Aquaculture 495 (2018) 247–254

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

In vitro oocyte maturation by radial nerve extract and early development of the black sea cucumber (*Holothuria leucospilota*)

Hoang Dinh Chieu[a,b], Saowaros Suwansa-ard[a], Tomer Abramov[a], Abigail Elizur[a], Scott F. Cummins[a,*]

[a] Genecology Research Centre, University of the Sunshine Coast, 90 Sippy Downs Drive, Sippy Downs, Queensland 4556, Australia
[b] Research Institute for Marine Fisheries (RIMF), 224 LeLai Street, HaiPhong City, Vietnam

ARTICLE INFO

Keywords:
Germinal vesicle breakdown
Fertilization
Maturation
Radial nerve extract
Sea cucumber

ABSTRACT

The popularity of sea cucumbers has led to overexploitation of wild populations and increasing need to develop their aquaculture. *In vitro* induction of oocyte maturation followed by fertilization represents an innovative approach that could help increase their production. The inducers of oocyte maturation are naturally synthesised within neural tissue, which in sea cucumbers includes longitudinal radial nerves and a circumoral nerve ring. In this study, we showed that reproductive maturation of the black sea cucumber (*Holothuria leucospilota* Brandt, 1835) in South-East Queensland, Australia, peaked in December, based on biometric analysis of gonads. Radial nerve extract (RNE; 233 μg/ml) prepared from reproductive mature females induced *in vitro* oocyte maturation at 98.9% efficiency, based on germinal vesicle breakdown. RNE-matured oocytes were fertilized (99%) with conspecific sperm and larvae developed to late auricularia stage (25 days post-fertilization). The NGIWYamide neuropeptide (cubifrin), which induces oocyte maturation in the sea cucumber *Apostichopus japonicus*, could not induce oocyte maturation in *H. leucospilota*. These results provide information that could be used for large-scale artificial breeding of *H. leucospilota*, and a platform towards the identification of the bioactive RNE factor(s).

1. Introduction

Sea cucumbers are commercially important species due to their high value in medicine and food. Their meat (body wall) is known to contain many beneficial nutrients, including lipids and almost no cholesterol, as well as molecules relevant to traditional medicine (Olivera-Castillo et al., 2013). Compounds derived from sea cucumber have been used as a treatment of gastritis and stomach ulcers, anemia, nervous breakdown, backache, and sexual dysfunction in men, among other things (Fahmy et al., 2015).

Several sea cucumber species are highly valued such as the sandfish (*Holothuria scabra*), black teatfish (*Holothuria nobilis*), Japanese sea cucumber (*Apostichopus japonicus*) and white teatfish (*Holothuria fuscogilva*). As a result, these have been overfished, leading to the rapid exhaustion of natural resources (Lovatelli, 2004). With reduced stocks, artificial breeding has been implemented in some countries to produce high quality seed for aquaculture, stock enhancement and sea ranching. For example, artificial breeding has been estblished for *A. japonicus* and *H. scabra,* primarily in Indo-Pacific countries (Purcell et al., 2012). To a lesser extent, artificial breeding and juvenile rearing has been implemented for several other sea cucumber species, including *H. fuscogilva* and the surf redfish *Actinopyga mauritiana* (Battaglene et al., 2002).

The black sea cucumber *Holothuria leucospilota*, has a very high level of protein (43.23–48.27%) and carbohydrates (44.62–48.56%) as well as the lowest level of total lipids (4.6%) of all sea cucumbers tested (Nahla, 2013). Thus, this species represents an excellent alternative choice for people who prefer low fat diets. Although it is also now being overfished from the wild, the large-scale artificial breeding and juvenile rearing of *H. leucospilota* has not yet been developed. To achieve this, a better knowledge of the animal reproductive cycle is required, which can be different between geographically separated populations (Kazanidis et al., 2014).

Spawning induction is a critical procedure for aquaculture. For sea cucumbers in hatcheries, this may include various approaches such as thermal stimulation, drying then rehydration, exposure to conspecific sperm or *Spirulina* (a blue-green algae) exposure, and a combination of the aforementioned treatments (Al Rashdi et al., 2012). However, their success is dependent upon recognising the animal's spawning season and that females have attained full maturity. An exciting new approach that could advance hatchery production is the use of reproductive neurohormones, which are hormones naturally produced by the animal

* Corresponding author at: Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Australia.
E-mail address: scummins@usc.edu.au (S.F. Cummins).

https://doi.org/10.1016/j.aquaculture.2018.05.032
Received 20 February 2018; Received in revised form 9 May 2018; Accepted 14 May 2018
Available online 19 May 2018
0044-8486/ © 2018 Elsevier B.V. All rights reserved.

to stimulate gonad maturation and spawning. Generally, neurohormones are produced in the neural tissue, which in the sea cucumber is divided into two major compartments: a circumoral nerve ring and five longitudinal radial nerve cords (RNCs) (Kato et al., 2009). Both the circumoral nerve ring and RNCs are known to contain gonadotropic neurohormones that induce final oocyte maturation, as determined by germinal vesicle breakdown (GVBD) (Fujiwara et al., 2010b; Kato et al., 2009). Excised oocytes will rarely spontaneously mature and become fertilizable (Maruyama, 1980), therefore, final oocyte maturation is essential and must occur prior to the fertilization process.

In *Apostichopus japonicus*, Radial Nerve Extract (RNE) at 3 mg/ml induces GVBD in 85% of immature oocytes (Katow et al., 2009). In the same species, a pentapeptide NGIWYamide (also called 'cubifrin'), purified from the RNE, could induce *in vitro* GVBD and ovulation of fully grown oocytes at < 1 pM and *in vivo* spawning at 10 nM, with an injected volume of 0.1% (v/w) (Kato et al., 2009). In addition, RNE can also induce maturation of fully grown oocytes in *H. leucospilota, Holothuria pervicax, Holothuria moebi,* and *Holothuria pardalis* (Maruyama, 1980), however, the bioactive neurohormone responsible for the maturation has not been elucidated. Concurrently, the subsequent development of larvae from RNE-matured oocytes has not been reported in sea cucumbers.

In this study, we investigated the reproductive cycle of *H. leucospilota* located in Australia's Sunshine Coast region (South-East Queensland). We then performed *in vitro* oocyte maturation using RNE followed by fertilization and larval rearing to late auricularia stage (25 days post-fertilization, dpf). In addition, we implemented the GVBD bioassay to check oocyte maturation of *H. leucospilota* with a synthetic NGIWYamide.

2. Materials and methods

2.1. Ethic statement

The collection and handling of the animals in this study was carried out in accordance with the guidelines for the care and use of laboratory animals at the University of the Sunshine Coast, Australia.

2.2. *Holothuria leucospilota* collection for analysis of reproductive biology

H. leucospilota (N = 15–25 individuals/sampling; both sexes) were collected monthly from Port Cartwright, Queensland, Australia from September 2016 to August 2017. The gender, total body weight, whole body length, Cuvierian tubules, gutted body weight, gonad wet weight and oocyte size were measured for biometric analysis. The gonad index (GI) was calculated separately for males and females as the ratio between the gonad wet weight and the gutted body weight according to the following formula: GI = [(gonad wet weight)/(gutted body weight)] × 100; where the gutted body weight is the body weight after removal of coelomic fluid, gonads, alimentary canal and respiratory trees (Conand, 1981; Gaudron et al., 2008; Guzmán et al., 2003; Navarro et al., 2012).

Classification of gonad maturation stages was based on previous reports (Drumm and Loneragan, 2005; Fujiwara et al., 2010a; Gaudron et al., 2008; Morgan and Neal, 2012): stage 0 (post-spawning gonad); stage I (immature gonad); stage II (growing gonad); stage III (advanced mature gonad); and stage IV (fully mature gonad). Since the reproductive cycle of other sea cucumbers were correlated with seawater temperature (Guzmán et al., 2003), monthly average sea temperature available from World Sea Temperatures (www.seatemperature.org) was used for our analysis.

2.3. Histological examination of radial nerve cord

The excised RNCs were collected and fixed in 4% paraformaldehyde solution [4 g paraformaldehyde in 100 ml of phosphate buffered saline (PBS), pH 7.4] at 4 °C, overnight. Samples were then washed in PBS and kept in 70% ethanol until paraffin histological processing using standard techniques (Suvarna et al., 2012). Tissue sections at 3–5 μm thickness were prepared using a microtome (ProSciTech company) and stained with Hematoxylin and Eosin (H&E) following standard procedures (Mayer, 1896; Putt, 1954). Sections were viewed and photographed using a Leica compound microscope (model DM5500 B; Leica Microsystems CMS GmbH, Germany) equipped with a CCD camera.

2.4. Radial nerve extract preparation and GVBD bioassay

Radial nerve cords were excised from the sea cucumbers collected during September and October (stage III), 2016. During dissection, the body wall was kept on ice, then RNCs were isolated and immediately frozen in liquid nitrogen before storage at -80 °C until used. To prepare RNE, RNC tissue (4.5 g) was thawed, then homogenised in 30 ml filtered artificial seawater (FASW). The homogenate was subsequently centrifuged at 2000 xg for 5 min at 4 °C. The supernatant (named RNE) was collected and total protein concentration determined by Spectrophotometer Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Finally, RNE was stored at -80 °C until use in GVBD bioassay.

For GVBD bioassay, ovarian tubules were dissected out from an individual *H. leucospilota* at gonad stage III (advanced mature, oocyte diameter > 150 μm) *via* a small incision to the dorsal bivium. Ovarian tubules were cut transversely into ≈5 mm long sections and transferred into tissue culture microplates containing 3 ml of FASW as a media. For RNE treatment, five concentrations were tested (50, 100, 150, 200, 233 and 466 μg/ml RNE in FASW). FASW without RNE was used as a control group. Each treatment was performed in triplicate. Briefly, tubules were incubated in FASW with/without RNE at 24 °C for 3 h, and then extruded oocytes and in-tubule oocytes were collected separately. These oocytes were observed under a stereo microscope (Nikon SMZ800N, Nikon CORP., Japan). The total number of mature and immature oocytes were recorded based on absence or presence of the germinal vesicle. The variation of maturation in oocytes in the different concentrations of RNE was tested by ANOVA with Tukey's HSD post-hoc testing (p-value cut-off 0.05).

2.5. Algal culture and larval rearing

Nannochloropsis oculata were continuously cultured in 3 L natural seawater containing f/2 nutrients (Guillard, 1975) at 26 ± 2 °C in a sterile 5 L Schott bottle under continuous cool white illumination with a photon flux density of ~100 μmol photons $m^{-2} s^{-1}$. Sterile air was bubbled through the media to provide gas exchange and circulation. *N. oculata* culture suspension in the stationary phase was harvested daily and the displaced volume was replaced with fresh growth media.

RNE-treated mature oocytes derived from the GVBD bioassay were fertilized with sperm stripped from a fully mature male *H. leucospilota* at a volume (ml) ratio of oocytes: spermatozoa = 4:1. The oocyte and sperm mixture was incubated at 24 °C for 10 min to allow fertilization. Fertilized eggs were then transferred for larval culture, reared at population density of 1000 larvae/L in 5 L beakers containing artificial seawater (32 ppm salinity) under standard lighting conditions (12 h:12 h light/dark) at 24 °C with aeration. Larvae were fed twice daily with the live *N. oculata* (final algae concentration 50,000–100,000 cells per ml). Developmental stages of larvae were assessed and photographed under a stereo microscope (Nikon SMZ800N) equipped with a CCD camera. At settlement stage, 25 dpf, the experiment was terminated.

2.6. NGIWYamide bioassay

The *H. leucospilota* NGIWYamide precursor sequence was identified using a BLASTp search (CLC Genomics workbench v.8.0) of a transcriptome assembly derived from *H. leucospilota* larval Illumina

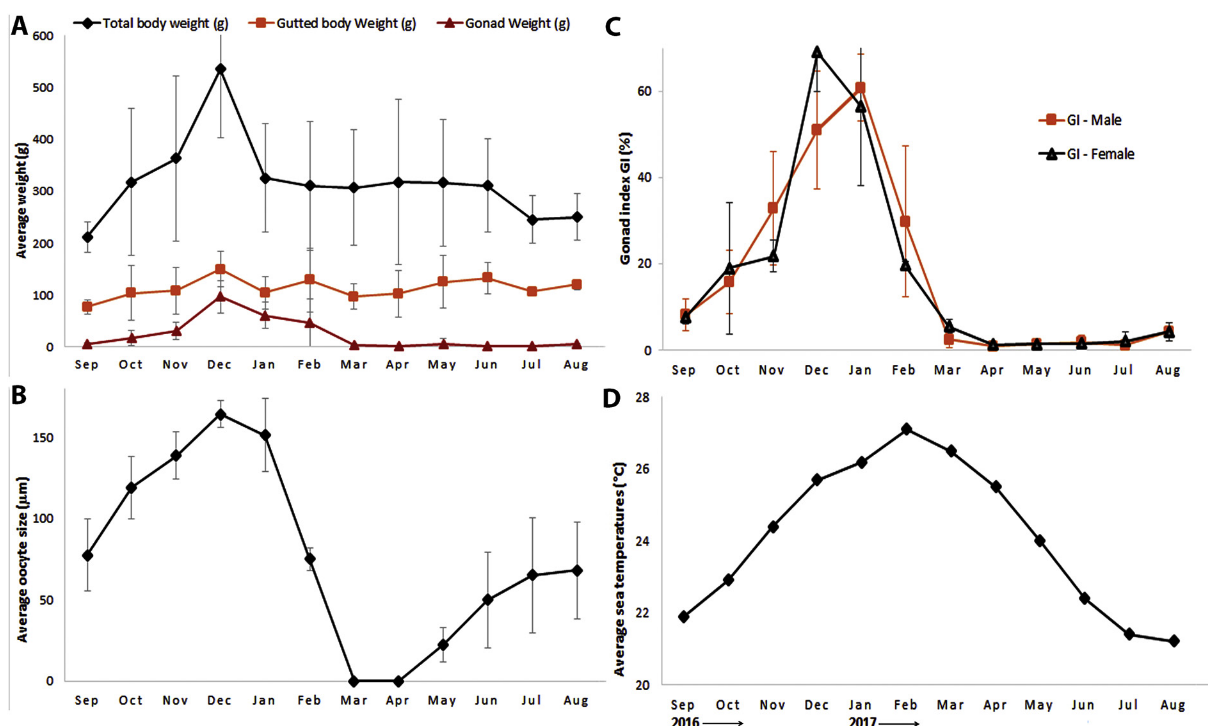

Fig. 1. Analysis of *Holothuria leucospilota* reproductive cycle located at Mooloolaba, Sunshine Coast, Australia. Graphs showing seasonal variation in (A) average weight (males + females), (B) oocyte size, and (C) gonad index (GI) of male and female from September 2016 to August 2017. Bars indicate standard derivation of mean. (D) Monthly average seawater temperatures were provided by World Sea Temperatures (www.seatemperature.org).

transcriptomes (SRA numbers: DRR023762 and DRR023763). A schematic representation of the *H. leucospilota* NGIWYamide precursor was prepared using the IBS software version 1.0.1 (Illustrator for BioSequence, the CUCKOO workgroup).

For bioassay, ovarian tubules were dissected out of *H. leucospilota* (stage IV) and treated as described for the GVBD bioassay, only synthetic NGIWYamide (ChinaPeptides Co.,Ltd) was the test compound, at 1 nM, 10 nM, 100 nM, 1 µM, and 10 µM. FASW was used as a negative control and RNE at 200 µg/ml was used as a positive control. Ovarian fragments/tubules were incubated in 300 µl of treatment solution with three replicates for 3 h at 24 °C.

3. Results

3.1. Reproductive cycle of H. leucospilota

In both sexes of *H. leucospilota*, the average total body weight, gutted body weight and gonad weight, increased gradually from September to a maximum in December (535.4 ± 133.1 g total weight, 149.9 ± 33.5 g gutted weight and 96.5 ± 31.3 g gonad weight) (Fig. 1A). From January, average weight for all parameters decreased gradually.

Oocyte size is a critical marker for gonad development. *H. leucospilota* oocytes were > 100 µm in diameter from October to January and reached a maximum size in December (164.3 ± 8.1 µm diameter) (Fig. 1B). Oocyte size was the lowest during March through to June, although some advanced mature oocytes (150–160 µm in diameter) were present in low numbers. The presence of new ovarian tubules without oocytes was reported in March and April, then gradually became populated with immature oocytes in May (22.5 ± 10.6 µm in diameter).

H. leucospilota GI increased rapidly from September (8.02 ± 3.68% in male and 7.58 ± 1.60% in female) and peaked in December for both female (69.08 ± 9.12%) and male (60.81 ± 7.69%) individuals (Fig. 1C). GI decreased rapidly by March and stayed consistently low until August (GI < 5.0%). The mean monthly sea temperature at the Sunshine Coast, exhibited a seasonal pattern peaking in February with a mean maximum monthly temperature of 27.1 °C and a minimum in August of 21.2 °C (Fig. 1D). During the period of spawning, seawater temperature was 27-28 °C and GI was at its highest, while GI was the lowest during the coldest seawater temperatures (21 °C–23 °C).

3.2. Radial nerve cords and GVBD bioassay with radial nerve extract

The position of the RNCs in *H. leucospilota* was confirmed by morphological and histological analyses. There are five RNCs travelling anteroposteriorly within the body wall, where each RNC is flanked by two longitudinal muscle bands (LMBs) (Fig. 2A). RNC are transparent in appearance and lie under a thin connective tissue layer continued with the body wall dermis. H&E-stained sections of the body wall show the anatomical and histological structures, including the RNC, LMB, radial water canal (RC) and body wall dermis (BD) (Fig. 2B, C). According to its histology, the RNC is composed of two closely apposed strips of neural tissue, an ectoneural neuroepithelium (EN) and hyponeural neuroepithelium (HN), divided by a thin connective tissue layer. The neuronal cell bodies were prominent at the outer region, while the nerve fibres occupy most of the inner region of the RNC. In addition, the EN and HN are protected by the epineural sinus and hyponeural sinus, respectively. The medial and lateral borders of the RNC include the RC and BD, respectively.

The RNC was isolated from the body wall and processed to obtain radian nerve extract (RNE) for assay; to determine its effect on oocyte extrusion from the ovarian tubule and oocyte GVBD. The number of extruded oocytes varied at the RNE concentrations tested (0, 50, 100, 150, 200, 233, and 466 µg/ml RNE), at 3 h post-incubation (Fig. 3). Bioassays showed that ovarian tubules incubated with 233 µg/ml RNE, extruded oocytes most prominently (Fig. 3A1-3, D). By comparison, approximately 50% of oocytes had been extruded with 466 µg/ml RNE (Fig. 3B1-3, D). The percentage of extruded oocytes peaked at 233 µg/ml RNE (79.5 ± 4.9%) and decreased at 466 µg/ml RNE

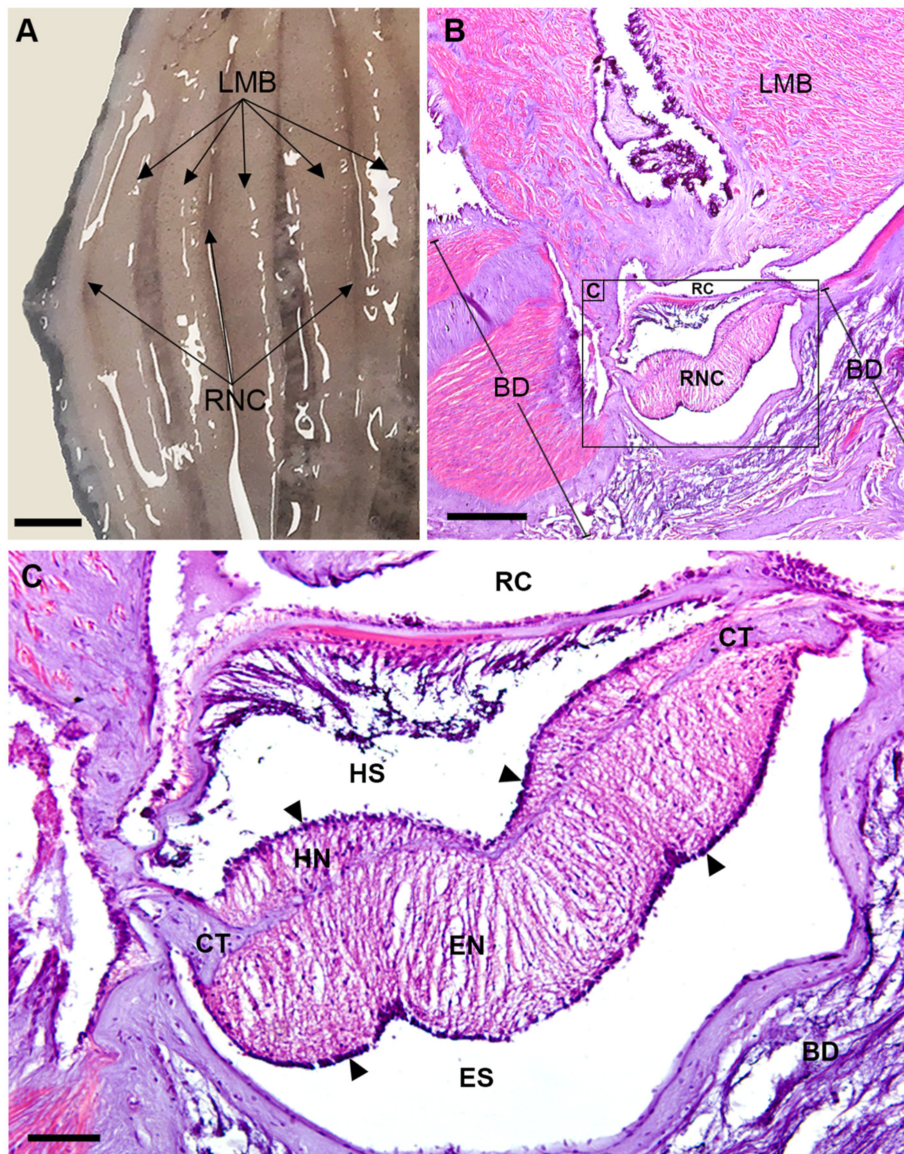

Fig. 2. Organization of the radial nerve cord (RNC) in *Holothuria leucospilota*. (A) Internal surface of body wall containing RNC and longitudinal muscle bands (LMBs). Scale bar indicates 1 cm. (B) Low magnification of the body wall (cross-section) showing the position of the RNC, LMBs, radial water canal (RC) and body wall dermis (BD). Scale bar indicates 1 mm. (C) Higher magnification of the RNC which is corresponded to the area in (B) (inset). A thicker ectoneural neuroepithelium (EN) and hyponeural neuroepithelium (HN) are separated by a thin connective tissue layer (CT). Arrowheads indicate the neuronal cell bodies. Surrounding EN and HN is the epineural sinus (ES) and hyponeural sinus (HS). ES and HS are bordered by the RC and BD, respectively. Scale bar indicates 200 μm.

(49.6 ± 3.3%). Very few oocytes were extruded in the control group which was treated with the filtered artificial sea water (FASW) (Fig. 3C1-3, D).

The progression of immature oocytes to mature oocytes was analyzed by presence or absence of germinal vesicle (Fig. 4A). At 3 h post-incubation, there was a significant increase in GVBD of extruded oocytes at all RNE-treated concentrations when compared with the controls ($p < 0.05$; Fig. 4B). Oocyte GVBD gradually increased from RNE concentrations of 50 to 233 μg/ml, but with no significant difference between 100, 150, 200 and 233 μg/ml. GVBD decreased significantly ($p < 0.05$) at RNE 466 μg/ml (Fig. 4B). In-tubule GVBD was very low at 50 and 100 μg/ml RNE and control groups (1.0–1.1%). These results show that in-tubule oocyte GVBD is interdependent and directly proportional to the percentage of extruded oocytes.

3.3. Fertilization and development of larvae from RNE-matured oocytes

RNE-matured oocytes (Fig. 5A) were collected and combined with *H. leucospilota* sperm *in vitro* (ratio 4 sperm:1 oocyte), resulting in 99% fertilization efficiency. Embryonic development at various stages from 2-cell stage were recorded hourly from 3 to 6 h, and at 24 h post-fertilization (Fig. 5, B-D). Gastrulae were first identified at 2 dpf (Fig. 5E), then the embryos gradually elongated and began to fold into the early auricularia larvae at 3 dpf. The early auricularia stage lengthened over 4–9 dpf (Fig. 5F). After that, *H. leucospilota* larvae developed into mid-auricularia stage, from 10 to 20 dpf (Fig. 5G) and late auricularia stage from 21 to 25 dpf (Fig. 5H).

During larval rearing, larvae were fed with live *N. oculata* and larval size was measured daily to assess growth and development. Gastrulae were 240–280 μm in length, whereas the early auricularia to mid-auricularia larvae were 280–350 μm in length. At the late auricularia stage, larvae did not increase in size and showed a tendency to decrease in length (~300 μm), in preparation for settlement.

3.4. NGIWYamide bioassay

The *H. leucospilota* NGIWYamide precursor (GenBank accession no. MG913385) contains an N-terminal signal peptide, 5 copies of the NGIWY peptide followed by a glycine residue, and 8 dibasic (‘KR’) cleavage sites (Fig. 6A).

A synthetic peptide corresponding to the amino acid sequence ‘NGIWYamide’ was used in a bioassay to assess stimulation of GVBD. Upon exposure to five concentrations of NGIWYa (1 nM, 10 nM, 100 nM, 1 μM, and 10 μM), ovarian tubules did not extrude oocytes and

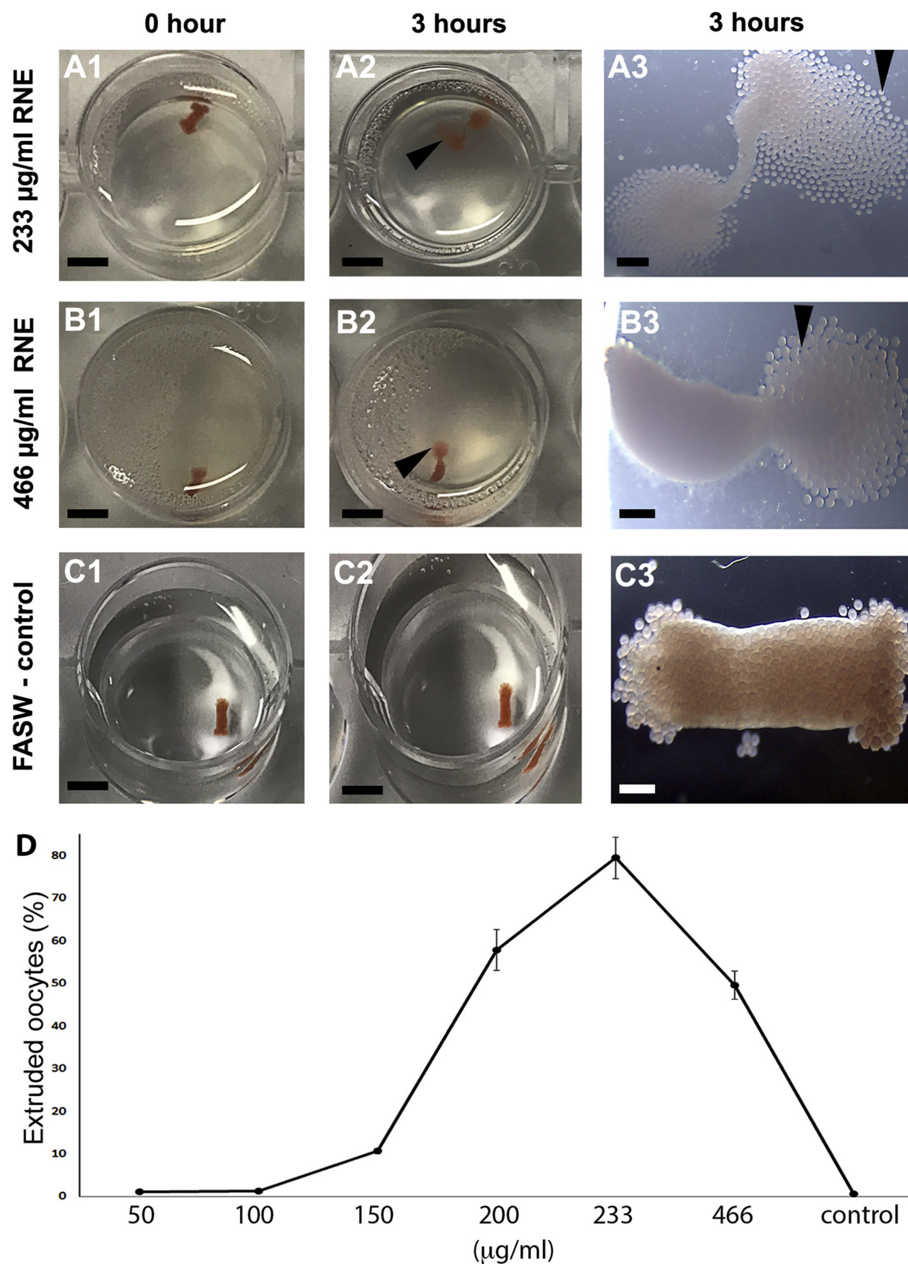

Fig. 3. Germinal vesicle breakdown (GVBD) bioassay using radial nerve extract (RNE) on *Holothuria leucospilota* ovarian fragments. The ovarian fragments were treated with RNE at 233 μg/ml (A1–3), 466 μg/ml (B1–3) and control groups (C1–3). A3, B3, and C3 show ovarian fragments at higher magnification which correspond to A2, B2, and C2, respectively. Scale bars: 5 mm in A1, A2, B1, B2, C1, C2 and 500 μm in A3, B3, C3. Black arrowheads indicate oocytes that were extruded from ovarian tubules. D: The extruded percentage of oocytes at five concentrations of RNE and control (FASW) group, at 3 h post-incubation.

little GVBD was observed (0.5–0.7% GVBD), which was similar to the negative controls using FASW (0.7–0.9% GVBD) (Fig. 6B, C). In positive controls (200 μg/ml RNE), ovarian tubule extrusion occurred and GVBD was observed in the majority of oocytes (98.7 ± 0.4%) (Fig. 6B).

4. Discussion

H. leucospilota is one of many sea cucumber species that are overfished in the wild due to its nutritional and medicinal value. Artificial breeding is now a common approach to help sea cucumber culture and thereby decrease the pressure on wild populations (Lovatelli, 2004). To date, artificial breeding of sea cucumbers has mostly relied upon traditional spawning induction during the spawning season, including thermal stimulation and conspecific sperm exposure, although its success relies on a variety of factors such as environmental conditions and gonadal maturation. For that reason, the use of a gonad-stimulating factor that can facilitate oocyte maturation for subsequent *in vitro* fertilization would represent a break-through new approach.

The increase in popularity of sea cucumbers has driven increasing numbers of reproductive-related studies. It is now clear that the duration of their spawning season varies significantly among different sea cucumber species, or even the same species separated by geographical location (Drumm and Loneragan, 2005; Guzmán et al., 2003; Hamel and Mercier, 1996; Martinez et al., 2011; Navarro et al., 2012). In our study, we investigated the variance of biological parameters related to reproductive maturation, including body weight, gonad weight, oocyte size, gonad stage, and GI, throughout an entire year, in order to determine the spawning season of *H. leucospilota* in the Sunshine Coast region of Australia. This knowledge is critical for the subsequent development of an artificial breeding approach. We found that spawning

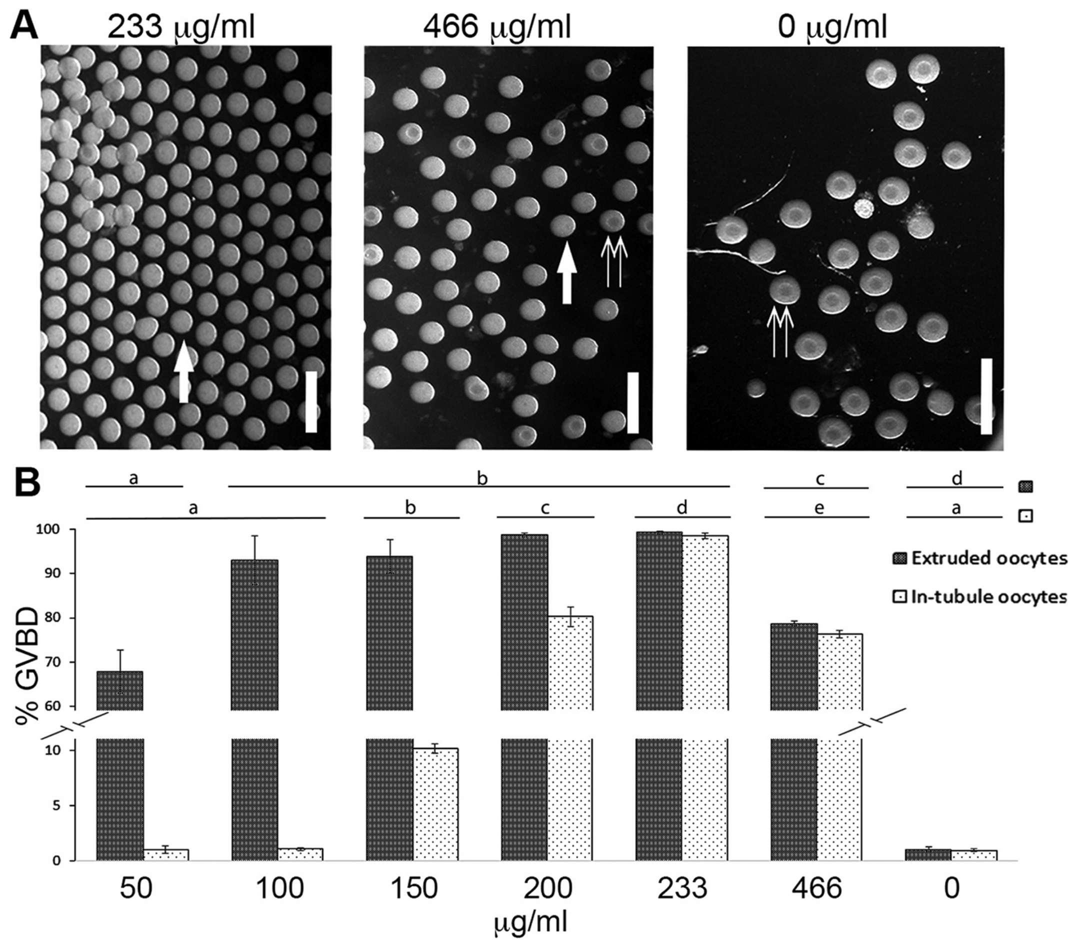

Fig. 4. Effect of radial nerve extract (RNE) on germinal vesicle breakdown (GVBD) and percentage of extruded oocytes at 3 h post-incubation. A: Micrographs showing oocyte GVBD at 233 μg/ml, 466 μg/ml RNE and compared to control group at 3 h post-incubation. Single white arrows indicate the oocytes with GVBD, while double white arrows the oocytes without GVBD (Scale bars indicate 500 μm). B: GVBD percentage of oocytes obtained from outside (extruded oocytes) and inside (in-tubule oocytes) the ovarian tubules, in RNE-treated and control (FASW) groups, at 3 h post-incubation. Bars with different letters (a, b, c, d, e) indicate significant difference within extruded oocyte groups and within in-tubule oocyte groups ($p < 0.05$).

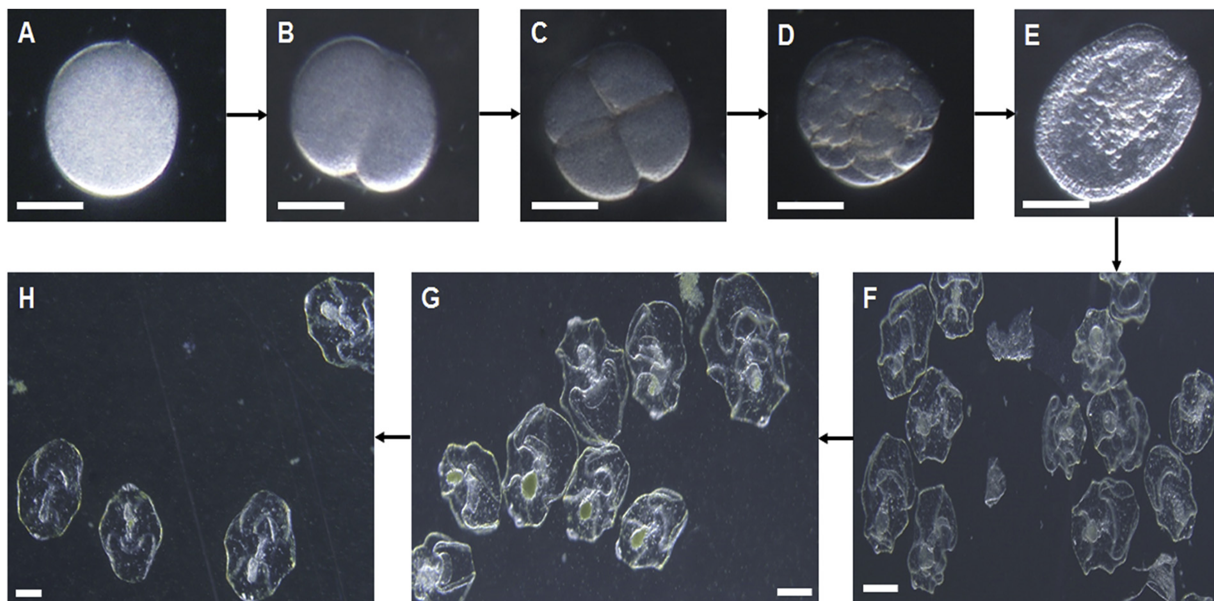

Fig. 5. Radial nerve extract (RNE)-induced oocyte maturation, fertilization and larval development in *Holothuria leucospilota*. (A) Mature oocyte following RNE-induced germinal vesicle breakdown. These oocytes were used for fertilization, then developed to a (B) two-cell embryo, (C) four-cell embryo, (D) multi-cell embryo at 1 dpf, (E) gastrula at 2–3 dpf, (F) early auricularia larvae at 4–9 dpf, (G) mid-auricularia larvae at 10–20 dpf, and (H) late auricularia larvae at 21–25 dpf. Scale bars represent 100 μm.

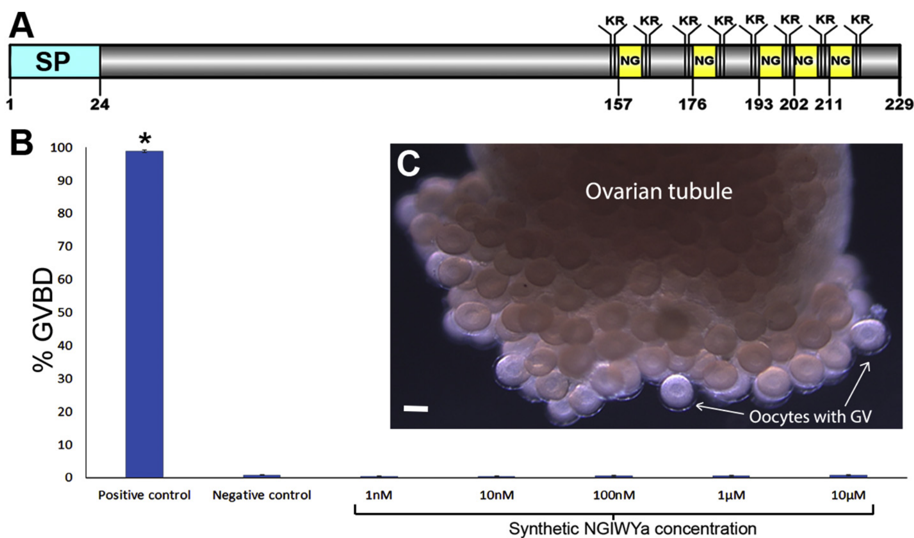

Fig. 6. Bioassay for oocyte extrusion and germinal vesicle breakdown (GVBD) in response to the synthetic NGIWYamide. A: Organization of *H. leucospilota* NGIWYamide precursor. (SP, signal peptide; KR, cleavage sites; NG, NGIWY mature peptide); B: A graph shows GVBD in response to five concentrations of synthetic NGIWYamide and positive control (RNE at 200 μg/ml). FASW was used as the negative control. Asterisk indicates a significant difference at $p < 0.05$; C: A photo representation of an ovarian tubule and oocytes with germinal vesicle (GV) at 3 h post-incubation. Scale bar indicates 100 μm.

of mature oocytes (150–175 μm in diameter) occurred throughout November to January. Oocyte size is related to the accumulation of yolk protein, which is essential for larval development post-fertilization (Fujiwara et al., 2010b).

The increase in GI observed for both male and female *H. leucospilota* correlated with an increase in seawater temperature from September to December. Meanwhile, the dramatic drop observed in GI during December–March was the result of increased spawning activity during this period. Collectively, we conclude that gonad development in *H. leucospilota* takes place during September to December and spawning occurs between December to early March. After the spawning period, gonad development is suspended up until August, which corresponds to a gradual decrease of seawater temperature.

Our study is consistent with the alignment of sea cucumber reproductive maturation and spawning based upon geographic region and seawater temperature. In *H. leucospilota* located in the Cook Islands, the GI peaks from November to December, just prior to a summer spawning period, from January until April (Drumm and Loneragan, 2005). Whereas, for *H. leucospilota* at La Réunion located in the Western Indian Ocean, spawning events occur two times, just prior to and just after maximum seawater temperatures (Gaudron et al., 2008). In other sea cucumber species such as *A. mauritiana* in Guam, the spawning period is relatively long, occurring during the periods of spring and summer (April to August) (Ramofafia et al., 2001). Meanwhile, *Isostichopus badionotus* and *Holothuria mexicana* in the Caribbean Panama can spawn throughout the year, since seawater temperature is consistently warm (> 26 °C) (Guzmán et al., 2003).

The first report of an oocyte maturation factor in sea cucumbers was in *A. japonicus*, where an *in vitro* assay showed that RNE at 3 mg/ml induced 85% oocyte GVBD at 2 h post-incubation (Katow et al., 2009). The existence of a GVBD factor in the sea cucumber RNCs was also supported in *H. leucospilota* (Maruyama, 1980, 1985), demonstrating ~25% GVBD at 500 μg/ml RNE, 1 h post-incubation, and 100% at 10 mg/ml RNE. Both the aforementioned studies used relatively high centrifugation to obtain the experimental RNE (26,000–45,000 x g) when compared to our study. In our study, RNE supernatant was collected at a relatively slow speed of 2000 xg and resulted in 98.9% GVBD at 233 μg/ml (see Figs. 3 and 4). Importantly, this slow centrifugation may help to increase the concentration of resultant active factor and is easier to replicate within a basic sea cucumber hatchery. However, the percentage of GVBD was decreased at a higher RNE concentration of 466 μg/ml (see Figs. 3 and 4). Certain inhibitors of oocyte maturation could cause this phenomenon when RNE volume is increased. In recent studies, holotoxin A_1 in sea cucumbers was suggested as an inhibitor of meiotic maturation (Aminin and Anisimov, 1990) and saponin extracted from sea cucumber body wall could decrease the oocyte growth and maturation (Moghadam et al., 2016). Therefore, RNE may contain several inhibitors of oocyte maturation, including holotoxin A_1, saponin or others which could impact into oocyte maturation at relatively high RNE concentration. An important technical point, however, is the rapid dissection and processing of RNCs, since this tissue is known to rapidly degrade after removal due to autolytic enzymes or non-enzymatic degradation (Hou et al., 2014).

The RNE derived from one sea cucumber species has been shown to be cross-species active on oocyte maturation in another sea cucumber species (Maruyama, 1985). In fact, RNE from a starfish can also induce GVBD in the sea cucumber *Australostichopus mollis* (Maruyama, 1986), suggesting that the maturation factor(s) is highly conserved across echinoderms. To date, the most well studied sea cucumber factor with relevance to GVBD is the NGIWYamide (cubifrin) of *A. japonicus* (Kato et al., 2009). This pentapeptide is released from the circumoral nerve ring and induces oocyte maturation and gamete spawning (Kato et al., 2009). The *H. leucospilota* NGIWYamide precursor gene transcript identified in our study, encodes five NGIWYamide. We showed that this peptide could not induce oocyte extrusion and maturation in *H. leucospilota*, suggesting that another peptide is required and should be further investigated. Another bioactive factor that could be considered is the 'gonad-stimulating substance-like molecule', a larger peptide that is also found in *A. japonicus* that can also induce oocyte maturation (Ahmed et al., 2011; Katow et al., 2009).

Artificial spawning of mature *H. leucospilota* was recently investigated using cold, dry and microalgae as stimulants, whereby resultant gametes were used in fertilization, larval development and juvenile growth (Huang et al., 2018). This is a helpful reference for *H. leucospilota* larval development, yet did not use RNE-induced maturation of oocytes. In our study, we demonstrated that *H. leucospilota* larvae from RNE-treated oocytes developed normally, although we did not culture them beyond late auricularia (25 dpf). In ovary samples taken during February and March, we observed the presence of both degenerated and newly regenerated ovarian tubules. Thus, in order to implement *in vitro* oocyte maturation by RNE at maximum efficiency, the ovaries could be excised non-destructively *via* small incision to the side of the sea cucumber, which would allow these animals to be reconditioned for the next spawning season.

The outcome of this study provides a useful approach to artificial breeding in the black sea cucumber, however, to circumvent the need to prepare RNE it will be helpful to determine the identity of the bioactive factor(s) within the RNE. To date, a general factor that can stimulate this in all sea cucumbers is not known. In order to help with hatchery production and conservation strategies, further research into other

factors, and in particular the reproductive neurohormones, is important. To achieve this, we propose that bioassay-guided analysis of the RNE components should be performed.

5. Conclusions

We provide foundation information related to the reproductive cycle of the black sea cucumber *H. leucospilota* located in South-Eastern Queensland, Australia. We have determined the timing of reproductive maturation and spawning, as well as evidence that radial nerve extract can induce *in vitro* oocyte GVBD; these eggs can be used for fertilization, leading to normal development up to late auricularia. Synthetic NGIWYamide does not induce oocyte maturation of *H. leucospilota.* Further research into the radial nerve extract biomolecules is recommended to identify the bioactive factor(s). Our results could be applied in large-scale culture of black sea cucumber hatcheries.

Acknowledgments

The authors gratefully acknowledge the financial support and laboratory facilities provided by the Genecology Research Centre, The Faculty of Science, Health, Education and Engineering and the Office of Research, University of the Sunshine Coast (USC) (807E9516), Australia. We also thank Dr. Luke Turner and Ms. Shahida Akter Mitu for their assistance in sample collection and dissection. This research was supported by USC-VIED PhD scholarship to Chieu Hoang Dinh, which is funded by Ministry of Agriculture and Rural Development, Vietnam and USC, Australia.

References

Ahmed, H.O., Katow, T., Katow, H., 2011. Spatiotemporal expression pattern of gonad-stimulating substance-like peptide of the sea cucumber, *Apostichopus japonicus*. Develop. Growth Differ. 53, 639–652.

Al Rashdi, K.M., Eeckhaut, I., Claereboudt, M.R., 2012. A Manual on Hatchery of Sea Cucumber *Holothuria scabra* in the Sultanate of Oman. Ministry of Agriculture and Fisheries Wealth, Aquaculture Centre, Muscat, Sultanate of Oman (27 pp).

Aminin, D.L., Anisimov, M.M., 1990. The influence of holotoxin A_1 to the Ca^{2+} transport and meiotic maturation of oocytes from the sea cucumber *Stichopus japonicus*. J. Evol. Biochem. Physiol. 26, 9–13.

Battaglene, S.C., Seymour, E.J., Ramofafia, C., Lane, I., 2002. Spawning induction of three tropical sea cucumbers, *Holothuria scabra*, *H. fuscogilva* and *Actinopyga mauritiana*. Aquaculture 207, 29–47.

Conand, C., 1981. Sexual cycle of three commercially important Holothurian species (Echinodermata) from the lagoon of New Caledonia. Bull. Mar. Sci. 31, 523–543.

Drumm, D.J., Loneragan, N.R., 2005. Reproductive biology of *Holothuria leucospilota* in the Cook Islands and the implications of traditional fishing of gonads on the population. N. Z. J. Mar. Freshw. Res. 39, 141–156.

Fahmy, S.R., Amer, M.A., Al-killidar, M.H., 2015. Ameliorative effect of the sea cucumber *Holothuria arenicola* extract against gastric ulcer in rats. J. Basic Appl. Zool. 72, 16–25.

Fujiwara, A., Unuma, T., Ohno, K., Yamano, K., 2010a. Molecular characterization of the major yolk protein of the Japanese common sea cucumber (*Apostichopus japonicus*) and its expression profile during ovarian development. Comp. Biochem. Physiol. A-Mol. Integr. Physiol. 155, 34–40.

Fujiwara, A., Yamano, K., Ohno, K., Yoshikuni, M., 2010b. Spawning induced by cubifrin in the Japanese common sea cucumber *Apostichopus japonicus*. Fish. Sci. 76, 795–801.

Gaudron, S.M., Kohler, S.A., Conand, C., 2008. Reproduction of the sea cucumber *Holothuria leucospilota* in the Western Indian Ocean: biological and ecological aspects. Invertebr. Reprod. Dev. 51, 19–31.

Guillard, R.R.L., 1975. Culture of Phytoplankton for Feeding Marine Invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals: Proceedings — 1st Conference on Culture of Marine Invertebrate Animals Greenport. Springer US, Boston, MA, pp. 29–60.

Guzmán, H., Guevara, C., Hernández, I., 2003. Reproductive cycle of two commercial species of sea cucumber (Echinodermata: Holothuroidea) from Caribbean Panama. Mar. Biol. 142, 271–279.

Hamel, J.-F., Mercier, A., 1996. Studie on the Reproductive Biology of the Atlantic Sea Cucumber *Cucumaria frondosa.* SPC Beche-de-mer Information Bulletin. Vol. 8. pp. 22–33.

Hou, H., Chen, T., Peng, Z., Zhang, Z., Xue, C., Li, B., 2014. Stability and degradation regulation of body wall gel of sea cucumber treated by high hydrostatic pressure. Trans. Chinese Soc. Agricult. Eng. 30, 316–322.

Huang, W., Huo, D., Yu, Z., Ren, C., Jiang, X., Luo, P., Chen, T., Hu, C., 2018. Spawning, larval development and juvenile growth of the tropical sea cucumber *Holothuria leucospilota*. Aquaculture 488, 22–29.

Kato, S., Tsurumaru, S., Taga, M., Yamane, T., Shibata, Y., Ohno, K., Fujiwara, A., Yamano, K., Yoshikuni, M., 2009. Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, *Apostichopus japonicus*. Dev. Biol. 326, 169–176.

Katow, H., Katow, T., Moriyama, A., 2009. Gonad-stimulating substance-like molecule from the radial nerve of the sea cucumber. Int. J. Develop. Biol. 53, 483–491.

Kazanidis, G., Lolas, A., Vafidis, D., 2014. Reproductive cycle of the traditionally exploited sea cucumber *Holothuria tubulosa* (Holothuroidea: Aspidochirotida) in Pagasitikos Gulf, western Aegean Sea, Greece. Turkish J. Zool. 38, 306–315.

Lovatelli, A., 2004. Advances in sea cucumber aquaculture and management. FAO 463, 440.

Martinez, M.I., Giménez, J., Penchaszadeh, P.E., 2011. Reproductive cycle of the sea cucumber *Psolus patagonicus* Ekman 1925, off Mar del Plata, Buenos Aires, Argentina. Invertebr. Reprod. Dev. 55, 124–130.

Maruyama, Y.K., 1980. Artificial induction of oocyte maturation and development in the sea cucumbers *Holothuria leucospilota* and *Holothuria pardalis*. Biol. Bull. 158, 339–348.

Maruyama, Y.K., 1985. Holothurian oocyte maturation induced by radial nerve. Biol. Bull. 168, 249–262.

Maruyama, Y.K., 1986. Induction of sea cucumber oocyte maturation by starfish radial nerve extracts. J. Exp. Zool. 238, 241–248.

Mayer, P., 1896. Hematoxylin and eosin (H&E) staining protocol. Mittheil. Zool. Station zu Neapel 12, 303.

Moghadam, F.D., Baharara, J., Balanezhad, S.Z., Jalali, M., Amini, E., 2016. Effect of *Holothuria leucospilota* extracted saponin on maturation of mice oocyte and granulosa cells. Res. Pharm. Sci. 11, 130–137.

Morgan, A., Neal, L., 2012. Aspects of reproductive ecology and benthic–pelagic coupling in the sub-antarctic sea cucumber *Pseudostichopus mollis* (Theel). Cont. Shelf Res. 43, 36–42.

Nahla, E.S.E.S.O., 2013. Nutritional value of some Egyptian sea cucumbers. Afr. J. Biotechnol. 12, 5466–5472.

Navarro, P.G., García-Sanz, S., Tuya, F., 2012. Reproductive biology of the sea cucumber *Holothuria sanctori* (Echinodermata: Holothuroidea). Sci. Mar. 76, 741–752.

Olivera-Castillo, L., Davalos, A., Grant, G., Valadez-Gonzalez, N., Montero, J., Barrera-Perez, H.A.M., Chim-Chi, Y., Olvera-Novoa, M.A., Ceja-Moreno, V., Acereto-Escoffie, P., Rubio-Piña, J., Rodriguez-Canul, R., 2013. Diets Containing Sea cucumber (*Isostichopus badionotus*) meals are Hypocholesterolemic in young rats. PLoS One 8, e79446.

Purcell, S.W., Hair, C.A., Mills, D.J., 2012. Sea cucumber culture, farming and sea ranching in the tropics: progress, problems and opportunities. Aquaculture 368-369, 68–81.

Putt, F.A., 1954. Histopathologic technic and practical Histochemistry. Yale J. Biol. Med. 27, 73.

Ramofafia, C., Byrne, M., Battaglene, S.C., 2001. Reproductive biology of the intertidal sea cucumber *Actinopyga mauritiana* in the Solomon Islands. J. Marine Biol. Assoc. United Kingdom 81, 523–531.

Suvarna, K.S., Layton, C., Bancroft, J.D., 2012. Bancroft's Theory and Practice of Histological Techniques E-Book. Elsevier Health Sciences.