM (Kato et al., 2009, Mita et al., 2009).

4.3.4.4 Assay of oocyte maturation activity of synthetic *H. scabra* neuropeptides

The crude RNE induced GVBD in a significant number (90%) of the *H. scabra* oocytes. In contrast, a relatively small number of oocytes showed GVBD when treated with the synthetic peptides; this was not significantly different than spontaneous GVBD observed in control samples. Univariate analysis of the GVBD assay revealed a significant effect of treatment $F_{198.226,\ 15}$ =11.252 P=0.000 but not for concentration $F_{24.395,\ 4}$ =1.385, P =0.239. Post hock analysis confirmed statistically that only RNE did induce significant GVBD (Fig. 4.24).
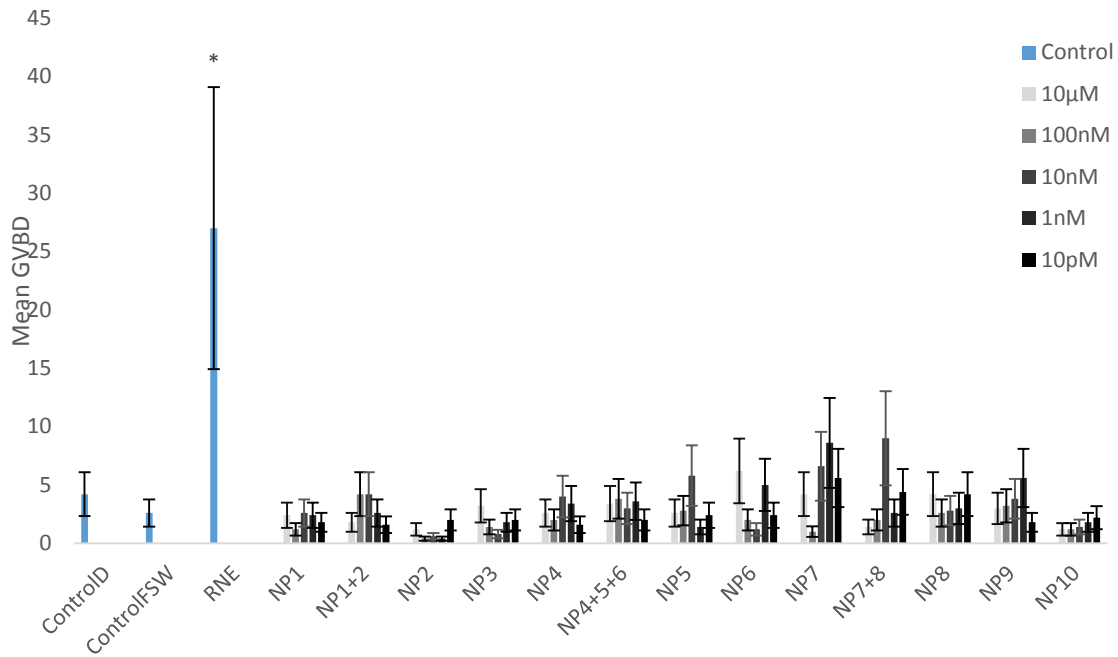
Figure 4.24: Maturation of *Holothuria scabra* oocytes incubated with synthetic peptides at various concentrations : 10 µM, 100 nM, 10 nM, 1 nM and 10 pM for 4 h. Maturation of oocytes was scored as mean number of oocytes that had undergone GVBD per treatment. Two negative controls (ControlD, and ControlFSW) and a positive control (RNE) are represented by blue bars. n = 5, error bars represent ± 1 S.E.*indicates significantly different GVBD at alpha 0.05.

An unexpected result of our assay of the synthetic neuropeptides was the lack of GVBD activity of the *H. scabra* cubifrin and cubifrin-like peptides (NP1 and NP2). *In vitro* assays of synthetic NGIWYa peptide triggered oocyte maturation in excised ovary fragments of *A. japonicus* (Kato *et al.,* 2009). In that experiment, presence of ovary wall tissue was necessary for the oocyte maturation activity, suggesting that the NGIWYa peptide was acting in a different manner to GSS. GSS from the radial nerve induces GVBD in follicle enclosed oocytes (Maruyama, 1985) acting directly on the follicle (Mita *et al.*, 2014). *In vivo* application of NGIWYa also triggered spawning in mature male and female *A. japonicus*, a function that could have beneficial application in aquaculture (Fujiwara *et al.*, 2010)*.

NGIWYa was first identified as a myoactive peptide controlling stiffness in the muscle and connective tissues of sea cucumber (Inoue *et al.*, 1999, Birenheide *et al.*, 1998). Similarly the sea star peptide NGFFYa has a contractile effect on cardiac stomach of the sea star *Asterias rubens* and may play a role in the feeding mechanism (Semmens *et al.*, 2013). The echinoid NGFFFa is also implicated in modulation of contraction of sea

urchin tissues (Elphick and Rowe, 2009). The authors propose that the peptides may be acting on muscular tissues through a receptor-mediated response directly or through triggering the release of other endogenous contraction factors. Similar questions do arise from the effects of NGIWYa on the sea cucumber ovary. Recent investigation of the NGFFFa peptide identified a NG peptide family receptor in sea urchins, a receptor with homology to the vertebrate neuropeptide S receptor (Semmens *et al.*, 2015). This finding will enable future receptor expression studies that will help answer questions on the mode of action of this peptide in reproductive processes.

Next, we tested a synthetic oocyte maturation associated peptide (SYHNPVDISGGYYLGSGVHRGTamide) identified after the purification and assay for GSS activity in RNE (see figure 4.20). This peptide did not induce significant GVBD in *H. scabra* oocytes after 4 h incubations at concentrations from 10 µM to 10 fM. Crude RNE induced GVBD in over 90% of oocytes while the highest rate observed for the synthetic peptide was 46% at a concentration of 10 µM which was not significantly higher than the 22% GVBD induced by the FSW control treatment (Fig. 4.25).
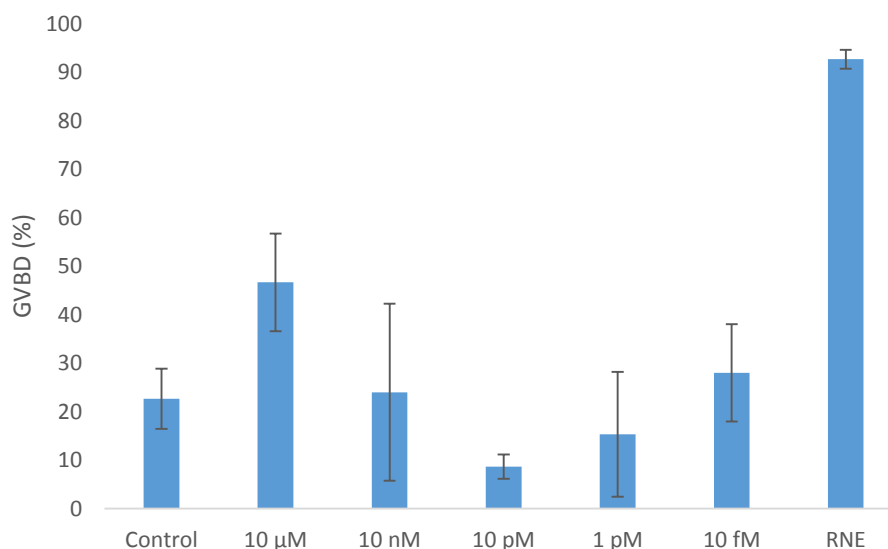
Figure 4.25: Analysis of GVBD in *Holothuria scabra* ovary following incubation in different concentrations of synthetic oocyte maturation associated peptide (SYHNPVDISGGYYLGSGVHRGTamide), control solution (FSW) and crude RNE (n=5).

It was surprising that the synthetic peptide did not induce GVBD given that a partial homologue had previously induced *in vitro* GVBD in *A. japonicus* (Kato *et al.*, 2009). Our model for the SYHNPVDISGGYYLGSGVHRGTamide peptide shows that it folds in such a way that the N-terminus may obscure the putative active C- terminus region

(Fig. 4.26). It is possible that some other interacting component may be required to modify the folding of the peptide in a way that could ensure the C- terminus is available for receptor binding. The N-terminal region of the peptide is highly conserved among holothurians suggesting it is important in some way for the function of the peptide. However, a seven amino acid peptide from the C- terminus of the peptide shows biological activity in *A. japonicus* ovary (Kato *et al.*, 2009). The lack of activity of the synthetic construct of the full length *H. scabra* homologue could indicate that a chaperone molecule interacting with the conformational structure of peptide is required to expose the receptor binding C-terminal peptide. In support of this, other peptides identified within the active RNE fractions matched with folding chaperone-like proteins, cyclophilin A and peptidyl-prolyl isomerase that may be involved in regulating the SYHNPVDISGGYYLGSGVHRGTamide peptide activity. Cyclophilin and peptidyl-prolyl isomerase catalyse the cis/trans folding of proteins at Proline residues (Fujita and Yoshida, 2001). Cis-trans isomerisation can be important for neuropeptide receptor-ligand binding (Pierson *et al.*, 2013, Lopez *et al.*, 2008).
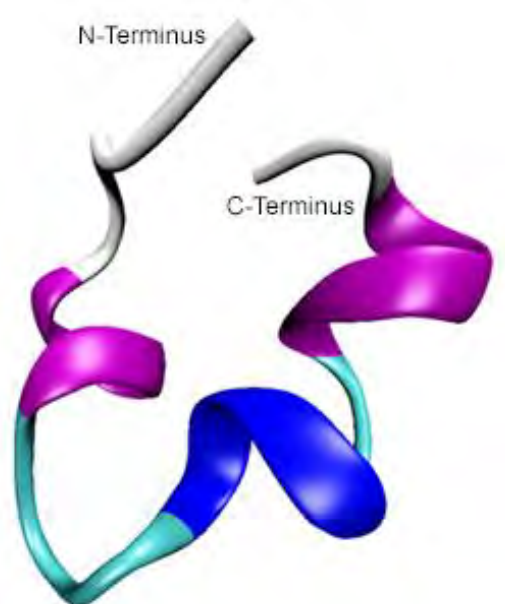
Figure 4.26: Model of the tertiary structure of the *Holothuria scabra* SYHNPVDISGGYYLGSGVHRGTamide peptide. This peptide is made up of two alpha helices Pro5 – Ill8 and Gly17-Arg20 (magenta) a 3_{10} helix Tyr12-Leu14 (dark blue) and two turns Ser9-Gly11, Gly15-Ser16 (light blue).

4.4 References

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5   General Conclusions

The availability of brood stock is important to aquaculture to ensure a consistent supply of genetic material for hatchery production. This is essential for the viability of commercial hatchery operations. The availability of mature sea cucumber from wild populations is highly variable; not only are there very large variations in reproductive cycles between species but also between different geographical locations within species (Tuwo, 1999, Rasolofonirina *et al.*, 2007, Ramofafia *et al.*, 2001, Muthiga, 2006, Morgan, 2000b, Mercier *et al.*, 2007, Krishnaswamy and Krishnan, 1967, Despalatovic *et al.*, 2004, Asha and Muthiah, 2008). Another issue is that overfishing is common in sea cucumber fisheries around the globe and therefore scarcity of adult animals in such fisheries limits the availability of brood stock for aquaculture (Eriksson *et al.*, 2012, Bell *et al.*, 2008). Specifically for *Holothuria scabra* aquaculture in Australia, whilst there are still healthy wild populations, the success rates of induced spawning of wild animals is usually quite low, primarily due to reproductive asynchrony within populations and often low numbers of mature individuals (particularly females) in collected brood stock (Battaglene *et al.*, 2002)*.* Brood stock domestication and reproductive conditioning is one way to improve the breeding outcomes for the sea cucumber aquaculture industry.

High market demand for sea cucumber products and concerns regarding overexploitation have motivated research into sea cucumber aquaculture (Lovatelli *et al.*, 2004). Although previous research has established methods for hatchery production and spawning (Pitt, 2001, Morgan, 1999, James, 1999, James *et al.*, 1994, Battaglene *et al.*, 1999, Battaglene and Seymour, 1998, Agudo, 2006), little is known about the requirements and preferred methods for culturing and conditioning brood stock resulting in continued reliance on wild stocks.

To address some of the major questions and knowledge gaps that hinder advancement of *H. scabra* aquaculture, the research presented here initially investigated basic animal husbandry methods such as anaesthesia, tagging and gonad sampling, so that knowledge obtained could then be applied to brood stock conditioning experiments.

Anaesthesia is an important tool because it eliminates the auto-evisceration response which is sometimes caused by rough handling. Regeneration after auto-

evisceration in *H. scabra* takes approximately one month (Mary Bai, 1994). Although the effect of auto-evisceration on the gonad is unknown it can lead to loss of tubules and probably results in a diversion of energy stores from gametogenesis to regeneration of the internal organs. It was determined that a 2% solution of 1.12 g L^{-1} menthol ethanol solution in seawater is a rapid anaesthetic for brood stock and totally prevents an auto-evisceration response which was sometimes caused by the biopsy procedure on non-anaesthetised animals (Chapter 2). Application of this anaesthetic protocol enabled for subsequent biopsy procedures on *H. scabra,* monitoring of gonad maturity and sexing of brood stock. Because it is usually easier to induce spawning with male *H. scabra* than females, the ability to reliably and carefully sex animals enables for the collection of relatively more females for brood stock conditioning. This would improve the success rates of subsequent induced spawning by increasing the number of females used. This is the first reported use of anaesthesia in a sea cucumber for reducing auto-evisceration during surgical biopsy. However others have reported the use of the same anaesthetic for reducing the variation of length measurement in juvenile *A. japonicus and H. scabra* (Watanabe *et al.*, 2012, Yamana *et al.*, 2005). In the Watanabe *et al.* (2009) study, a 2% solution of menthol ethanol (1.12 g L^{-1}) solution was preferred for juvenile *H. scabra* although the authors also showed that a 4% solution was also effective. In our study solutions over 2% were not appropriate for anaesthetising adult *H. scabra.* We suggest this may have been due to warmer temperatures in our experiments or an increased sensitivity to the anaesthetic solution for adults.

Biopsies are an important tool for determining the reproductive stage of a cultured animal. This is particularly important for *H. scabra* brood stock as they can be at different stages of reproduction throughout the year. We trialled two different methods for biopsy of gonad tissue; a surgical biopsy where a section of gonad was excised through an incision in the body wall and a needle biopsy where a sample of gonad was extracted using a 16 G needle and a syringe. Both procedures were aided by anaesthetic. Although the less invasive needle biopsy method was effective in determining mature individuals, it showed decreased accuracy, particularly with animals that had small or reduced gonads. The surgical biopsy method was proven to be an accurate way to sample gonad tissue even in reduced condition, i.e. when the animals were in indeterminate or spent stages. Where definite assessment of gonad is required,

the surgical method is recommended but for rapid assessment of maturity or gender the needle biopsy is preferred. The latter method is very good for increasing the success rate of induced spawning by enabling pre-selection of mature brood stock. In two studies on reproduction of sea cucumbers Hamel and Mercier (2007, 1999) used a surgical biopsy method to obtain gonad samples. Both studies using two different species found the surgical method to be effective and did not result in adverse effects on animal health and rarely caused auto-evisceration even without anaesthesia. The authors also justify the use of a surgical method over a needle biopsy due to increased accuracy of assessment of the gonad (Hamel and Mercier, 1996). *H. leucospilota and C. frondosa* were the species used in the trials and a suture was used to close the incision post biopsy. Due to the tough and stiff body wall of *H. scabra* suturing is very difficult as the connective tissue in the body wall is resistant to penetration with a suture needle so suturing was not appropriate for our study. Morgan (2000) also recommended needle biopsy for assessing maturity of *H. scabra* brood stock but warned that it may cause auto-evisceration.

It is essential for industry to identify individual brood stock to enable management of breeding. This is especially important when progeny are to be used for stock augmentation such as restocking or stock enhancement. Yet, tagging still remains an issue for sea cucumber research and stock monitoring in aquaculture. For manageable numbers in tank based conditioning systems or ponds that can be monitored regularly, the glue scar method developed in this research was found to be very effective for individual identification (Chapter 2). At least six individuals per tank could be tagged for brief periods of at least 4 weeks or longer, if re-tagging is applied. Other studies which assess the individual marking or tagging of adult sea cucumbers found that retention varies with tag type and the species tagged. For example, a tagging trial with *P. californicus* showed retention varied with tag type and tagging also affected behaviour with tagged animals moving significantly more than control animals. The tag type with the highest retention in *P. californicus* was T-bar type with a retention of 70% after 4 months (Cieciel *et al.*, 2009). T-bar type tags were also relatively effective for *C. frodosa* with 65% retention after 140 days (Kirshenbaum *et al.*, 2006). However, for *C. frondosa* a fluorescent visible implant elastomer was the most effective of the trialled tag types with 80% retention after 140 days in the same study. Using a method of

marking similar to ours Sheill (2006) tagged *H. whitmaei* by scratching an identifying number into the dorsal tegument. The tags healed quickly and were useful in identifying animals in the wild for up to three weeks. As Cieciel *et al.* (2009) noted for *C. frondosa*, *H. whitmaei* also moved significantly more for about 3 days after tagging (Shiell, 2006). In the only other study on tagging adult *H. scabra*, apart from our study, T-bar tags had a retention rate of 90% after 5 weeks (Morgan, 2000). Morgan (2000) described autotomy of body wall tissue affected by the insertion of the tag lead to the loss of the tags. The animals in this study were also afflicted by a disease. While the cause of the disease is not clear the wound created by the T-bar type tag could allow opportunistic pathogens to affect the stock. For this reason T-bar tags were not trialled in our study.

Once these methods were established, conditioning experiments were designed to investigate culture conditions and their effects on *H. scabra* brood stock (Chapter 3). In particular, the effects of light level, water filtration and nutrient addition on growth and gonad development. The underlying aim was to identify conditions and systems to effectively condition brood stock *H. scabra* that could increase hatchery production.

Results of this studies brood stock culture experiments found that overall system design was more important than light levels and water filtration (Chapter 3). Stocking densities were deliberately kept low for these experiments to prevent confounding results due to density dependent factors. The combination of low animal density, and the space required to optimally conduct these types of experiments with *H. scabra,* resulted in the numbers used in the experiments limiting the statistical power of inferences which could be made. In an attempt to address this issue during the conditioning experiments, the experimental system was redesigned with many small tanks (0.8 m^2). The early experiments in our investigations showed that the tank system was influencing the condition of the animals. However, mass loss and poor survival was observed in both trials using the small highly replicated system. Modifications of this system by increasing light levels and increasing the nutrients levels in the tanks through removing water filtration did not improve the performance of brood stock. Despite the poor growth of the *H. scabra* used in the smaller tank trials, useful results were obtained regarding the effects of light levels and water filtration.

This study found that light level and water filtration did not have an effect on the growth of the adult *H. scabra* i.e. did not change the observed mass loss. However, given

the demonstrated improved growth rates of juveniles cultured under increased sunlight it was expected to have similar effect for adults due to an increase in benthic primary productivity (Battaglene *et al.*, 1999). Light level and water filtration also had no detectable effect on the gonad maturation based on brood stock gonad size. We speculate that tank design may have been a factor, masking the effects of light level due to insufficient space. Nevertheless the experiment was useful to rule out light and filtration as factors affecting results of the earlier study assessing the gonad biopsy method (Chapter 3). Based on the suboptimal performance in this initial system, a second system was developed to conduct further experiments.

To investigate the hypothesis that organic matter levels in the sediment could improve growth and to assess the influence of addition of organic matter on gonad conditioning, a larger system was used. Organic nutrient addition was not beneficial to gonad development or growth in brood stock. The experiments show that brood stock can be successfully conditioned over one month on sediment with or without additional organic matter. Conditioning will be able to improve sea cucumber aquaculture production but requires a great deal of tank space (i.e. >1 ind. per m^2). The large amount of space required to condition *H. scabra* brood stock adds significant financial cost to land based hatchery operations. Optimising the return of fertilised gametes from brood stock conditioning space may be key to the success of a *H. scabra* hatchery.

Although gonad development was observed in all experiments, the level of synchronicity was low and larger numbers would still be required for efficient hatchery operations. Previous brood stock studies have highlighted the difficulties with maintaining and feeding adult *H. scabra* (Morgan, 2000a, Pitt, 2001, Pitt and Duy, 2005). After our research, we can recommend that at low density culture it is possible to maintain *H. scabra* adults with reduced mass loss. This finding will permit future experimentation on brood stock culture which is an important first step towards domestication which will still require development of feeds and optimisation of culture conditions. We investigated the fertilisation of substrate with organic matter as a substitute for feeding. Organic matter was chosen because of anecdotal evidence for it improving the growth rate of juvenile *H. scabra* in tanks and ponds. In tank based feeding experiments assessing growth rates and survival of juvenile *H. scabra* the addition of ground dynamic lifter resulted in higher growth rate and survival for the

juveniles compared to the addition of other substances such as dried spirulina powder and inorganic fertiliser (Knauer, *unpublished data*).

The potential gains generated through future research into brood stock conditioning and spawning for aquaculture of this species are important as typical spawning events currently occurring in hatcheries are producing much less than the estimated fecundity for this species. For example, spawning of 2 million eggs per female is typical in the hatchery compared to fecundity estimates of up to 20 million per female (Hamel *et al.*, 2001). With domestication of brood stock and development of manufactured diets for *H. scabra,* the potential gains to productivity are very large. The development of conditioning systems and methods presented in this thesis will enable greater confidence in designing conditioning experiments with *H. scabra* to address these constraints to the advancement of the tropical sea cucumber aquaculture industry*.*

H. scabra is one of the most commercially valuable sea cucumber species (Conand, 2008), yet, as with many echinoderms, little is known about the endogenous mechanisms controlling reproduction. Previous studies in sea stars and sea cucumbers have identified a potent gonad stimulating substance (GSS), with analogous activity to the vertebrate gonadotropins, which is expressed in the neural tissues (Mita *et al.*, 2015, Mita, 2013, Strathmann and Sato, 1969, Maruyama, 1985, Katow *et al.*, 2009). GSS has since been characterised as a relaxin-like neuropeptide for sea stars but the GSS and other reproductive peptide hormones involved in sea cucumber reproduction were not characterised. In this study, for the first time, an investigation into the neuropeptide signalling molecules of *H. scabra* was undertaken using a transcriptomic and peptidomic approach (Chapter 4). The list of neuropeptides and putative reproductive neuropeptide hormones presented here increases our understanding of the peptides involved in molecular signalling in sea cucumbers. Additionally, two peptide hormones were identified which have potential for use in aquaculture as reproductive stimulants and breeding technologies. First a homologue to sea star GSS was identified for the first time in a holothurian. This significant finding will require additional research to determine if the homologue has a gonad stimulatory affect in holothurians as it does for asteroids. Due to the complex tertiary structure of the peptide dimer, it is difficult to synthesise and therefore it was outside the scope of this study to conduct bioassays using this

neuropeptide. Secondly an oocyte maturation associated peptide (OAMP) was identified following assay of radial nerve tissue extracts. This peptide has sequence similarities with a smaller peptide which had some gonadotropic activity in *A. japonicus* (Kato *et al.*, 2009). However, in our assays using a synthetic derivative of this OMAP, no GVBD activity was detected above expected spontaneous levels. This result was unexpected given that MS detected the native peptide in the active fraction of the radial nerve, and a similar peptide has previously been shown to have *in vitro* GVBD activity in *A. japonicus (Kato et al., 2009).* We hypothesise that some protein interaction or post-translational folding may be regulating the activity of this peptide *in vivo*. Further investigation of both neuropeptides is planned in our lab to investigate the role of each in the final stages of oocyte development and spawning in *H. scabra.*

A suite of other neuropeptides were found in the *H. scabra* radial nerve transcriptome including NGIWYamide which, interestingly, also was not able to induce GVBD *in vitro* in our trials using a synthetic peptide, while the same peptide is a potent inducer of GVBD and spawning in other sea cucumber species (Fujiwara *et al.*, 2010, Kato *et al.*, 2009).

In summary, the research presented in this thesis is an important initial step in understanding neuropeptide signalling in holothuroids. The *H. scabra* radial nerve transcriptome and preliminary neuropeptide list will enable future work to focus on the role of holothurian neuropeptides in earlier stages of reproduction, such as gametogenesis and puberty, as well as neuropeptide signalling in general.

5.1 References

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Supplementary S1

3. Analysis Methods

3.1 Output Statistics

Raw Sequence Data

Image data output from sequencing machine is transformed by base calling into sequence data, which is called raw data or raw reads and stored in fastq format.

```
@EC6IFL8AAXX:1:17:1012:19200#GGCAAT/1
CCACTGTCATGTGAACATCACAGAGACATTTCTTGA
+
hhhhhhhhhhhhhhhhhhhhhhhhhhhaaaaaaaaa
```

The line 1 and 3 are sequences name generated by the sequencing machines; line 2 is sequence; line 4 is quality letters, of which each letter correspond to a base in line 2; we calculate the sequencing quality of each base in line 2 by subtracting 64 from the ASCII value of the letter in line 4 (sequencing quality value). For example, the ASCII value of c is 99, so the corresponding sequencing quality value is 35. Sequencing quality values range from 2 to 35. Follow table shows the brief relationship between sequencing error rate and sequencing quality value. Denote E as sequencing error rate and sQ as sequencing quality value, then we have:

$$sQ = -10\lg E$$

Brief relationship between sequencing error rate and sequencing quality

Sequencing error rate(E)	Sequencing quality	Corresponding character
5%	13	M
1%	20	T
0.1%	30	^
0.01%	40	h

Data process

Raw reads produced from sequencing machines contain dirty reads which contain adapters, unknown or low quality bases. These data will negatively affect following bioinformatics analysis.

Therefore, dirty raw reads are discarded:

1. Remove reads with adaptors
2. Remove reads with unknown nucleotides larger than 5%
3. Remove low quality reads (The rate of reads which quality value <= 10 is more than 20%)
4. Get the clean reads

Software	Version	Web Site	Parameters
filter_fq	Internal software	-	-

Clean Reads

The following analysis is based on clean reads, which are generated by filtering raw reads.

3.2 Assembly

Transcriptome de novo assembly is carried out with short reads assembling program - Trinity. Trinity combines three independent software modules: Inchworm, Chrysalis, and Butterfly, applied sequentially to process large volumes of RNA-seq reads. Trinity partitions the sequence data into many individual de Bruijn graphs, each representing the transcriptional complexity at at a given gene or locus, and then processes each graph independently to extract full-length splicing isoforms and to tease apart transcripts derived from paralogous genes. Briefly, the process works like so:

Inchworm Assembles the RNA-seq data into the unique sequences of transcripts, often generating full-length transcripts for a dominant isoform, but then reports just the unique portions of alternatively spliced transcripts.

Chrysalis Clusters the Inchworm Contigs into clusters and constructs complete de Bruijn graphs for each cluster. Each cluster represents the full transcriptonal complexity for a given gene (or sets of genes that share sequences in common). Chrysalis then partitions the full read set among these disjoint graphs.

Butterfly Then processes the individual graphs in parallel, tracing the paths that reads and pairs of reads take within the graph, ultimately reporting full-length transcripts for alternatively spliced isoforms, and teasing apart transcripts that corresponds to paralogous genes.

The result sequences of trinity is called Unigenes. When multiple samples from a same species are sequenced, Unigenes from each sample's assembly can be taken into further process of sequence splicing and redundancy removing with sequence clustering software to acquire non-redundant Unigenes as long as possible. Then do gene family clustering, the Unigenes will be divided to two class. One is clusters, which the prefix is CL and the cluster id is behind. In one cluster, there are several Unigenes which similarity between them is more than 70%. And the other are singletons, which the prefix is Unigene.

In the final step, blastx alignment (evalue < 0.00001) between Unigenes and protein databases like NR, Swiss-Prot, KEGG and COG is performed, and the best aligning results are used to decide sequence direction of Unigenes. If results of different databases conflict with each other, a priority order of NR, Swiss-Prot, KEGG and COG should be followed when deciding sequence direction of Unigenes. When a Unigene happens to be unaligned to non of the above databases, a software named ESTScan will be introduced to decide its sequence direction. For Unigenes with sequence directions, we provide their sequences from 5' end to 3' end; for those without any direction we provide their sequences from assembly software.

3.5 Unigene Metabolic Pathway Analysis

KEGG is a database that is able to anaylze gene product during metabolism process and related gene function in the cellular processes. With the help of KEGG database, we can further study genes' biological complex behaviors, and by KEGG annotation we can get pathway annotation for Unigenes.

Software	Version	Web Site	Database	Release	Parameters
Path_finder	Internal version	http://www.genome.jp/	KEGG	Release 63.0	Default

Referece:

[1] Kanehisa M, Araki M, et al. KEGG for linking genomes to life and the environment. Nucleic Acids Res. 2008, 36(Database issue): D480-4.

* Assembly process

Software	Version	Web Site	Parameters
Trinity	release-20130225	http://trinityrnaseq.sourceforge.net/	--seqType fq --min_contig_length 100 --min_glue 3 --group_pairs_distance 250 --path_reinforcement_distance 85 --min_kmer_cov 3
TGICL	v2.1	http://sourceforge.net/projects/tgicl/files/tgicl%20v2.1/	-l 40 -c 10 -v 20
Phrap	Release 23.0	http://www.phrap.org/	-repeat_stringency 0.95 -minmatch 35 -minscore 35

Reference:

[1] Grabherr MG, Haas BJ, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology. 2011, 29(7):644-52.
[2] Iseli C, Jongeneel CV, et al. ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. Proc Int Conf Intell Syst Mol Biol. 1999:138-48.

4/31

3.3 Unigene Function Annotation

KEGG database contains systematic analysis of inner-cell metabolic pathways and functions of gene products. It helps studying complicated biological behaviors of genes. With KEGG annotation we can get Pathway annotation of Unigenes.

COG is a database where orthologous gene products are classified. Every protein in COG is assumed to to evolve from an ancestor protein, and the whole database is built on coding proteins with complete genome as well as system evolution relationships of bacteria, algae and eukaryotic creatures. Unigenes are aligned to COG database to predict and classify possible functions of Unigenes.

NT is non-redundant NCBI nucleotide database, with entries from all traditional divisions of GenBank, EMBL, and DDBJ excluding bulk divisions (gss, sts, pat, est, and htg divisions. wgs entries are also excluded.

Software	Version	Web Site	Database	Release	Parameters
BLAST	v2.2.26+x64-linux	http://blast.ncbi.nlm.nih.gov/Blast.cgi	NT	release-20130408	-F F -e 1e-5 -p blastn
			NR	release-20130408	-F F -e 1e-5 -p blastx
			KEGG	Release 63.0	
			Swiss-Prot	release-2013_03	
			COG	release-20090331	

3.4 Unigene GO Classification

We can get GO functional annotation with nr annotation. Gene Ontology (GO) is an international standardized gene functional classification system which offers a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe properties of genes and their products in any organism. GO has three ontologies: molecular function, cellular component and biological process. The basic unit of GO is GO-term. Every GO-term belongs to type of ontology.

With nr annotation, we use Blast2GO program to get GO annotation of Unigenes. Blast2GO has been cited by other articles for more than 150 times and is widely recognized GO annotation software. After getting GO annotation for every Unigene, we use WEGO software to do GO functional classification for all Unigenes and to understand the distribution of gene functions of the species from the macro level.

Software	Version	Web Site	Database	Release	Parameters
Blast2GO	v2.5.0	http://www.blast2go.com/b2ghome	GO	release 2012-08-01	Default

Reference:

[1] Conesa A, Götz S, et al. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics.2005, 21(18): 3674-6.
[2] Ye J, Fang L, et al. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res .2006,34(Web Server issue): W293-7.