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# New Technologies to Improve Sydney Rock Oyster Breeding and Production


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# Contents

## Tables



## Figures

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# Abbreviations

6-DMAP: 6-dimethylaminopurine

APGW: APGWamide

BP: family-based breeding program

CB: cytochalasin B

cDNA: complementary DNA

CRC-P: Cooperative Research Centres Projects

DEG: differential gene expression

DIIS: Department of Industry, Innovation and Science

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DPI: Department of Primary Industries

EBV: estimated breeding value

GnRH: gonadotropin-releasing hormone

H&E: hematoxylin and eosin

HPLC: High Performance Liquid Chromatography

MS: mass spectrometry

N: number

NP: neuropeptide

NS: normal saline

NSW: New South Wales

PCR: polymerase chain reaction

PFA: paraformaldehyde

PSFI: Port Stephens Fisheries Institute

PVP: polyvinylpyrrolidone

QX: Queensland unknown disease

RPGW: RPGWamide

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

RNA: ribonucleic acid

RT-PCR: reverse-transcription polymerase chain reaction

SCS: Southern Cross Shellfish

SOCo: The Select Oyster Company

SRO: Sydney Rock Oyster

TK: tachykinin

UoN: The University of Newcastle

USC: University of the Sunshine Coast

WM: winter mortality

YC: year class

# Executive Summary

Hatchery production of Sydney Rock Oysters (SROs, *Saccostrea glomerata*) is a costly and high risk activity for the breeding program and industry exacerbated by factors such as: reliance on hatchery conditioning, low fertilisation success using strip-spawned gametes, extended larval rearing period compared to Pacific Oysters (*Crassostrea gigas*), and variable settlement rates. This project, one of a number that comprised the Future Oysters Coopoerative Research Centre project (Future Oysters CRC-P), was developed through discussions with the SRO industry hatchery sector and was designed to target specific hatchery production challenges. Four fundamental components were identified for research and development:

1. Production of tetraploid SROs for triploid hatchery production
2. Decreasing the time frame and increasing the reliability of hatchery conditioning
3. Producing a spawn inducing factor(s)/pheromone to trigger natural release of gametes
4. Physiological process that occurs following oocyte release to extend the duration of viability through the use of benchtop storage media

Each component listed above is designed to either decrease hatchery operation costs, increase production reliability or increase the market for hatchery produced seed. The highest priority for further development of the SRO Breeding Program (BP) is increasing the success rate of single pair mated crosses. Research for production of tetraploid SROs, producing a spawn inducing factor and investigating oocyte viability will produce valuable information that can be directly applied to increase the success rate of single pair mated crosses.

Tetraploid inductions were performed by Southern Cross Shellfish (SCS) and the NSW Department of Primary Industries (NSW DPI). Tetraploid inductions were not successful in producing a batch of tetraploid SRO that could be made available for commercial hatchery production of triploids using tetraploid male and triploid female crosses. Eleven attempts were made and the major challenges encountered was low egg numbers, asynchronous embryonic development in strip-spawned oocytes after fertilisation, poor development and poor larval survival. A very small number of spat were successfully settled from 2 trials, however, no tetraploids were found in these batches when oysters had reached a size where tissue could be taken to determine ploidy level.

Although the original milestones related to the tetraploid research were not achieved, there have been some significant outcomes from this Future Oysters CRC-P project with respect to production of triploid SROs and making these available to industry. Successful chemical triploid batches produced for the CRC-P tetraploid work have resulted in distribution of triploids from QX disease-resistant broodstock to industry. NSW DPI used this opportunity to commercially evaluate triploids and measured QX disease resistance and oyster growth performance. Although no QX disease occurred during field evaluations, triploid oysters had significantly faster growth rates compared to a group of selected 2017 year class (YC) families and non-selected oysters.

SROs generally require 10 weeks of hatchery conditioning to obtain suitable gametes for a successful spawning. Hatchery conditioning requires significant hatchery resources in terms of algae, energy and labour over this period. Reducing the hatchery conditioning period and improving hatchery conditioning reliability with respect to producing ready-to-spawn broodstock reduces the financial impost for SRO hatcheries.

Results from three independent trials performed by the University of the Sunshine Coast (USC), revealed that administering the individual forms of buccalin and APGWamide stimulated gonad conditioning in SRO. Comparing the stimulatory effect of two different forms of APGWamide, APGWa and RPGWa, USC found that the APGWa form was more potent than the RPGWa form. For buccalin, the buccalin-G

form showed a better performance than the buccalin-A form in most of the reproductive activities assessed. Considering the stimulatory effects of APGWa and buccalin-G on SRO conditioning, their stimulatory effects appear to be comparable. Hence, either APGWa or buccalin-G can be used on SRO broodstock for both breeding runs and commercial spat production. With respect to peptide delivery, we found that using cocoa butter to allow a slow release of peptide was not appropriate as it caused high mortalities. Injection of peptides is therefore preferable and multiple injections are expected to help in maintaining the level of the peptides in the oyster's circulatory system in order to successfully control gonad conditioning. Yet, delivery of peptide by injection is relatively difficult and uncontrollable since different individual oysters could receive different amount of the neuropeptide per injection. To overcome this problem, delivery using other techniques such as, oral delivery by using peptide-encapsulated algae, should be considered and tested in the future.

Spawning inducing factor/pheromone was found to be present in SRO sperm by USC. SRO sperm was isolated and proteins semi-purified, giving two major extract groups - the intrinsic and extrinsic sperm membrane proteins. Further purification was performed using RP-HPLC, resulting in multiple fractions of sperm membrane proteins. Crude extracts and RP-HPLC fractions were tested in a spawning induction bioassay, in which fully mature oysters were treated with the crude extract or fraction, prior to observation of spawning activity over the period of 2 h. We found that proteins extracted from the extrinsic sperm membrane, but not the intrinsic sperm membrane, could successfully induce spawning in the SRO. Further purification of positive extrinsic membrane fraction S3 minute 41-45 led to sub-fractions that were also tested, resulting in two positive sub-fractions (at 6-10 min and 11-15 min). The MS analysis of each revealed 8 proteins, including: aminopeptidase N; calmodulin; 60 kDa SS-A/Ro ribonucleoprotein; protein bark beetle-like; helicase; failed axon connections homolog; nascent polypeptide-associated complex subunit alpha; and, a novel protein. We propose that one, or more of these proteins may play a critical role in stimulating spawning of the SRO.

Another objective of this project related to a straightforward method for benchtop storage of SRO gametes. This work was done by The University of Newcastle (UoN) and required an understanding of the causes of oocyte degradation and to develop improved storage protocols for oocytes that extend the holding period in vitro after strip-spawning gametes. Specifically, the objective reported on here was to evaluate up to three methods for the improved benchtop storage of SRO gametes. This objective was achieved, but further work will continue to evaluate further approaches to gamete storage as part of a continuing PhD project. Typically, SRO oocytes show the best fertilisation rates if fertilised within 24 h of stripping; afterwards, the success decreases exponentially. Results in this experiment suggest that vitality can be improved if these characteristics are altered in the media, and when additives are included (such as antibiotics and polyvinylpyrrolidone (PVP)). This is a significant step in extending the life of a cell for breeding purposes, effectively extending the window in which quality testing and fertilisations can be conducted.

Research is ongoing for tetraploidy induction techniques (SCS), neuropeptide delivery to broodstock (USC), spawn inducing factors (USC) and oocyte storage for SROs (UoN). Further research is required to incorporate all findings from this research into routine operations of the SRO BP. However, outcomes from this work are already providing benefits for this breeding program, notably technical improvements to the fertilisation process. This has increased family production success from 27% to 45%. This outcome is relevant for the SRO BP and commercial hatcheries as it reduces the time it takes to create families and the numbers of valuable broodstock required for a breeding or commercial hatchery run.


## Keywords

Sydney Rock Oyster, *Saccostrea glomerata*, hatchery, tetraploidy, triploid, conditioning, neuropeptide, buccalin, APGWamide, spawn inducing factor, oocyte viability.

# Introduction

## Background

The Australian aquaculture industry is an important fisheries sector which contributes over $70 million to the New South Wales (NSW) economy. Oyster production is the main aquaculture activity in NSW and from 2018-2019, it was calculated to be worth more than $58 million and is increasing in value yearly (NSW Department of Primary Industries 2019). The Sydney Rock Oyster (*Saccostrea glomerata*; SRO) is the native oyster species and contributes more than $53 million production value in NSW. The SRO mass selection breeding program was established by NSW Department of Primary Industries (DPI) in 1990 in response to a 60% production decline after the mid-1970s caused by disease, water quality declines and competition from the faster growing Pacific Oyster. Mass selection methods successfully developed oyster lines with superior growth and resistance to QX disease as well as reducing the impacts caused by Winter Mortality (WM) disease (Nell 2006; Dove et al. 2013a; Dove et al. 2013b).

The production of triploids has become increasingly important to the oyster industry worldwide. Triploid oysters have superior growth and can be marketed outside the normal windows of sale compared to diploid counterparts which can be used to extend the season of oyster sales (Allen et al. 1988). NSW DPI investigated triploid and tetraploidy induction of SROs in the 1990s. SRO triploids were produced using cytochalasin B (CB) after 50% of eggs had released the first polar body post fertilisation (Nell et al. 1996). CB was more effective than 6-dimethylaminopurine (6-DMAP) as it resulted in higher levels of survival in larvae and triploidy percentage in the treated batches post settlement (Nell et al. 1996). Tetraploidy induction in SROs through blocking of both polar bodies in diploid eggs fertilised with diploid sperm and blocking polar body 1 in eggs from triploid oysters fertilised with sperm from diploids was investigated (Nell et al. 1998).

This research was unsuccessful due to low numbers of tetraploid larvae surviving through to metamorphosis and no tetraploids found in batches where metamorphosis and settlement did occur (Nell et al. 1998). However, chemically induced SRO triploid batches were distributed to oyster growers for commercial performance evaluations. Grower feedback was positive and results clearly showed that SRO triploids grew faster than diploid oysters (Hand et al. 1998a) and had significantly lower mortality (12%) compared to diploids (35%) when exposed to WM disease (Hand et al. 1998b). Furthermore, the growth improvements from mass selection breeding were additive to the growth advantage offered by triploids (Hand et al. 2004).

During the mass selection program the trade-off for superior growth and disease resistance was reduced meat condition affecting oyster marketability (Dove and O'Connor 2012). Mass selection did not enable selection for other commercially important traits in combination with disease resistance and faster growth. Additionally, mass selection over six generations caused a substantial loss of genetic diversity in selected lines to the extent that this method of breeding is not a viable long term option (In et al. 2016). Consequently, family-based breeding was recommended for the SRO BP by two separate reviews in 2002 and 2012 (Benzie et al. 2002; Rye 2012). The primary reasons to change from mass selection methods to a family-based model were: improved selection methods, better estimates of genetic gains and future improvement as well as control over inbreeding.

In 2014, annual family production commenced at the Port Stephens Fisheries Institute (PSFI), NSW DPI to enable multi-trait selection. Founder families were created from the mass selection lines and wild oysters selected for shell shape. In 2016, a Fisheries Research and Development Corporation (FRDC) funded project (2015-230) commenced developing operational and industry-focused family based breeding, with the goal to allow industry to access oysters with superior disease resistance, growth and meat condition (SOCo et al. 2019). A subsequent related Future Oysters CRC-P project (Dove et al. 2020) focussed on increasing genetic resistance of SRO families to disease. This was

done by doubling the numbers of families each breeding run and reducing the breeding cycle for QX disease (Dove et al. 2020).

Hatchery production of SROs is a costly and high risk activity for the breeding program and industry exacerbated by factors such as: reliance on hatchery conditioning, low fertilisation success using strip-spawned gametes, extended larval rearing period compared to Pacific Oysters, and variable settlement rates. More effective hatchery conditioning that reduces the 10-week period increases spawning success and reduces costs to the breeding program. Additionally, methods that increase fertilisation success using strip-spawned gametes or enable naturally released gametes to be used instead of strip-spawned gametes would significantly increase family success rate and yields from each family produced each breeding run. Fertilisation using naturally spawned SRO gametes results in high development rates (>90%) and low levels of larval deformities compared to strip-spawning where only 27% of the matings performed using strip-spawned gametes produce a single pair-mated family (Dove et al. 2020). Increasing the success of family matings is the highest priority for research for the SRO BP.

In molluscs, reproduction, including gametogenesis and sexual maturation, is regulated by NPs (Geraerts et al. 1991). In SROs, the effects of those NPs on gonad conditioning and spawning have been investigated (In et al. 2016), showing that buccalin and APGWamide are the most potent NPs that help stimulate gonad conditioning. In natural conditions, these peptides are produced and function in a cocktail form – a mixture of different bioactive peptides. The current study aimed to investigate the effect of administering multiple forms of buccalin and APGWamide in assays to observe stimulation of gonad conditioning in SRO. In addition to this, experiments investigated different methods that can be used to effectively deliver peptides to SROs. Knowing the optimal form of these NPs and the best method to deliver the NPs to oysters would provide a more effective strategy towards implementation into large-scale breeding programs for SRO.

Several factors are known to be involved with spawning activity in SROs, including environmental factors (e.g., water temperature, tides and currents) and pheromones (Bernard et al. 2016). Recently, a spawning inducing factor/pheromone was found in the sperm of the pearl oyster, *Pinctada maxima*, which successfully encourages natural gamete release (Taylor et al. 2018). In this study, we aim to identify the spawning inducing pheromone(s)/factors in SRO sperm. This is of particular interest to the SRO BP to: reduce the number of broodstock sacrificed during breeding runs; increase the success rate of each cross by using naturally released gametes; and, use high performing individual oysters for crosses in subsequent generations.

This Future Oysters CRC-P project was developed through discussions with the hatchery sector and was designed to target specific hatchery production challenges. The four areas of research and development covered in this project were tetraploid induction of SROs, improving hatchery conditioning methods as well as investigation of a spawn inducing factor for SROs and a benchtop storage media to extend SRI oocyte viability.

This project's aims were to decrease hatchery operation costs, increase hatchery production reliability and increase the market for hatchery produced seed by focusing on the highest priority for the further development of the SRO BP, that is increasing the success rate of single pair-mated crosses. This was addressed by research targeted at: production of tetraploid SROs, production of a spawn inducing factor and improved oocyte viability.

## Need

Given the need to reliably produce families annually that are resistant to disease and other environmental threats and the economic benefits that derive from improved seed, it is important that oyster breeding is optimised. Commercial hatchery production of SROs is more difficult compared to other species such as Pacific Oysters and Eastern Oysters (*Crassostrea virginica*). More challenges

are encountered when producing single pair mated SRO families using strip-spawned gametes due to fertilisation deficiency in certain crosses, low rates of success when fertilisation occurs and low levels of larval development in the 24 h period post fertilisation. This compares poorly with very high fertilisation and development rates in the initial 24 h period when natural SRO gametes are used.

A related Future Oysters CRC-P (2016-802) study aimed to accelerate genetic gains for key traits in the SRO BP. One strategy employed by this project to increase gains was to produce more families in each annual breeding run. This project was very successful in doubling the numbers of SRO families that can be produced in a single breeding run. However, to achieve 60-80 families in a single breeding run large numbers of valuable broodstock were sacrificed. This is due to only 27% of single pair mated crosses successfully producing a family that can be incorporated into the SRO BP. Additionally, broodstock available for a breeding run is limited due to all oysters used requiring hatchery conditioning because breeding runs are performed out-of-season.

The ability to store gametes for a couple of days means that fertilisations that fail in the early stages of development (< 24 h) can be quickly repeated without sacrificing additional broodstock. Reducing the length of time and increasing the reliability of the hatchery conditioning process means that oysters are in a better reproductive state at the time of spawning increasing the family success rate. Improved understanding of SRO gamete quality, optimal fertilisation conditions and embryonic development from strip-spawned individuals will increase family production success.

Enhancement of SRO breeding is achieved through numerous stepwise improvements to protocols, facilities, husbandry, and staff training. Methods that enable the use of naturally released gametes as opposed to strip-spawned gametes increases fertilisation success rates and larval yield as well as conserves valuable broodstock. Additionally, benchtop storage of oocytes can increase their viability and reduce the number of broodstock needed for a breeding run. This serves to increases the reliability of breeding runs which safeguards the SRO BP.

Since the original tetraploid SRO work done by NSW DPI in 1990s, significant improvements have been made to hatchery techniques for SROs to facilitate reliable commercial production (O'Connor et al. 2008) and to pave the way for a single pair-mated breeding program (Dove et al. 2019). Tetraploids produced from disease-resistant SRO families is new research that, if successful will advance the performance of selected SROs for industry profitability. Tetraploids for industry allow reliable triploid SRO hatchery production without direct application of harmful chemicals that can cause consumer backlash as well as increase on-farm growth performance and marketing opportunities for SRO growers.

# Objectives

Access to triploid SRO provides industry with stock that has marketable attributes when diploid oysters are not in season as well as faster growth rates and improved resistance to WM disease. This project endeavours to produce tetraploids from QX disease-resistant SRO families for industry. The tetraploid induction method will utilise triploid oocytes. Therefore the first step for this research is production of triploid SROs using chemical induction methods. Once fecund triploid SROs can be sourced a series of tetraploid inductions can be performed in order to obtain tetraploid stock.

Hatchery production of SROs is more costly compared to Pacific Oyster hatchery production due to different methods, a longer larval cycle and slower growth rates of spat. The optimal time for consignments of spat to go to oyster growers is during early spring to maximise growth over the ensuing period of warm water temperatures. This reduces the overall time to market for SRO seed. Spring hatchery production of SROs requires hatchery conditioning of broodstock which takes approximately 10 weeks. The SRO BP also undertakes annual breeding runs in spring (October), before SROs have attained their reproductive peak, and relies on hatchery conditioning to do this. This increases the costs of hatchery runs for both breeding and commercial spat production. Reducing the period of hatchery conditioning will reduce overall costs of early season production of SROs.

Methods to increase the fertilisation success in SROs will reduce hatchery costs further. SRO broodstock obtained from the estuary in peak reproductive condition have a strip-spawning success rate of less than 40%. When strip-spawning is performed out-of-season the success rate is reduced to approximately 27% after hatchery conditioning has been used to prepare broodstock for reproduction. Attaining sufficient numbers of families for a breeding run (> 50) takes approximately 15 days of strip-spawnings and sacrifices large numbers of valuable and limited broodstock. Improving spawning success to attain single pair mated crosses will have time and cost savings for the SRO BP as well as hatchery operators, and at the same time improve BP reliability through increased yields in families. This can be achieved through the development of reliable methods that:

- improves broodstock conditioning effectiveness
- induces a single oyster to naturally release gametes into seawater, and
- enable preservation of gametes from broodstock without the complicated freezing and thawing steps needed for cryopreservation.

Given the need to reliably produce families annually that are resistant to disease and other environmental threats and the economic benefits that derive from hatchery produced selected seed, it is important that oyster breeding is optimised.

The major objectives of this project were:

1. 20% of industry with access to triploid SRO;
2. to reduce complete hatchery operation costs by 15% through a reduction in time for oyster conditioning; and
3. increase the SRO BP reliability through improved conditioning methods and increased fertilisation success rates.

The following four project aims were formulated from these major objectives and form the four components of research for this project:

1. develop optimised protocols for tetraploidy SRO production and produce a batch of tetraploids for commercial production of triploid SROs (Section 1).

2. reduce the overall hatchery conditioning period for SROs using neuropeptides (Section 2).

3. investigate methods to induce natural release of gametes for SROs to increase fertilisation success rates (Section 3).

4. develop benchtop storage media to hold SRO gametes during breeding run spawnings so that broodstock aren't wasted and important single pair crosses can be redone in the case that the initial attempt was not successful (Section 4).

# 1. Optimised protocols for tetraploid SRO production

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## 1.1 Introduction

The production of triploids has become increasingly important to the oyster industry worldwide. Triploid oysters have superior growth and can be marketed outside the normal windows of sale compared to diploid counterparts which can be used to extend the season of oyster sales (Allen et al. 1988). Assessment of mated Pacific Oyster triploids (ie. tetraploid x diploid cross) compared with direct chemical induced triploids (CI triploids) indicated mated triploids outgrew CI triploids by 14% whole wet weight gain and 11% shell height increase (Wadsworth et al. 2019).

NSW DPI investigated triploid and tetraploidy induction of SROs in the 1990s. This research found that SRO triploids grow faster than diploid oysters in whole wet weight and shell height increase (Hand et al. 1998a). Additionally, triploid SROs in four estuaries affected by winter mortality (Pambula Lake, Georges River, Brisbane Water and Port Stephens) had significantly lower mortality (12%) compared to diploids (35%) (Hand et al. 1998b). Triploid growth trials also showed that the growth improvements from mass selection breeding were additive to the growth advantage offered by triploids (Hand et al. 2004). The growth, marketing and WM disease resistance advantages offered by triploid SROs have the potential to improve productivity of oyster businesses using triploid stock.

The SRO triploids were produced using cytochalasin B (CB) after 50% of eggs had released the first polar body post fertilisation (Nell et al. 1996). CB was more effective than 6-dimethylaminopurine (6-DMAP) as it resulted in higher levels of survival in larvae and triploidy percentage in the treated batches post-settlement (Nell et al. 1996). Tetraploidy induction in SROs through blocking of both polar bodies in diploid eggs fertilised with diploid sperm and blocking polar body 1 in eggs from triploid oysters fertilised with sperm from diploids was investigated (Nell et al. 1998). This research was unsuccessful due to low numbers of tetraploid larvae surviving through to metamorphosis and no tetraploids found in batches where metamorphosis and settlement did occur (Nell et al. 1998).

Presently, there are two main techniques used for the production of triploid oysters, direct chemical induction (CB) or 6-dimethylaminopurine (6-DMAP) to block either meiosis I or II by inhibiting polar body extrusion (Stanley et al. 1984; Desrosiers et al. 1993) or mated triploid production involving crossing a tetraploid male with a diploid female. Production of tetraploid oysters involves finding a fertile triploid female oyster, strip-spawning the oocytes, fertilising the oocytes with a diploid male and blocking expulsion of polar body I using CB or 6-DMAP (Gou and Allen 1994; Gou et al. 1996). Pertinent to the SRO BP is that selectively bred diploid oysters can be crossed directly with tetraploid (with the use of CB) to produce more tetraploid oysters incorporating the desirable genetic traits without the necessity of triploid production step (McCombie et. al. 2005). Although, Allen (2012) states that there are significant issues to overcome with this method especially outside of application to Pacific Oysters.

Since the time that the original tetraploid SRO work was done in 1990s, significant improvements have been made to hatchery techniques for SROs to facilitate reliable commercial production (O'Connor et al. 2008) and to pave the way for a single pair-mated breeding program (Dove et al. 2019). Tetraploids produced from disease-resistant SRO families is new research that, if successful will further improve the performance of selected stock for industry profitability. This chapter contains the series of experiments to attempt to produce a batch of SRO tetraploids that industry could access for hatchery production of triploid SROs. Selected QX disease-resistant broodstock was used

for these experiments as this is the primary trait under improvement in the SRO BP and the use of genetically similar broodstock has the potential to benefit synchronicity of meiosis development in gametes from different parents.

The process requires production of chemically induced triploids as a source of oocytes to cross with diploid males. Chemical inductions using CB to produce triploid SROs were successful and produced triploid oysters that were available to industry to commercially assess. This provided an opportunity to evaluate growth and survival of triploid SROs in a QX disease affected estuary of NSW. This section describes the method used to induct tetraploidy in SROs and data from a survival and growth trial run in the Hawkesbury River using chemically induced triploid SROs.

## 1.2 Method

### *1.2.1 SRO tetraploid inductions*

The materials and methods used were based on those developed by Guo and Allen (1994) and further improved on by Eudeline et al. (2000). The initial step was producing triploid SROs using the process described in Allen et al. (1989). The broodstock for triploid inductions was sourced from QX disease-resistant families from the SRO BP.

The method for tetraploid induction adopted by Eudeline et al. (2000) involved blocking 1$^{st}$ polar body expulsion of the cross between a female triploid and male diploid oyster. Either CB or 6-DMAP can be used for this process. Timing is critical in both administering and removing the chemical used to inhibit polar body extrusion. Eleven tetraploid inductions of SROs were conducted from March 2017 to March 2019 (Table 1.1) using primarily CB.

A solution of CB dissolved in 100% dimethyl sulfoxide (DMSO) (ie. 10 mg CB in 10 mL of DMSO) was prepared and aliquoted into 1mL volumes and store in the freezer. The final treatment dose for SRO larvae was between 0.5 and 1 mg DMSO $L^{1}$ in fresh filtered seawater (FFSW) (Allen et al. 1989) and 350 µmolar 6-DMAP (molecular weight 163.18) in FFSW (Peachy and Allen 2016) can be used to inhibit polar body extrusion. A small quantity of DMSO (final concentration of a 1 mL $L^{-1}$ (0.1%) solution for rinsing) is required in the rinsing process for CB treatment only.

The following items are required for tetraploid inductions: a knife to open oysters; 20 µm and 100 µm rinsing screens; fresh filtered seawater (equilibrated to 27 to 28°C); scalpel blades (or other implement for assaying gonad tissue); compound light microscope; individual containers to strip gametes into (plastic food containers work well); and a 1mL adjustable micropipette and Sedgwick rafter slide to quantify egg numbers. Two samples of fertilised eggs preserved in a 1.5 mL centrifuge tube are also required to assess for polar body II extrusion.

Suspected triploid oysters are opened and examined for the presence of oocytes. Oysters found to have sufficient oocytes were assessed for ploidy with a Sysmex Partec CyFlow ploidy analyser 11-01-1002 before use. When examining the oysters for gametes and during the strip-spawning process care was taken to ensure no male gametes are inadvertently added to oocytes prior to the treatment process. Once oysters were opened, a small sample of the gonad tissue was taken with a clean scalpel blade to determine the presence of male or female gametes. Male and female oysters were then separated.

Oocytes from individual oysters were stripped using a clean scalpel and a wash bottle filled with FFSW into a plastic container and rinsed through a 100 µm screen to remove large pieces of tissue. The oocytes are then rinsed and retained on a 20 µm screen to remove remaining debris. Further information on the strip-spawning technique for SROs is contained in Dove et al. (2019). Oocytes were examined using light microscopy for evidence of fertilisation before treatment, if any fertilisation had occurred (i.e. polar body extrusion or cell division) the oocytes are discarded. If no fertilisation is evident then oocytes are counted using a micropipette and a Sedgwick rafter slide. The

tetraploid induction process can be carried out on an individual basis if sufficient oocytes are obtained from a single oyster (Eudeline et al. 2000) or pooled if too few oocytes are acquired (Gou and Allen 1994).

Oocytes are then left to water harden in the FFSW for approximately 30 min. Oocytes are again checked for any accidental fertilisation. The oocyte densities should not exceed 50 million $L^{-1}$. During the 30 minute period, males are set aside and checked for sperm motility by placing a small amount of sperm on a microscope slide and adding a drop of FFSW.

The primary aim is to block 1st polar body extrusion in a triploid female fertilised with a diploid male's sperm. Therefore, the timing of application and removal of the chemical (CB or 6-DMAP) is critical. Containers with oocytes are adjusted to 90% of the FFSW final volume. The remaining 10% of the FFSW is added with the chemical to make the final volume and desired treatment concentration (see above for concentration required). The chemical should be pre-prepared so that it can be added in the correct volume to the FFSW containing the oocytes.

The oocytes were fertilised with motile sperm recording the time at which the sperm was added and the mixture then gently stirred. A small sub-sample of the oocytes were then taken to check that sufficient sperm has been added and then the fresh samples of oocytes examined at regular intervals to the time point when the 1st polar body is observed in one or two individuals. When this occurred, a sample of the oocytes was taken and placed in two separate plastic vials, sealed and returned to the vessel containing the oocytes. Once the oocyte sample was taken, the chemical in solution was immediately added and stirred gently, with the time that the chemical was added recorded.

After approximately 10 minutes and then at regular intervals, a small sample was taken from the vials to determine the time point for when the 2nd polar body extrusion starts to occur. The time period to the 2nd polar body extrusion in tetraploid inductions is longer than that observed in normal diploid crosses as per Eudeline et al. (2000). As soon as the first 2nd polar body is observed in an oocyte in the untreated sample vial immediately strain oocytes on a 20 µm screen retaining the seawater for chemical disposal. The treated oocytes are then rinsed back into a volume of FFSW containing 0.1 mL of dimethylsulfoxide (DMSO) $L^{-1}$ for 20 min and then transferred to a larval rearing vessel and resulting larvae were cultured using the techniques documented in O'Connor et al. (2008). If 6-DMAP is used to block polar body I release, oocytes can be rinsed in seawater and placed directly into the standard larval rearing vessel.

### *1.2.2 Hawkesbury River triploid performance evaluation*

Chemical triploid inductions using CB produced a batch of triploids using QX disease-resistant broodstock. These oysters were used for tetraploid inductions and were also distributed to industry for commercial assessment. NSW DPI evaluated the QX disease resistance of this batch by placing these oysters in the Hawkesbury River in July 2018. Oysters were placed at Coba which experiences recurrent outbreaks of QX disease in order to expose QX disease-resistant triploids (QXR3n) to QX disease so that survival and growth rates could be compared to non-selected control oysters (Control) and 2017 year class SRO families (YC2017). The 2017 year class SRO families were a pooled sample of oysters from 2017002, 2017012, 2017025, 2017036, 2017073, 2017074, 2017076 and 2017078 which had an average EBV of 55% for QX survival and 7% for weight. Control oysters were obtained from a hatchery run of non-selected SROs produced by Aquafarms Queensland P/L oyster hatchery and nursery reared by Geoff Diemar in Port Stephens. The average starting whole weight (± SE) of YC2017, QXR3n and Control were 0.2 ±0.001 g, 0.1 ± 0.008 g and 1.6 ± 0.04 g, respectively. Due to the different size and whole weights of oysters at the start of the experiment (Figure 1.1) instantaneous growth rate (IGR) using whole weight data was used to compare growth of YC2017, QXR3n and Control groups. Growth rate based on whole weight (g) was calculated as follows (Askew 1978):

$$IGR = (\ln W_t - \ln W_0)/\text{duration}$$

Where: $W_t$ = final mean weight of oysters, and $W_0$ = initial mean weight of oysters and duration units are days. The experiment ran for 380 days, starting on 16 July 2018 and concluding on 31 July 2019.

A one factor analysis of variance (ANOVA) was used to investigate differences between the oyster type (fixed factor) on condition index data from a subsample of oysters collected in February 2011, March 2011, May 2011 and June 2011. One factor (oyster type) analysis of variance (ANOVA) was used to compare instantaneous growth rate (calculated from whole oyster weight) and mortality data measured in July 2019. Homogeneity of variances was confirmed prior to analyses using Cochran's C test. Where significant differences ($P < 0.05$) were detected, pairwise comparisons of means were conducted using the Tukey HSD multiple comparison procedure. Means shown in the text are mean ± SE.

## 1.3 Results

### *1.3.1 Tetraploid inductions*

The results of the 11 tetraploid induction trials are listed in Table 1.1. All induction attempts failed to produce enough numbers of tetraploid larvae and settled spat. The implications of this were no tetraploid SROs were then available to perform a triploid hatchery run where a male tetraploid is used to fertilise eggs from diploid SROs. Success was achieved in producing chemically-induced triploid SROs using CB. The level of triploidy in these batches exceeded 90% (Figure 1.1). More than three million triploid SROs were produced which were distributed to oyster growers and held for additional tetraploid inductions in the future. These oysters were evaluated in a QX disease affected estuary (Coba, Hawkesbury River) for survival and growth.

### *1.3.2 Hawkesbury River triploid performance*

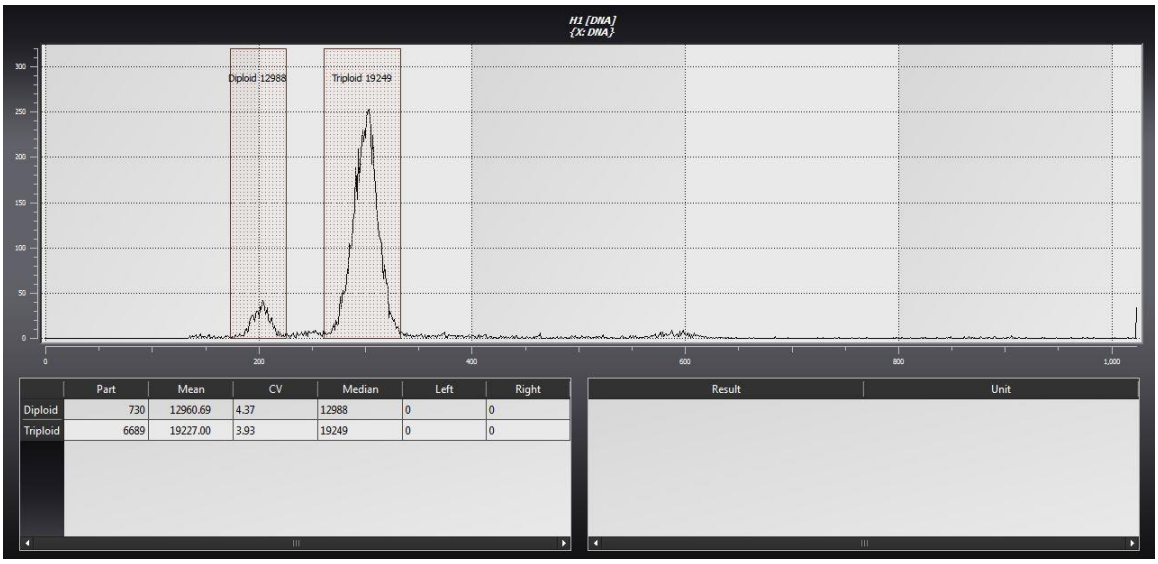
Mortality over the duration of experiment was 7.7 ± 1.8% for YC2017, 7.8 ± 2.2% for QXR3n and 11.9 ± 1.8% for Controls. No QX disease was detected during the experiment and there was no difference in oyster mortality between the three oyster types, YC2017, QXR3n and Control oysters ($F = 1.67$; $df$ 2/7; $P > 0.05$).

At the conclusion of the experiment (31/7/19) YC2017, QXR3n and Control oysters weighed 28.1 ± 2.0 g, 31.8 ± 2.1 g and 32.7 ± 1.9 g, respectively (Figure 1.2). Growth rates of QXR3n, YC2017 and Control oysters are shown in Figure 1.3. A significant difference was detected for IGR ($F = 1401.39$; df 2/7; $P < 0.001$). The growth rate of QXR3n oysters was significantly faster than YC2017 oysters (Figure 1.2). Additionally, the growth rate of YC2017 oysters was significantly greater than Control oysters (Figure 1.3).

**Table 1.1:** Trials to induce tetraploid Sydney Rock Oysters.

| Induction trial | Date | Number of triploid females | Number of diploid males | Result |
|---|---|---|---|---|
| 1 | 22/03/2017 | 2 | 1 | Slow development post fertilisation<br>Low numbers of eggs with polar body extrusion<br>Low recovery of larvae post treatment<br>Larval phase not completed |
| 2 | 16/11/2017 | 3 | 1 | No larvae obtained |
| 3 | 22/11/2017 | 3 | 1 | Slow polar body extrusion<br>Low recovery of larvae post treatment<br>Larval phase not completed |
| 4 | 18/01/2018 | 2 | 1 | Low recovery of larvae post treatment<br>Larval phase not completed |
| 5 | 24/01/2018 | 2 | 1 | Low recovery of larvae post treatment<br>Small numbers of spat settled<br>Spat reared through to adults for tetraploid assays |
| 6 | 6/02/2018 | 2 | 1 | No larvae obtained |
| 7 | 5/02/2019 | 3 | 1 | Low recovery of larvae post treatment<br>Larval phase not completed |
| 8 | 15/02/2019 | 4 | 2 | Low recovery of larvae post treatment<br>Larval phase not completed |
| 9 | 19/02/2019 | 5 | 1 | Low recovery of larvae post treatment<br>Larval phase not completed |
| 10 | 20/02/2019 | 4 | 1 | Low recovery of larvae post treatment<br>Larvae pooled with previous treatment |
| 11 | 28/02/2019 | 5 | 2 | Low recovery of larvae post treatment<br>Larval phase not completed |

**A.**




**B.**

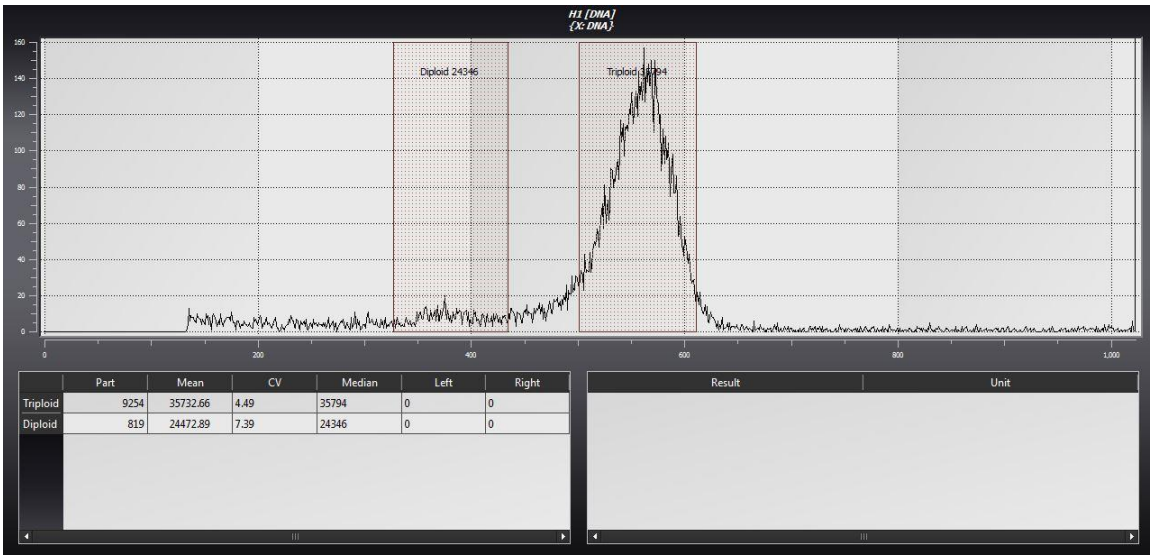



**Figure 1.1:** Flow cytometry results for QXR3n Sydney Rock Oysters with a triploidy level of 90% (A) and for a second commercial triploid batch produced by Southern Cross Shellfish with a triploidy level of 91% (B).

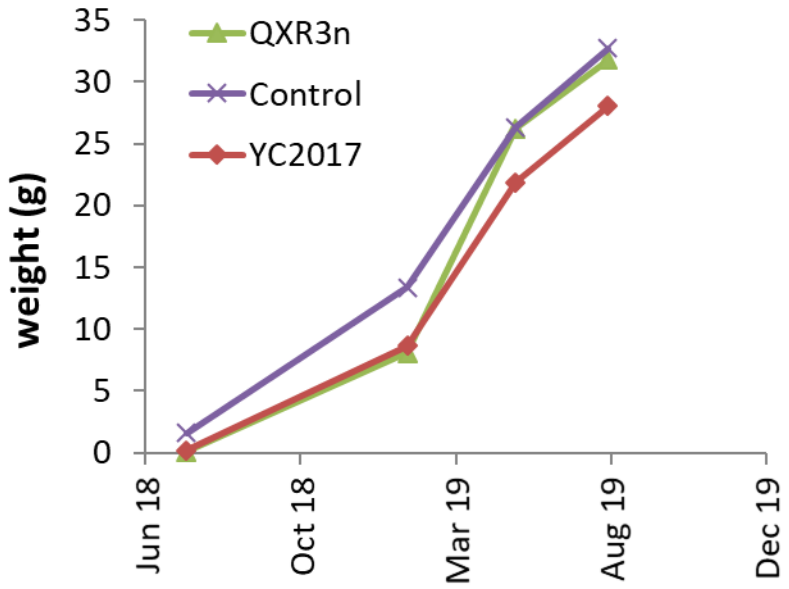



**Figure 1.2:** Whole weight growth of YC2017, QXR3n and Control Sydney Rock Oysters at Coba, Hawkesbury River from 16 July 2018 to 31 July 2019.

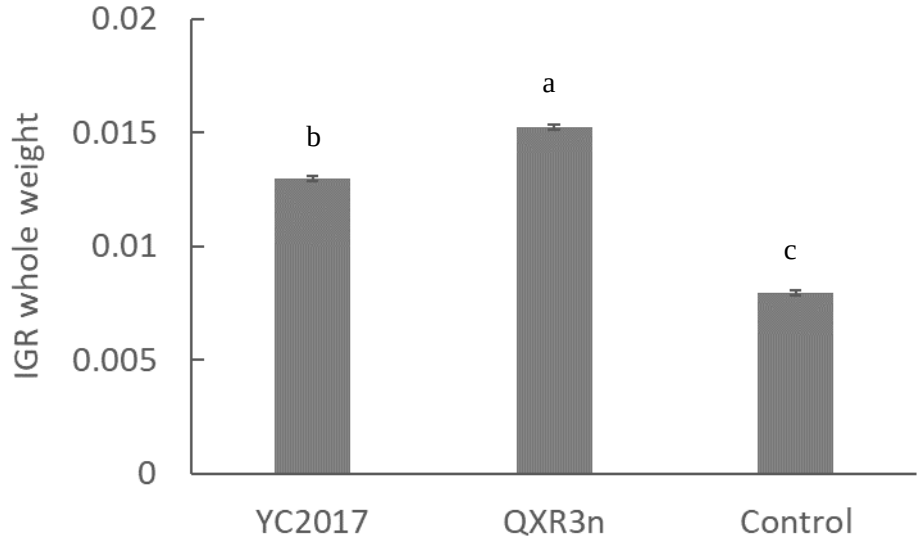



**Figure 1.3:** Instantaneous growth rates of YC2017, QXR3n and Control Sydney Rock Oysters at Coba, Hawkesbury River between 16 July 2018 and 31 July 2019.

## 1.4 Discussion

Tetraploid induction attempts for this project did not successfully produce tetraploid oysters, however, further attempts by Southern Cross Shellfish are planned in the future in the hope of successfully obtaining adult tetraploids. The major challenges have been low egg numbers, asynchronous development in strip-spawned oocytes after fertilisation, poor development and poor larval survival. A very small number of spat were successfully settled from 2 trials however no tetraploids were detected in these batches when oysters had reached a size where tissue could be taken to determine ploidy level.

There has been success in producing chemically-induced triploid SROs using CB. The level of triploidy in these batches was 90% or higher. Over three million triploid SROs were produced and either distributed to oyster growers for commercial growout or held for additional tetraploid inductions in the future. Chemically induced triploids were produced from selected families with high levels of QX disease resistance. Triploid oysters from this batch were evaluated at Coba in the Hawkesbury River by NSW DPI to monitor survival and growth through the QX disease season. No QX disease was detected throughout this experiment. However, the growth rate of the triploid oysters was significantly faster than a group of selected YC2017 families and non-selected oysters.

## 1.5 Conclusion

The tetraploid inductions were not successful in producing a batch of tetraploid oysters that could be made available for hatchery production of triploid SROs using tetraploid male and triploid female crosses. Eleven attempts were made and the major challenges encountered were low egg numbers in triploid SROs, asynchronous embryonic development in strip-spawned oocytes after fertilisation, poor development and poor larval survival. A very small number of spat were successfully settled from 2 trials, however, no tetraploids were found in these batches when oysters had reached a size large enough to assay tissue. Further tetraploid inductions are planned by Southern Cross Shellfish in the 2019/2020 reproductive season. Although the original milestones related to the tetraploid research have not been achieved, there have been some significant outcomes from this Future Oysters CRC-P project with respect to production of triploids and making these available to industry. Successful chemical triploid batches produced for the CRC-P tetraploid work have resulted in the distribution of triploids to industry. NSW DPI used this opportunity to commercially evaluate this triploid stock to measure QX resistance and growth performance. Although no QX disease occurred during field evaluations, triploid oysters had significantly faster growth rates compared to a group of selected YC2017 families and non-selected oysters.

# 2. Neuropeptide induction of sexual maturation in Sydney Rock Oysters

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## 2.1 Introduction

The peak reproductive period for SROs generally occurs from late November through to early April throughout NSW (Dove and O'Connor 2012). Obtaining ripe, ready-to-spawn broodstock outside of this period is a challenge for hatcheries producing SROs. Hatchery reproductive conditioning can be used to perform out-of-season breeding runs but is expensive due to the time and resources required. Broodstock conditioning is routinely used for the SRO BP to reduce the QX disease resistance breeding cycle to one year. Broodstock conditioning is also used by commercial hatcheries to produce seed for industry at a time when increasing estuarine water temperatures in spring provide a significant growth advantage for juvenile SROs reducing the overall time to market.

Hatchery conditioning is a process by which broodstock are held under specific temperature- and food-controlled conditions to promote gametogenesis. SROs generally require 10 weeks of hatchery conditioning to obtain suitable gametes for a successful spawning. Hatchery conditioning requires significant hatchery resources in terms of algae, energy and labour over this period. Reducing the duration of the hatchery conditioning period and improving hatchery conditioning success with respect to producing ready-to-spawn animals reduces the financial impost for SRO hatcheries.

Neuropeptides (NPs) are peptides that are synthesized and released by modified neurons, called neurosecretory cells (Burbach 2011). Their mode of function ranges from being a neurotransmitter, where a NP acts on other neurons, to a neurohormone, where a NP acts on target cells located further away from the NP release site (Burbach 2011). NPs play important roles in neuroendocrine control of many physiological activities, for example, growth, reproduction and stress responses (Burbach 2011; Nässel and Larhammar 2013). In vertebrates, neuroendocrine control of reproduction is well established, and the major reproductive regulatory axis involves the gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus and leads to the release of the gonadotropins from the pituitary.

Mollusc reproduction, including gametogenesis and sexual maturation, is also regulated by NPs (Geraerts et al. 1991) although the regulatory axis used in this phyla has not been established (Pantelouris 1967; Morishita et al. 2010; Byrne et al. 2017);). However, it is very clear that various NPs play a critical role in regulating reproduction in molluscs including the GnRH, buccalin, APGWamide, RFamides and egg-laying hormone (ELH) (Cosmo and Polese 2013).

In the SRO, the effects of those NPs on gonad conditioning and spawning have been investigated (In et al. 2016), showing that buccalin and APGWamide are the most potent NPs that help stimulate gonad conditioning. Multiple forms of buccalin and APGWamide, with minor variations in their amino acid sequence, are derived from a single precursor protein. The multiple forms were combined for the assays, yet it is likely that one specific form is more potent. Therefore, the current study aimed to investigate the effect of administering the individual forms of buccalin and APGWamide in assays to observe stimulation of gonad conditioning in SRO. In addition to this, experiments investigated

different methods that can be used to effectively deliver peptides to SROs. Knowing the optimal form of these NPs and the best method to deliver the NPs to oysters will provide a more effective strategy towards implementation into large-scale breeding programs for SRO.

Three gonad maturation assays were performed for this project. Iterative changes were made to the method for each subsequent assay based on the results from previous experiments including In et al. (2016). The following sections describe methods and results for gonad maturation assay I, II and III. The outcomes of all gonad maturation experiments are discussed and summarized in the conclusion at the end of this section.

## 2.2 Method: Gonad Maturation Assay I

### *2.2.1 Gonad conditioning assay*

Male and female SROs were injected with APGW and buccalin peptides (50 µg injection$^{-1}$) at day 0 and 10 (Figure 2.1A). Control group oysters were injected with sterile water without any peptide. The development of the gonad was then observed every 10 days after first injection. Hence, five oyster gonads were sampled at days 10, 20 and 30, for gene expression (stored at -80 °C) and histology (fixed in 4% paraformaldehyde overnight and subsequently processed through a tissue processor). Upon arrival, oysters were cleaned of fouling organisms and whole weight, shell weight and wet tissue weight were measured. The weight data were used to calculate wet weight condition index (CI) according to the formula: [wet tissue weight / (whole weight – shell weight)] x 100 (Lawrence and Scott 1982).

### *2.2.2 Spawning assay and Fertilisation efficiency test*

At day 30 post-injection, all oysters were collected and transported from PSFI, NSW DPI to the USC, Queensland. Upon arrival, ten oysters from each group were anesthetized by immersion in $MgCl_2$ (50g $L^{-1}$) for 3 h and then sampled. Oysters were injected with either distilled water (control), 40 ug APGW, or 40 ug buccalin (this concentration was used according to In et al. (2016). Each individual was then kept in a 250 mL container filled with seawater (33 ppt, 22 °C, fan ventilated) (Figure 2.1B and Figure 2.2). The spawning activity was observed every 1 h until 6 h post-injection, and then at 24 h post-injection. At day 45 post-injection, a fertilisation efficiency test was performed by using sperm stripped from a mature male oyster for fertilisation with oocytes obtained from injected female oysters (Figure 2.1B and Figure 2.3).

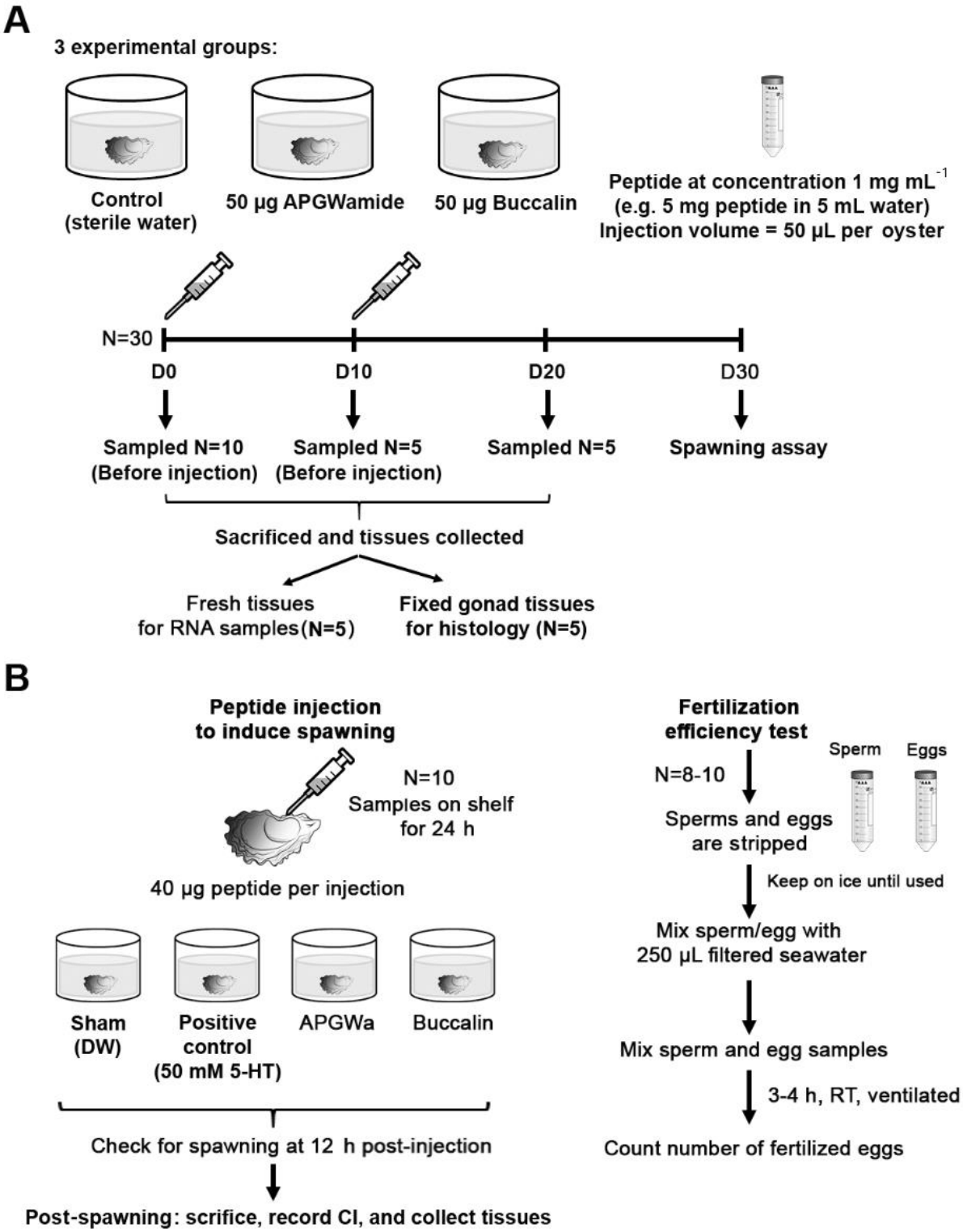


**Figure 2.1:** Experimental design for gonad conditioning and spawning assays. (A) Experimental design for gonad conditioning. (B) Experimental design for spawning assay and fertilisation efficiency test.

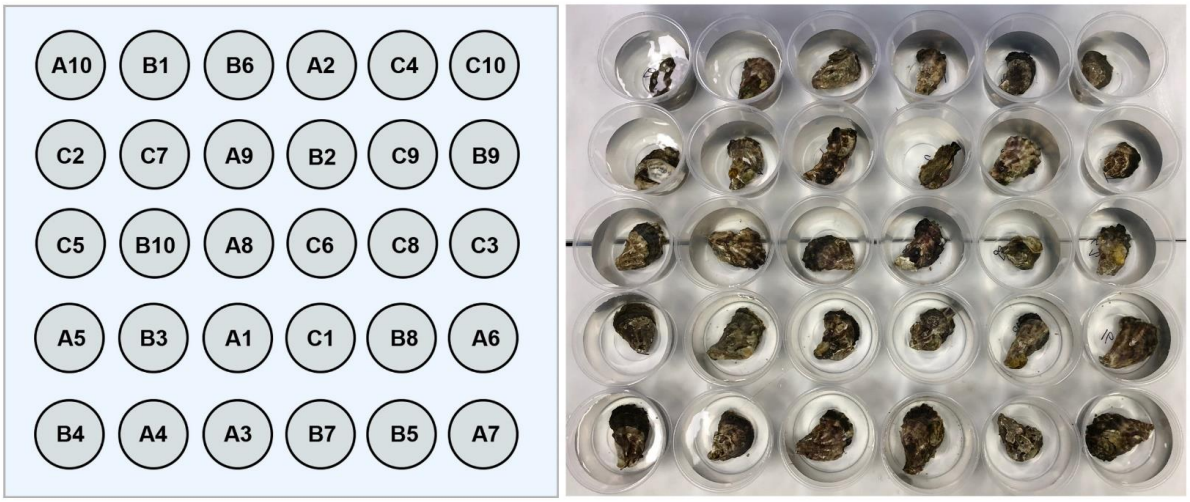


**Figure 2.2:** Experimental setup of spawning assay. Oysters from different treatments were kept individually within the spawning chamber containing 250 mL seawater and placed randomly before the injection was performed. A1-A10, APGW-treated oysters; B1-B10, buccalin-treated oysters; C1-C10, sterile water-injected oysters (negative controls).

For sperm preparation, sperm with high motility from mature male(s) was selected for fertilisation with eggs obtained from treated females. To prepare the sperm, male oysters showing fully developed testis (occupying ~70% of shell cavity), with white creamy colour and visible veining appearance (O'Connor et al. 2008), were sacrificed. Sperm was stripped using a scalpel and/or a pipette, and then transferred to a sterile microtube, which was kept on ice. Five microliters of sperm sample were taken and observed under light microscope (40X and 100X) to check motility. Sperm samples with high motility from different males were pooled to a final 1-2 mL volume and kept on ice until used. For egg preparation, eggs were stripped from each female, transferred to a sterile microtube, and kept on ice until use. Two microliters of egg sample were taken and observed under light microscope (40X and 100X) to check maturity. Only mature eggs (> 40 µm) were used for the fertilisation test. Before fertilisation, sperm was mixed with FSW to a dilution of 1:250 [sperm : FSW (V/V)] to provide a density of $10^5$ sperm $mL^{-1}$. Sperm was incubated at room temperature (RT) for 5-10 min to activate motility. Sperm samples that showed relatively low motility could be activated by incubation in alkaline FSW [pH of FSW can be adjusted by adding ammonium chloride ($NH_4Cl$)], at room temperature - this was used for fertilisation when necessary. Motility after activation was observed under light microscope to ensure sperm quality before use. Mature eggs were suspended in FSW at the density of 25,000 oocytes $mL^{-1}$. To allow fertilisation, the same volume of sperm and eggs (1 mL) were transferred into a 24-well plate and mixed thoroughly. The mixture in each well was observed under light microscope in order to check the egg and sperm ratio, which should be at 1:4 (egg : sperm). A 24-well plate containing eggs and sperm mixture was kept at RT and ventilated by electric fan. Fertilized eggs were determined by the presence of the first polar body, which should occur at 15-20 min post-fertilisation, and the cell division of embryos at 1-3 h post-fertilisation (unfertilized eggs tend to be degraded or partially damaged or broken). Fertilized eggs and embryos were counted at 3 h post-fertilisation for 3-6 oysters per treatment, including control, APGWa, and buccalin. The workflow of fertilisation efficiency test is summarized in Figure 2.3.

Fertilisation efficiency for each individual female was performed in duplicate for each egg preparation stripped from an individual female (Figure 2.4). The eggs were fertilized with sperm pooled from three mature testis with high sperm motility. Observations were performed under light microscope at 15 min and 15 h post-fertilisation.

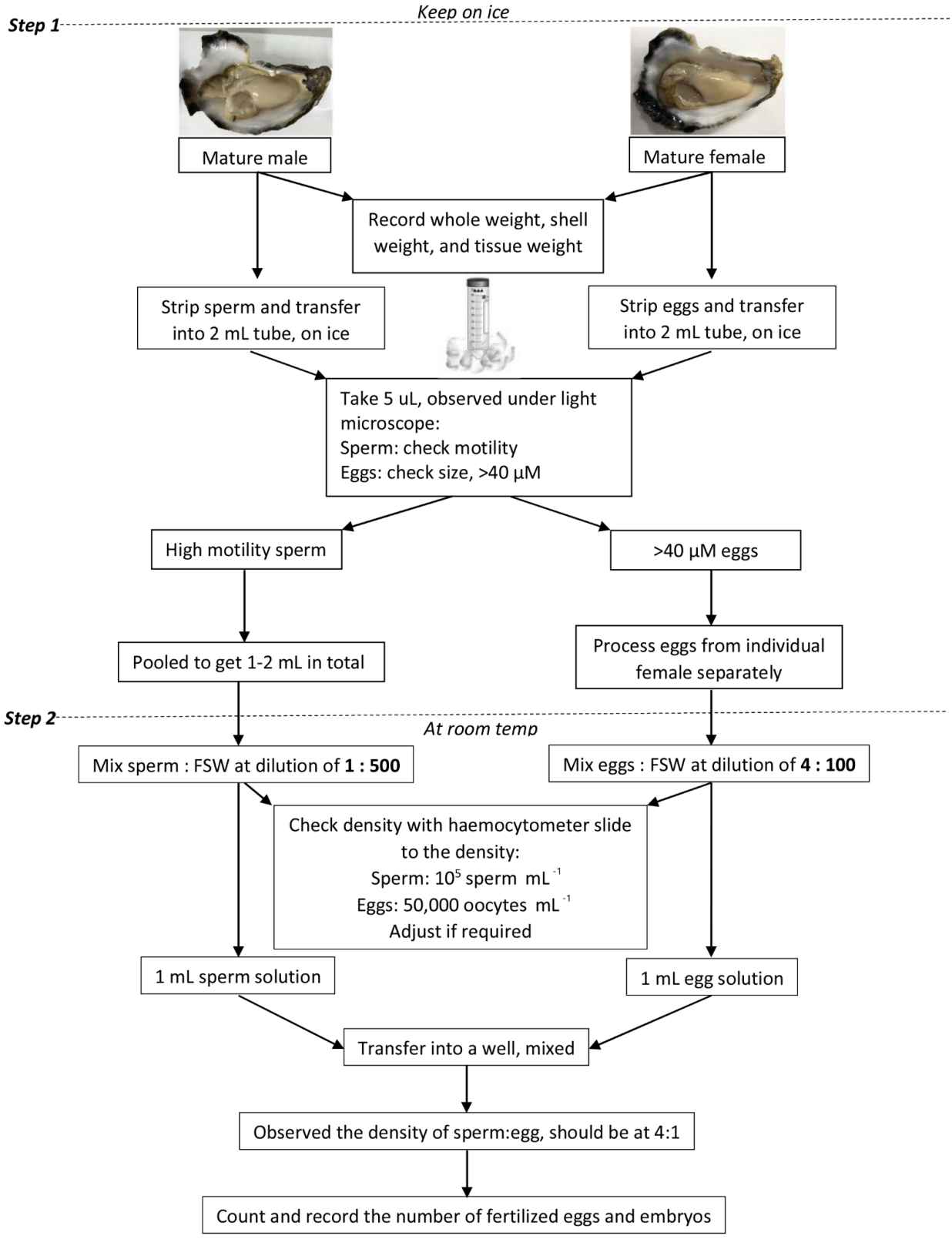


**Figure 2.3:** Workflow summary of fertilisation efficiency test.

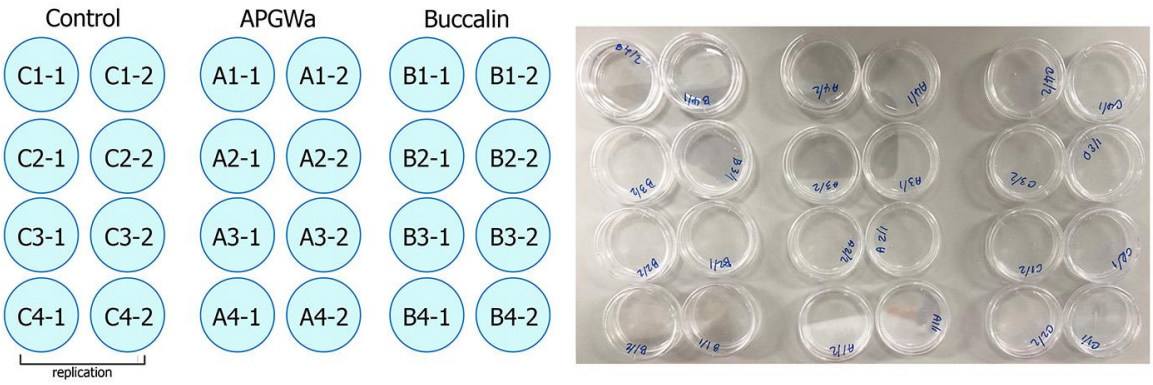


**Figure 2.4:** Experimental setup for fertilisation efficiency. Eggs stripped from four females per treatment, including control (C), APGWamide (A), and buccalin (B) were separately placed and mixed with sperm suspension within a cell-culture dish.

## 2.3 Results and Discussion: Gonad Maturation Assay I

### *2.3.1 Sex ratio and condition indices*

At day 0, 10 oysters from each group were sacrificed and assessed for whole body weight, shell weight, soft tissue weight, and sex. The sex ratio (female : male) was 40%:60% in all groups. At day 10, the sex ratio within the control group was 60%:40%, whereas in APGW- and buccalin-treated groups were 80%:20% and 40%:60%, respectively (Figure 2.5). At day 20 post-injection, the sex ratios within the control and APGW-treated groups were 80%:20%, whereas the sex ratio was at 60%:40% within the buccalin-treated group. At day 30, the sex ratios were 50%:50% in the control and buccalin-treated groups and 60%:40% in the APGW-treated group (Figure 2.5).

CI of gonads were recorded at day 0, 10, 20, and 30 post-peptide injection. At day 0, the CI in all groups were not significantly different. At day 10, we found that the CI within the APGW-treated group had increased to 517.11± 63.06 when compared to the CI at day 0 (475.13±54.63), and this was significantly higher than the CI of the control group (Figure 2.6). However, the CI in APGW-treated group at days 20 and 30 post-peptide injection were not significantly different to those of the control group. Meanwhile, the CI in buccalin-treated group at day 10, 20 and 30 were similar to those of control group. The appearance of representative oysters in each treatment at day 30 post-peptide injection is shown in Figure 2.7. Therefore, we concluded that administration of APGW could slightly induce gonad maturation at day 10 post-peptide injection (after receiving 1 injection of APGW at day 0), while the treatment of buccalin did not show any effect on gonad maturation. This is in contrast to the previous study by our group (In et al. 2016) in which the stimulatory effects of APGW and buccalin on gonad maturation were pronounced. There are factors that were potentially different between the current and previous study, including the peptide delivery (i.e. peptide pellet implantation versus peptide injection) and the reproductive stage of oysters when the bioassay started. Also, in the current study there were two peptide injections (at days 0 and 10), which may not be sufficient to induce full gonad development. Hence, increasing the number of peptide injections (or dose) should be considered in a future study.

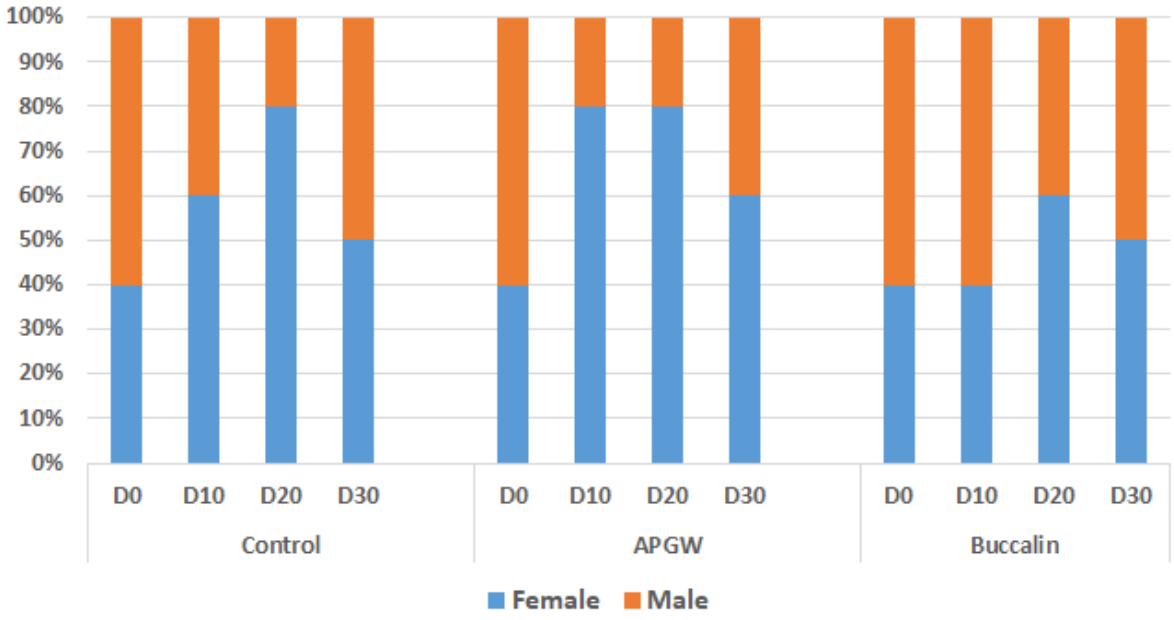


**Figure 2.5:** Sex ratio of experimental groups. The histogram shows the sex ratio between male and female oysters in different treatments, including control, APGW-treated, and buccalin-treated groups, at days 0, 10, 20, and 30, post-injection.

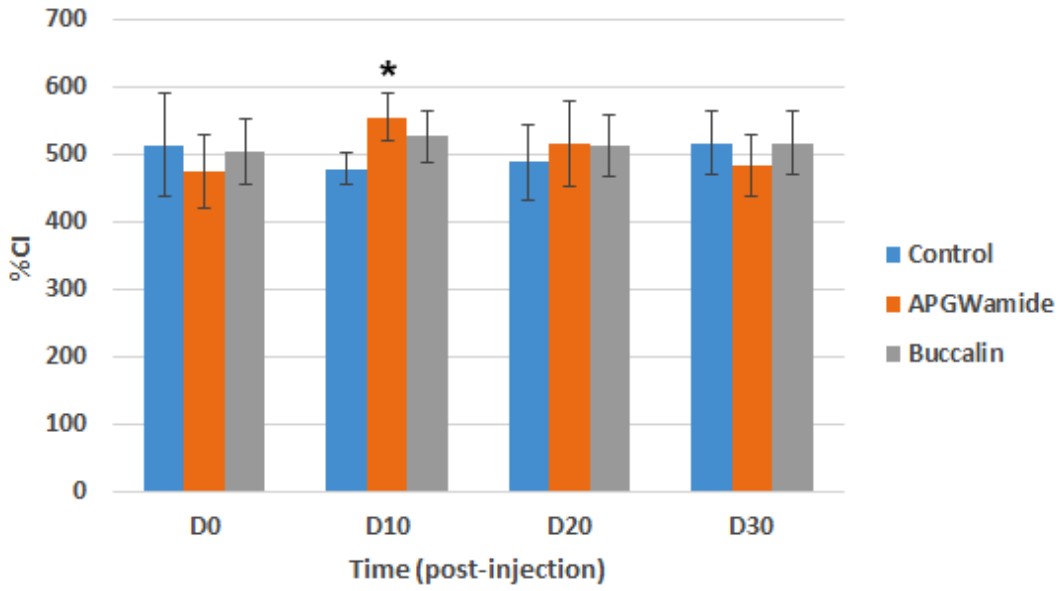


**Figure 2.6:** Condition index. The histogram shows condition index of Sydney Rock Oysters at various days in different treatments. Asterisk indicates a statistical difference to the control group within the same day of collection ($p \leq 0.05$).

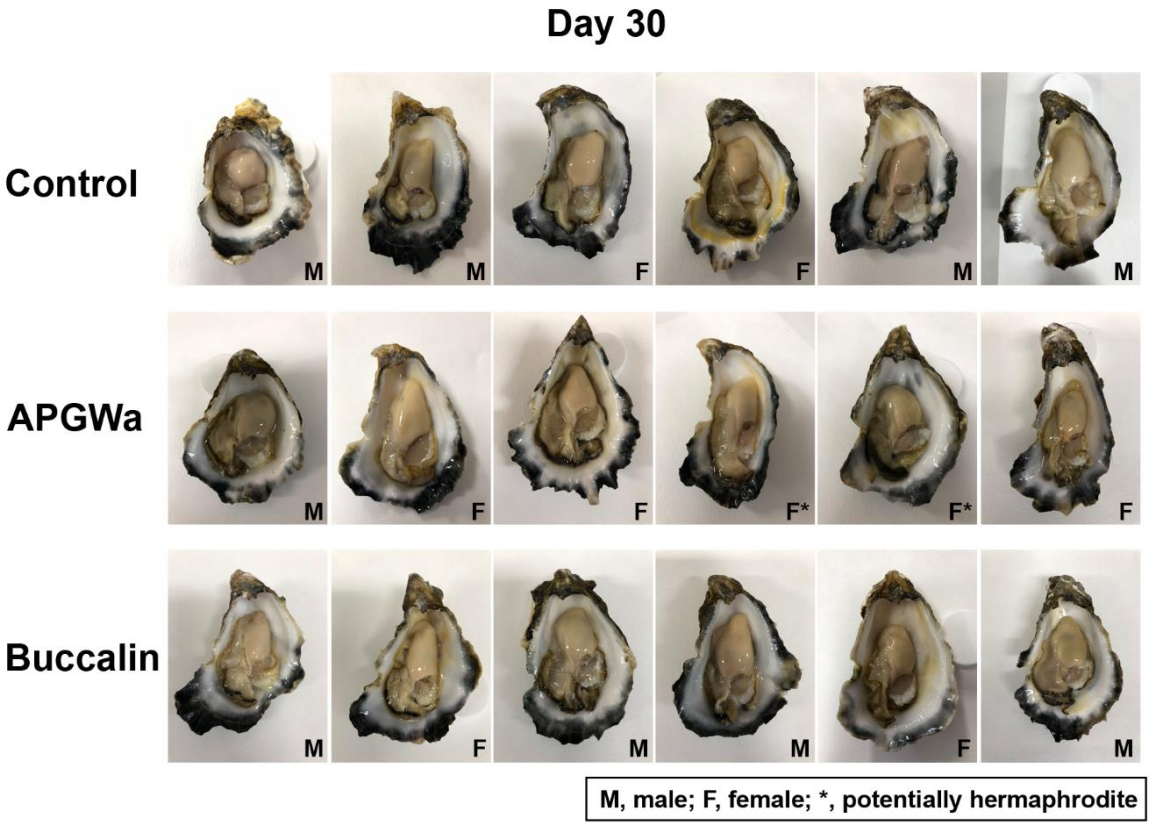


**Figure 2.7:** Appearance of the oyster gonads at day 30 post-peptide injection.

### *2.3.2 Spawning assay and Fertilisation efficiency test*

Oysters from Bioassay I were used for Bioassay II. No spawning was observed within 24 h post-peptide injection for any treatment. Following an additional peptide injection, there was again no induced spawning in any treatment. Therefore, we concluded that the oysters in all groups did not reach the fully mature stage at day 30 post-peptide injection, and hence the spawning could not be induced by APGW and buccalin injections.

At day 45 of Bioassay I, 8-10 oysters per treatment were sacrificed for gamete collection and their CI recorded. Three sperm samples (one from each treatment, average CI was 562.33 ± 85.76; Figure 2.8 histogram) showed high motility after incubation in FSW for at least 3 min at RT. The sperm samples were then pooled before use. Mature oocytes with diameter > 40 µm were stripped from four females per treatment. The average CI of females were not different between control (495.22 ± 39), APGW (512.02 ± 129.74), and buccalin (465.96 ± 33.60) treatments. After combining egg and sperm suspensions, sperm showed movement towards eggs. However, no 1$^{st}$ polar body could be observed at 15 min after egg-sperm contact. Possible development of embryos was then examined at 15 h post-fertilisation. Figure 2.8 shows the result of when 5 µL of mixture was taken at 15 h post-fertilisation and observed under light microscope. From the results, no fertilisation had occurred in any group. Hence, we concluded that the eggs from females in all groups were not fully mature and ready for fertilisation at day 45 post-peptide injection.

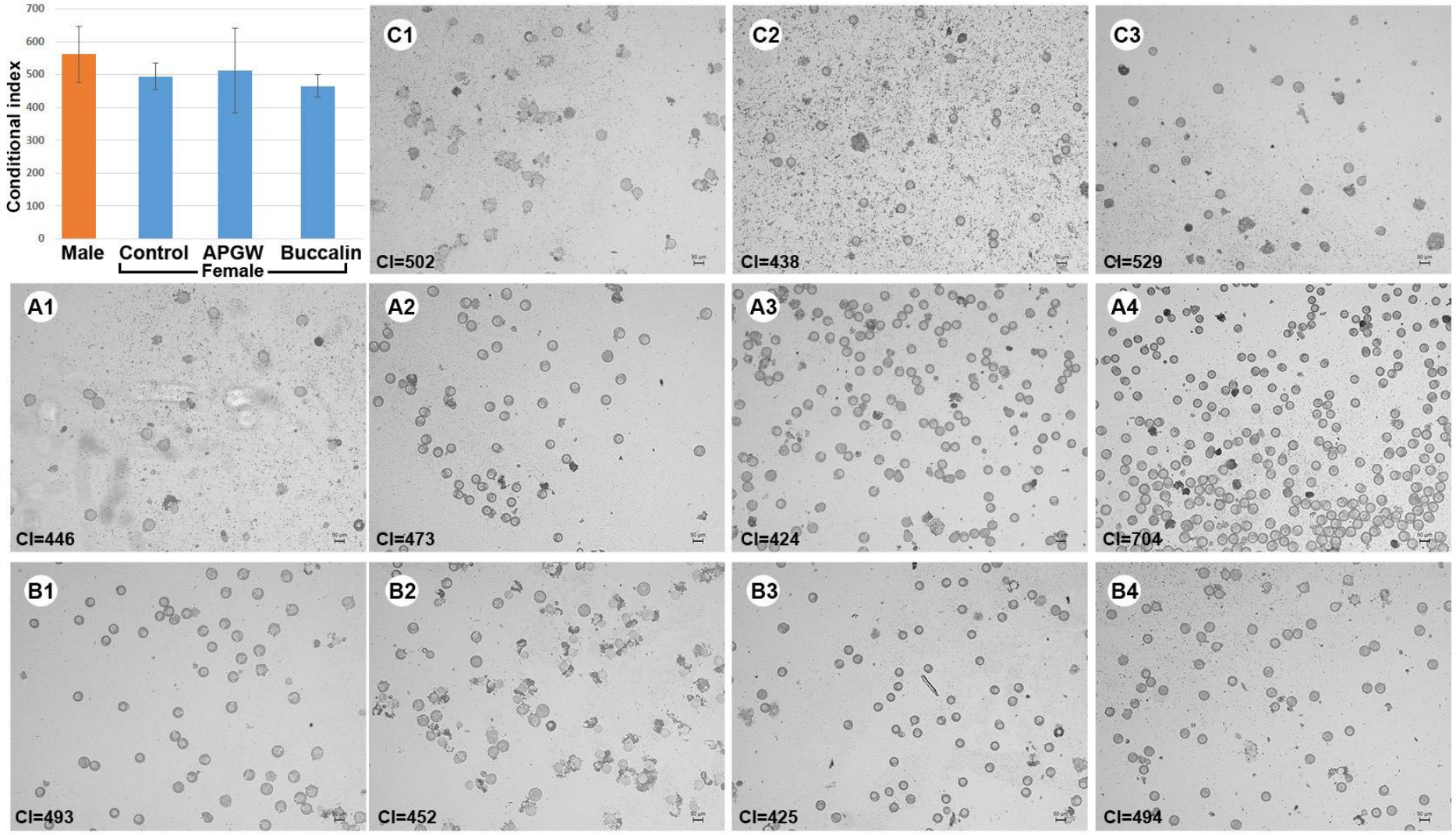
Conditional index
700
600
500
400
300
200
100
0
Male
Control
APGW
Buccalin
Female
C1
CI=502
C2
CI=438
C3
CI=529
A1
CI=446
A2
CI=473
A3
CI=424
A4
CI=704
B1
CI=493
B2
CI=452
B3
CI=425
B4
CI=494

**Figure 2.8 (previous page):** Eggs post attempted fertilisation after 15 h. The histogram shows the average condition index (CI) of males (sperm donors) and females in various treatments. Control (C) 1-C3 are the eggs obtained from female oysters in the control group. APGW (A) 1-A4 and Buccalin (B) 1-B4 are the eggs obtained from female oysters in the APGWamide and buccalin treatments, respectively. The CI for each female from which the eggs were stripped is also provided (*bottom left of the panel*). (*Magnification: 50X*)

## 2.4 Method: Gonad Maturation Assay II

In assay I, we found a positive effect of the NPs APGWamide and buccalin on gonad conditioning following 2 injections, yet further weekly injections did not advance conditioning beyond controls. This suggested that the concentration of peptides administered might not be optimal. It is also noteworthy that injections provide a short burst of peptide activity, rather than a sustained, slow release into the hemolymph. We hypothesized that a sustained concentration of NP in the hemolymph might be critical to optimally stimulate gonad maturation in the SRO. Hence, the injection of cocoa butter-NP mixture, which has been shown to allow slow release (Nagasawa et al. 2015), was implemented.

Oysters with regressive stage of gonads were used for experimental tests to assess gonad conditioning following administration of various reproductive-related peptides (Table 2.1). Oysters were transferred to the QX hatchery, PSFI, NSW DPI. Oyster shells were filed at an area of close proximity to the adductor muscle, in order to provide an open site for peptide injection. Oysters were then acclimatized for 2 weeks prior to peptide injection. For peptide delivery, a cocoa butter-NP mixture was used to allow for the sustained release of peptide into the animal's circulatory system (Nagasawa et al. 2015). For cocoa butter-NP mixture preparation, see the '*Peptide preparation for injection*' section in the Appendices. Peptide administration was performed (N=50 oysters per group) on day 0 and, if necessary, day 21 (Figure 2.9). The mortality rate was checked daily. For the assessment of CI, the oysters were sampled at day 0 (before peptide injection) and then once a week for 5 weeks, and then transferred to USC.

**Table 2.1:** List of peptides used in gonad conditioning assay.

| Peptide | Sequence | Hydrophobicity | Concentration used (ug oyster$^{-1}$) |
|---|---|---|---|
| Buccalin-A | ALDRYSFFGGL-NH2 | 45.45% | 20 |
| | ALDKYGFFGGI-NH2 | 45.45% | |
| Buccalin-G | GLDRYSFMGGI-NH2 | 36.36% | 20 |
| | GLDRYGFAGSL-NH2 | 36.36% | |
| Tachykinin (TK) | YGFAAMR-NH2 | 57.14% | 20 |
| | ARFFGLR-NH2 | 57.14% | |
| | FRFTALR-NH2 | 57.14% | |
| RPGWamide | RPGW-NH2 | 50% | 20 |
| | KPGW-NH2 | 50% | |
| APGWamide | APGW-NH2 | 75% | 20 |

## 2.5 Results and Discussion: Gonad Maturation Assay II

The experiment was terminated at 21 days due to a high mortality rate after peptide injection. This compares to very low or no mortality in previous peptide injection trials using the peptide solution without cocoa butter. Mortality was observed from 3 days post cocoa butter-NP mixture injection, then to a maximum at 9-10 days post-injection (Figure 2.10A). To address this high mortality occurred, the experimental design was modified by reducing the observation duration to 21 days and omitting the second peptide injection at day 21. After sampling, we found that the peptide-cocoa butter mixture caused irritation and infection to the oyster tissues (Figure 2.10B), confirming that the use of cocoa butter caused the mortality in this experiment. Hence, we suggest that the use of cocoa butter to allow for a sustained release of peptide is not suitable for the SRO, despite this technique being used successfully in the Yesso Scallop, *Patinopecten yessoensis* (Nagasawa et al. 2015).

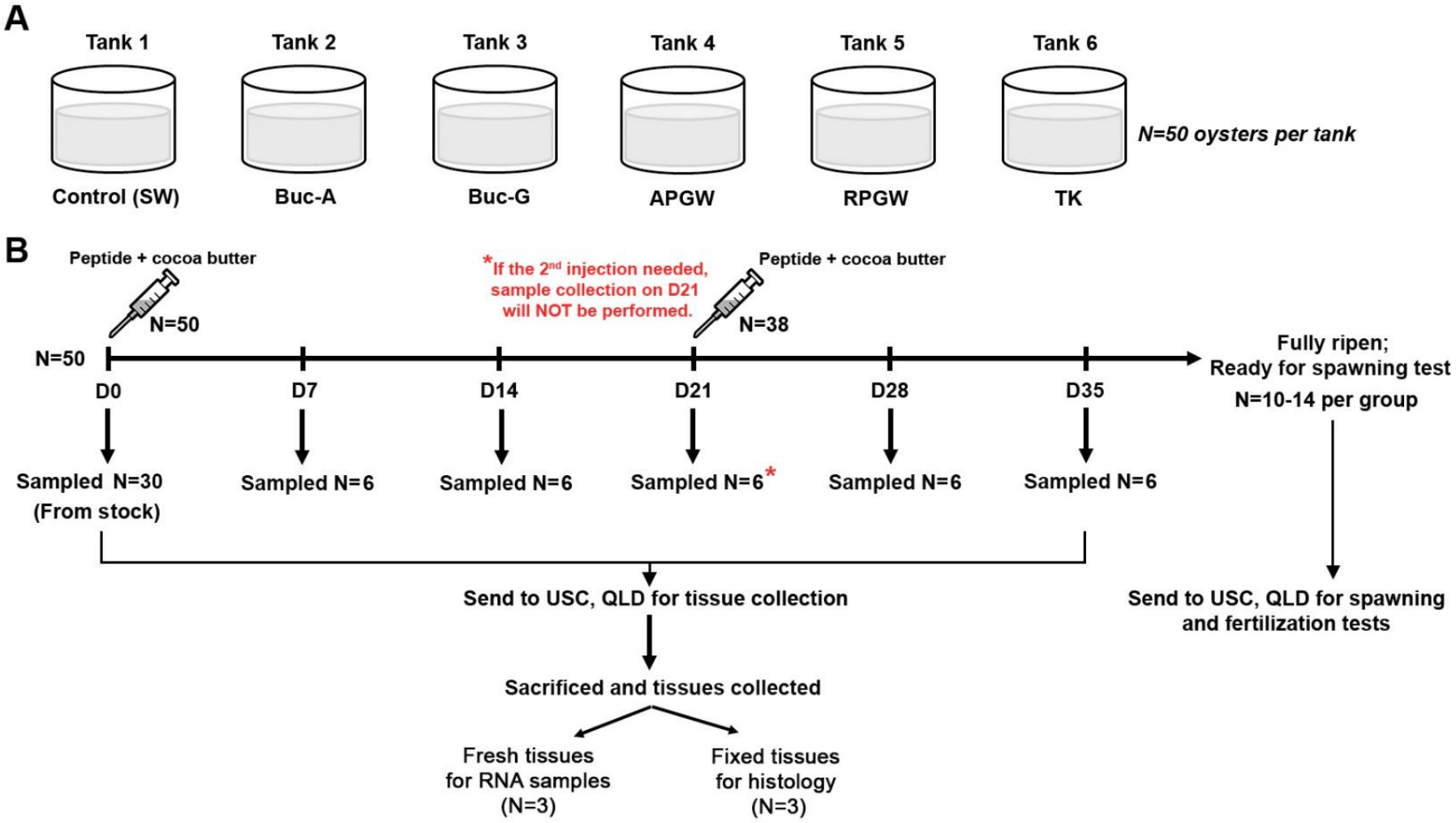
A
Tank 1
Tank 2
Tank 3
Tank 4
Tank 5
Tank 6
N=50 oysters per tank
Control (SW)
Buc-A
Buc-G
APGW
RPGW
TK
B
Peptide + cocoa butter
N=50
*If the 2nd injection needed, sample collection on D21 will NOT be performed.
Peptide + cocoa butter
N=38
N=50
D0
D7
D14
D21
D28
D35
Fully ripen;
Ready for spawning test
N=10-14 per group
Sampled N=30
(From stock)
Sampled N=6
Sampled N=6
Sampled N=6*
Sampled N=6
Sampled N=6
Send to USC, QLD for tissue collection
Send to USC, QLD for spawning and fertilization tests
Sacrificed and tissues collected
Fresh tissues
for RNA samples
(N=3)
Fixed tissues
for histology
(N=3)

**Figure 2.9 (previous page):** Maturation assay. (A) Six groups (6 peptides and 1 negative control) were tested in separate tanks (N=50 per group). (B) Peptide injections were performed on day 0. At day 0, 50 oysters were sampled from the stock and transported back to USC for gonad condition index (CI) record and tissue collection. If the 2nd peptide injection was required on day 21, the remaining oysters in each group were injected with peptide and placed back to the culture tanks. Sampling was not performed at that time. At day 42, the remaining oysters were collected and posted to USC for spawning and fertilisation tests.

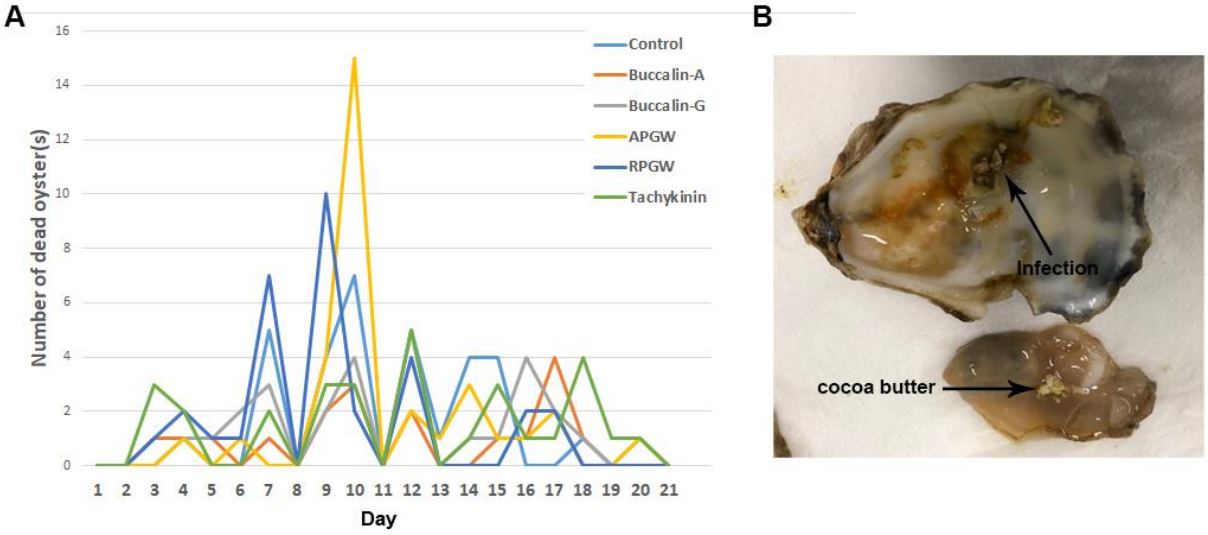


**Figure 2.10:** Mortality after peptide-cocoa butter treatments. (**A**) A line graph shows the number of dead oyster(s) (Y-axis) per day (X-axis). (**B**) The oyster injected with peptide-cocoa butter mixture at day 21. The remnant of cocoa butter and the site of infection are indicated.

The average CI at day 0 in different treatments ranged from 398 to 498, while the average CI of all samples at day 0 was 437.55 ± 77.10 (dashed line, Figure 2.11). After peptide injection, spawning was observed (likely stress-induced), indicating that some of the oysters were still at a spawning stage, which corresponded to the appearance of ripe gonads in some oysters (about 30%). At day 21, the average CI in the control, buccalin-A, RPGW, and tachykinin treatments were not significantly different, while the average CI in the buccalin-G and APGW treatments were significantly higher than the average CI of the control group (at $P \leq 0.05$). Since the first peptide injection at day 0 caused spawning in all treatments (predominantly in the control and tachykinin groups), the lower average CI at day 21 was not unexpected. However, the average CI in the buccalin-G and APGW treatments at day 21 were slightly higher than the average CI of all animals at day 0 (437.55 ± 77.10), suggesting that the treatment of buccalin-G and APGW could potentially promote/maintain gamete production in the oysters that were at a spawnable stage. The sex ratio of the oysters at days 0 and 21 were at 8:2 and 7:3 of female: male, respectively.

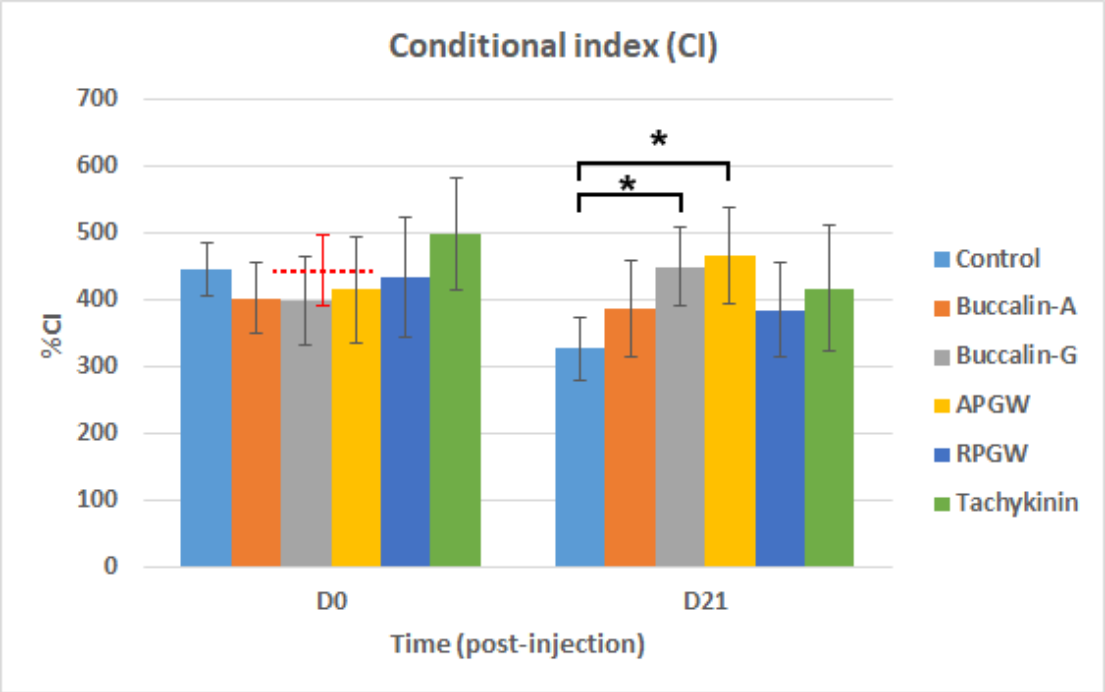


**Figure 2.11:** Condition index of the gonads at days 0 and 21, using various peptide treatments. The red dashed line indicates the average CI of all animals sampled at day 0. Asterisks indicate the statistically different at $p \leq 0.05$.

## 2.6 Method: Gonad Maturation Assay III

### *2.6.1 Introduction*

In assay II, the injection of cocoa butter caused infection in SRO tissue and therefore the mortality rate was high, suggesting that this technique was not appropriate. In this assay, peptide delivery was performed by injection into the adductor muscle. To provide a sufficient amount of peptide in order to achieve a better gonad conditioning, peptide injection was performed three times over the course of the experiment, at days 0, 7, and 14.

### *2.6.2 Sydney Rock Oysters*

SRO with regressive phase gonads (N=450 individuals; average weight 43.23 ± 6.04) were obtained from Port Stephens, NSW. The animals were then transferred to the oyster hatchery at PSFI, NSW. To provide the site for peptide injection, oyster shells were filed, approximately 0.5 cm in diameter, at the ventral side at close proximity to the adductor muscle. The animals were then acclimatized for two weeks prior to the initiation of experiments. The experiment was performed according to routine conditioning protocol for SROs (O'Connor et al. 2008). Briefly, the oysters were cultured in seawater (30-33 ppt) at 23 °C, under 12-12 h dark-light cycle, and fed with microalgae twice daily.

### *2.6.3 Preparation of peptides*

In the current study, the effect of peptide administration on gonad conditioning in SROs was investigated. Five different peptides were tested, including buccalin-A (combining of ALDRYSFFGGLamide and ALDKYGFFGGIamide forms), buccalin-G (combining of GLDRYSFMGGIamide and GLDRYSFMGGIamide forms), APGW (APGWamide), RPGW (RPGWamide) and tachykinin (TK; combining of YGFAAMRamide, ARFFGLRamide and

FRFTALRamide forms) (Table 2.2). Stock peptide solutions were prepared at a concentration of 2 mg $mL^{-1}$ in sterile water and kept at -20 °C until use. For injection, 20 µg peptide(s) in a total volume of 50 µL per injection per individual was prepared from the peptide stock solution using normal saline (NS) as a diluent.

**Table 2.2:** List of peptides for *in vivo* bioassay in Sydney Rock Oysters.

| Peptide | Sequence | Modification | Hydrophobicity | Concentration used (µg $injection^{-1}.oyster^{-1}$) |
|---|---|---|---|---|
| Buccalin-A | ALDRYSFFGGL-NH2 | Amidation | 45.45% | 20 |
| | ALDKYGFFGGI-NH2 | Amidation | 45.45% | |
| Buccalin-G | GLDRYGFAGSL- NH2 | Amidation | 36.36% | 20 |
| | GLDRYSFMGGI-NH2 | Amidation | 36.36% | |
| APGWamide | APGW-NH2 | Amidation | 50% | 20 |
| RPGWamide | RPGW-NH2 | Amidation | 75% | 20 |
| Tachykinin (TK) | YGFAAMR-NH2 | Amidation | 57.14% | 20 |
| | ARFFGLR-NH2 | Amidation | 57.14% | |
| | FRFTALR-NH2 | Amidation | 57.14% | |

### *2.6.4 In vivo bioassay*

Oysters were divided into 6 groups (70 individuals per group), including control (sham), buccalin-A, buccalin-G, APGW, RPGW, and tachykinin (TK) treatments (Figure 2.12A). For peptide administration, oysters (N=70) were injected peptides at a concentration of 20 µg $injection^{-1}.individual^{-1}$ (for list of peptides, see Table 2.2) for 3 times, at days 0, 7, and 14 (Figure 2.12B). At day 0, thirty oysters were sampled and used for gonad assessment in order to determine the stage of gonad development before treatments. During the experiment, samples (N=10) were sampled at days 7, 14, 21, 28 and 35, and subsequently sent to USC for tissue collection. Notably, the oysters sampled on days 7 and 14 were removed from the treatment tanks before the additional peptide injection into the remaining oysters.

Similarly to what described in the previous section, whole weight, shell weight and wet tissue weight were measured and subsequently used for CI calculation. Fresh tissues, including visceral ganglia, mantle, gonad and hepatopancreas, were collected and kept in RNAlater solution, before being stored at -80 °C until use. For further gonad histology examination, a transverse tissue cross-section (~4-5 mm thick) was cut from the anterior of the oyster (through the gonad, intestine, digestive diverticula, stomach, and labial palps) at the point where the palps intersected the gills. Tissues were immediately fixed in pre-chilled 4% paraformaldehyde (PFA; 4% PFA in 0.1 M phosphate buffer saline) for 24 h. At day 42, remaining oysters were collected and sent to USC for fertilisation test.

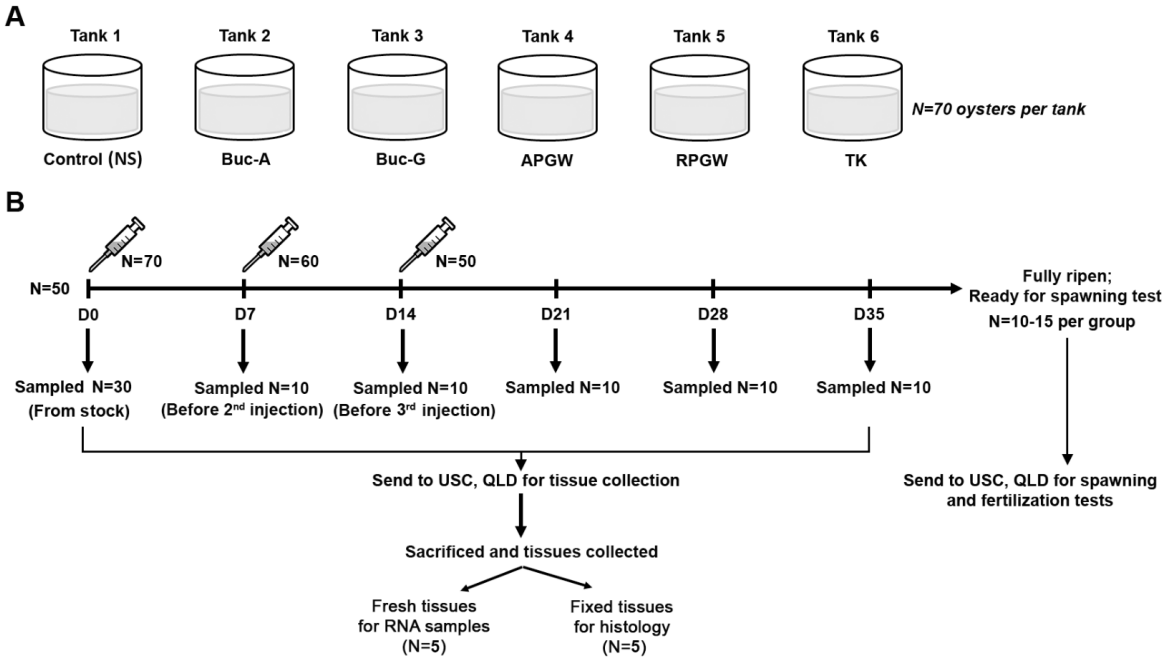


**Figure 2.12:** Maturation assay. (A) Six groups, including 6 peptide treatments and 1 sham control [injected with normal saline (NS)] were tested in separate tanks (N=70 per group). (B) Peptide injection was performed on day 0, 7, and 14. At day 0, 30 oysters were sampled from the stock and transported back to USC for gonad condition index (CI) record and tissue collection. At days 7 and 14, before peptide injection, 10 oysters per group were sampled, and sent to USC by post. At days 21, 28 and 35, 10 oysters were sampled from each group and posted to USC. At day 42, the remaining oysters were collected and posted to USC for spawning and fertilisation tests.

### *2.6.5 Fertilisation efficiency test*

At day 42, the remaining oysters were collected and sent to USC for fertilisation test in order to investigate the fertilisation efficiency of the eggs after peptide treatment. Fertilisation efficiency was performed according to the aforementioned procedure.

### *2.6.6 RNA extraction and expression of genes involved in gametogenesis*

Total RNA was extracted from frozen tissues, including visceral ganglia and gonad, using Tripure solution, following the manufacturer's protocol. The amount of total RNA as well as its quality extracted from each tissue collected from each individual was measured by the Nanodrop-2000 machine. One microgram of total RNA was used for further complementary DNA (cDNA) synthesis, using Tetro-cDNA synthesis kit (Bioline, US). cDNAs from various tissues and treatments were then used for investigation of gene expression by reverse-transcription polymerase chain reaction (RT-PCR). The expression of genes associated with gametogenesis and gonad maturation in bivalves was examined (Table 2.3). To obtain the nucleotide sequences of target genes, BLAST search against in-house SRO transcriptomes was performed on CLC Main Workbench program (version 10) by using the protein sequences of target genes obtained from other bivalves as queries. Nucleotide sequences of SRO target genes were then used for primer design, using Primer3 webtool (http://bioinfo.ut.ee/primer3-0.4.0/). Specific primers (Table 2.3) were subsequently used for RT-PCR, using previously synthesized cDNA as templates.

**Table 2.3:** List of target genes in Sydney Rock Oysters and their specific primers used for RT-PCR.

| Genes | Forward primer (5'→3') | Reverse primer (5'→3') | Amplicon size (bp) |
|---|---|---|---|
| Vasa-homolog | AGGAGTTGGGTACTCCTCCC | TAACCAGTCTGGGACTTCTTGC | 617 |
| Vitellogenin (oogenesis) | GGCGGGAGGACATACAACAA | CCAACGAAGGTGTTCCGAGA | 432 |
| SoxH/Sox30/Sry (spermatogenesis) | AGTTGCTGAGCCATCGACAA | CTCTGTGGCTCGGATTTGGT | 427 |
| Protein regulator of cytokinesis 1 (Prc1) | CCCCGTCATTGAGGCACTAA | TGCGGAGTTTATTGGGGGTC | 659 |
| Bloom syndrome protein homolog (Mus309) | TGACAGTGTGGCTTCAGCAT | TGGTGCTTAACGAGTTGCCT | 479 |
| Centromere protein F (Cenpf) | CTTCACAGAAGCCTCCAGCA | TCCAGAGCCCCTTAGTCCTC | 480 |
| 18S (internal control gene) | TCCGCTGAATTATCAACATGGCT | ATTTACCGTCCTTGACGTCCTT | 300 |

### *2.6.7 Histological examination of gonads*

Fixed tissues were processed through our routine tissue processing protocol. In brief, tissues were processed through a serial dilution of alcohol for dehydration, and then histolene solution (twice, 30 min each) for clearing. Finally, the tissues were infiltrated with paraffin (twice; 1 h each). Subsequently, tissues were embedded in paraffin block and sections (7 µm thick) were prepared. Tissue sections were stained with hematoxylin and eosin (H&E) staining following a routine protocol, and permanently mounted with permount solution. Sections were then visualized under a compound microscope equipped with a CDC camera to obtain digital images of the gonad. Oyster sex was determined, and stages of gonadal development were classified according to Dinamani (1974) for SROs. Dinamani's (1974): phase I is a ripening period; phase II is when oysters are fully ripe; phase III occurs immediately after spawning; phase IV was when phagocytes were present (i.e. gonad regression) but sex is still determinable; and, phase V is a regressive phase where gonial cells are indifferent.

Quantitative histology, based on the proportion of stomach area occupied by gonad tissue, was also performed for the gonad samples collected on days 0 (pre-treatment) and 28 (post-treatment). Briefly, the images of gonad histology at 5x magnification were used and the area of the stomach occupied by gametes was measured using Image J software, and subsequently compared to the total area of the stomach (measured in picture elements) (Royer et al. 2008; Dove and O'Connor 2012). The proportion of the stomach area occupied by gametes was then expressed as percentage gonad area.

### *2.6.8 Transcriptome analysis of visceral ganglia after neuropeptide administration*

#### *2.6.8.1 Sydney Rock Oysters and neuropeptide administration*

Oysters at regressive/early gametogenesis stage were used in this experiment. An average CI was 499.96 ± 74.30. Experiment was divided into 6 groups following different injections, including NaCl (as sham control), serotonin (5-HT), APGWamide, buccalin, GnRH, and tachykinin groups. The sequences of the peptides, as well as concentration per injection, is provided in Table 2.4.

**Table 2.4:** List of experimental groups and peptide/chemical reagent used in each group.

| **Treatment** | **Peptide sequence / chemical formula** | **Concentration per injection (µg in a total volume of 50 µL)** |
|---|---|---|
| Control | 0.9% NaCl | - |
| 5-HT | $C_{10}H_{12}N_2O$ | 88.1 |
| APGWamide | RPGW-NH2 | 20 |
| | KPGW-NH2 | |
| | SPGW-NH2 | |
| | APGW-NH2 | |
| Buccalin | ALDRYSFFGGL-NH2 | 20 |
| | ALDKYGFFGGI-NH2 | |
| | GLDRYSFMGGI-NH2 | |
| | GLDRYGFAGSL-NH2 | |
| GnRH | qNYHFSNGWQP-NH2 | 20 |
| TK | YGFAAMR-NH2 | 20 |
| | ARFFGLR-NH2 | |
| | FRFTALR-NH2 | |

To investigate the effect of various peptides on differential expression of gene of interest in the visceral ganglia (VG), VG tissues (N=6) were collected 24h post-injection. In addition, VG were collected from intact oysters (N=6) before injection and used as the samples at 0 h (pre-injection) in order to provide the information of background gene expression prior to experimental treatment. Before peptide injection, oysters were placed overnight out of water to allow a fast response in anesthetisation which was performed by immersing oysters in 50 g $L^{-1}$ $MgCl_2$ solution for approximately 1h or until oysters were completely anesthetized. Subsequently, oysters were injected with peptide solution (50 µL in total volume) via the adductor muscle, before being placed back into seawater. At 24 h post-injection, oysters were sacrificed, and tissues collected and placed in ice-cold *RNAlater* solution. For long term storage, tissues in *RNAlater* solution were kept at -80 °C until used for RNA extraction.

#### *2.6.8.2 Total RNA extraction and transcriptome sequencing*

Total RNA was extracted from VG tissues by a routine phenol-chloroform extraction using TriSure reagent (Bioline, USA). VG from 2 individuals within the same group was pooled for total RNA extraction (hence, there were 3 total RNA samples in each group at each time point). Quantity and quality of RNA were evaluated by the Nanodrop-2000 and the Agilent 2100 expert bioanalyzer using RNA 6000 Nano kit. Total RNA samples were sent to Novogene, Hong Kong, for cDNA library construction and sequencing. For transcriptome assembly, clean reads obtained from transcriptome sequencing were processed following our transcriptome analysis pipeline, using CLC Genomics Workbench (version 11). In brief, clean reads were trimmed before used for read mapping against the genome sequence of SRO which has been previously generated by our group (Powell et al. 2018). Gene expression analysis was performed and differential gene expression between control and neuropeptide-treated groups was tested. Statistically significant gene expression was tested using Student's T-test. Finally, expression of neuropeptide genes, including GnRH, buccalin, APGWamide, and tachykinin, in all groups was examined.

## 2.7 Results and Discussion: Gonad Maturation Assay III

### *2.7.1 Condition indices and histology*

In the current trial, the effects of five different neuropeptides, including buccalin (2 forms; buccalin-A and -G), APGWa, RPGWa, and tachykinin, on gonad conditioning of the SRO were examined using an *in vivo* bioassay. As negative controls, saline was injected. The regressive stage oysters were administered with the neuropeptides or saline by injection, once a week for 3 consecutive weeks (days 0, 7 and 14). Observation of gonad conditioning was performed once a week (N=10 oysters per group per week) and tissue was collected for histological examination and gene expression analysis, for 5 weeks. At the end of the experiment (42 days post-treatment), mature gametes were stripped from male and female oysters, and used to assess fertilisation efficiency.

Thirty oysters were sacrificed at day 0 for tissue collection, including for RNA extraction and histology. CI and sex ratio were recorded and shown in Figure 2.13 and Figure 2.14, respectively. Samples were then collected once a week, including at days 7, 14, 21, 28, and 35. We found that gonad development in all neuropeptide treatments and negative control were significantly increased at day 35 post-treatment, although there was a degree of variation in gonad development within the same treatment, as indicated by variable gonad CI and quantitative histology (Figures 2.13, 2.15, 2.16 and 2.17). Among various neuropeptide treatments, oysters treated with buccalin-G showed a trend of higher CI at day 35 post-treatment than that of the control group, although it was not statistically different due to variation of CI among the samples collected. Results from the fertilisation test indicated that the fertilisation rate of the eggs stripped from females treated with buccalin-G were significantly higher than that of the control group (75% in buccalin-G treatment compared to 20% in controls) (Figure 2.18). The variation in gonad development among individuals was possibly due to the efficiency of delivery method. Neuropeptide administration was performed by injection into the adductor muscle, via a small gap between the shell valves (by filing technique) at the ventral side, in close proximity to the adductor muscle. Precise injection of neuropeptide into the centre of the adductor muscle was relatively difficult and uncontrollable due to variation in size and location of adductor muscle among individuals. Hence, different individuals might have received a different amount of neuropeptide per injection, ultimately leading to a different degree of gonad development.

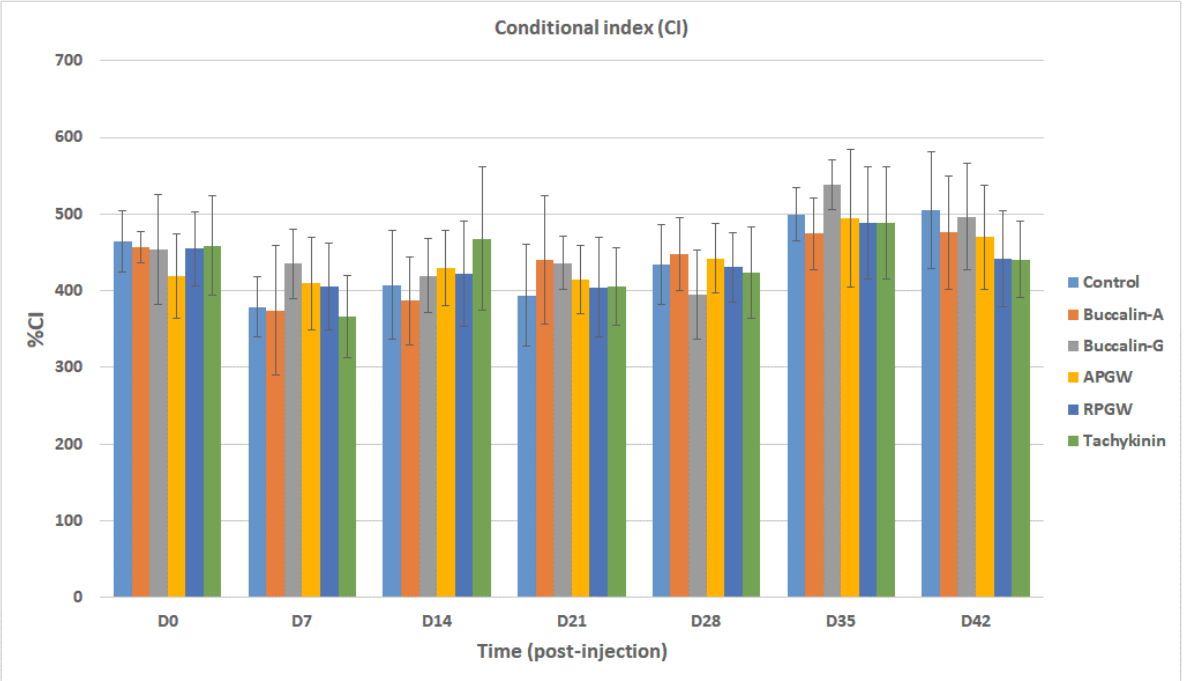


**Figure 2.13:** Condition index. The histogram shows condition index of the Sydney Rock Oysters at various days in different treatments.

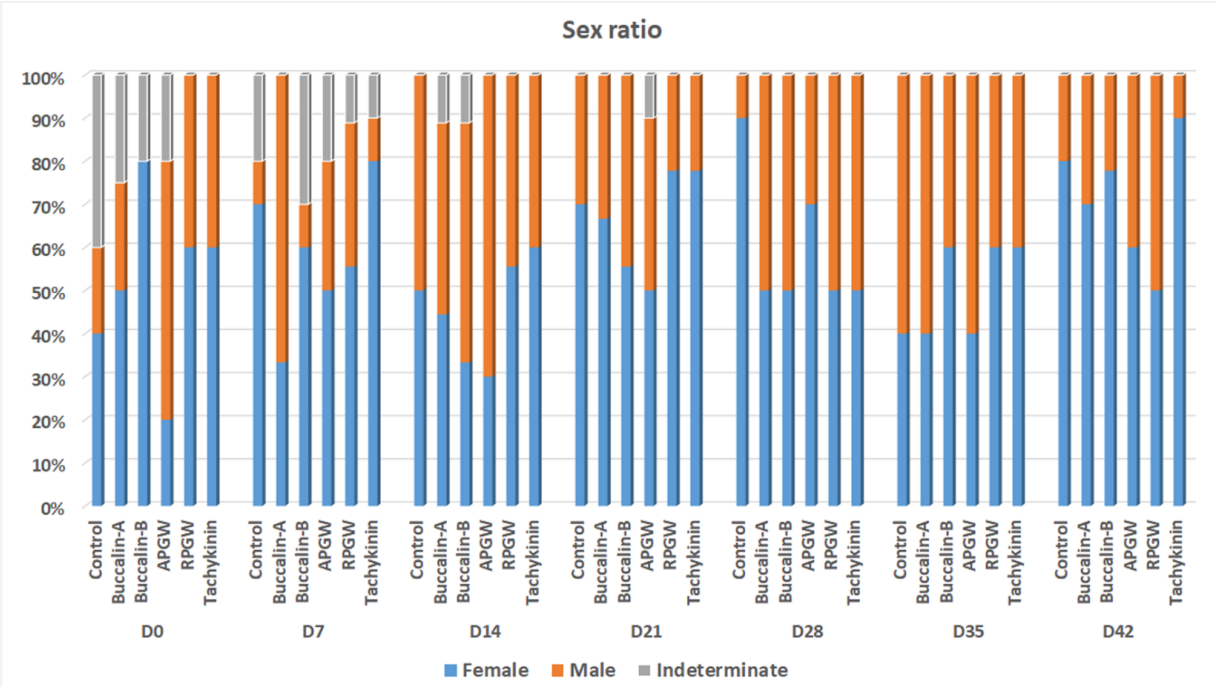


**Figure 2.14:** Sex ratio. The histogram shows the sex ratio between male and female Sydney Rock Oysters in different treatments from day 0 to day 42.

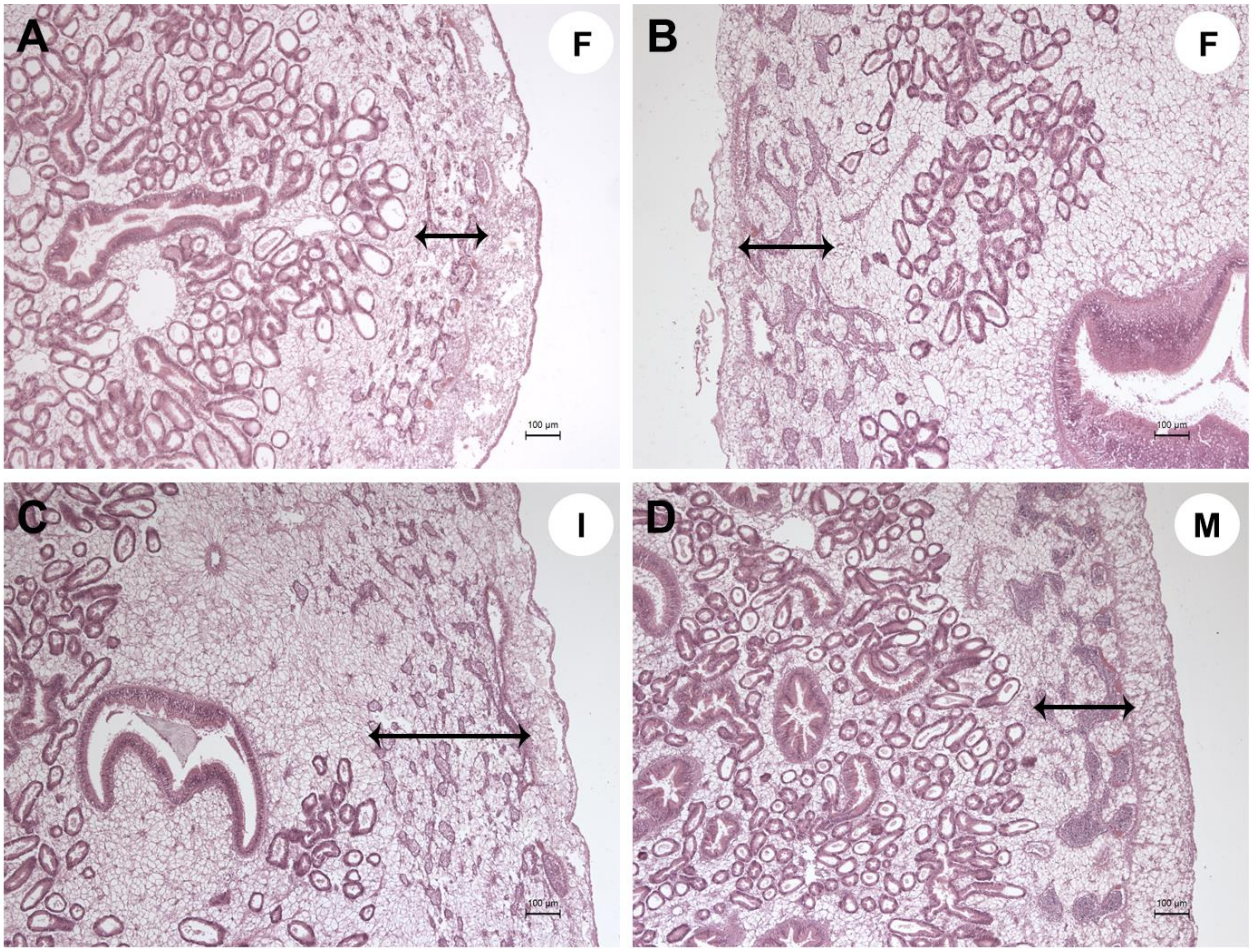


**Figure 2.15:** Histology of Sydney Rock Oyster gonads at day 0 (pre-treatment). (A), (B) Histology of female gonads (early oogenesis; phase II). (C) Histology of indeterminate gonad (phase V; phase II). (D) Histology of male gonad (early spermatogenesis). Abbreviations: F, female; M, male; I, indeterminate sex. Arrows indicate gonad regions where gametes were present.

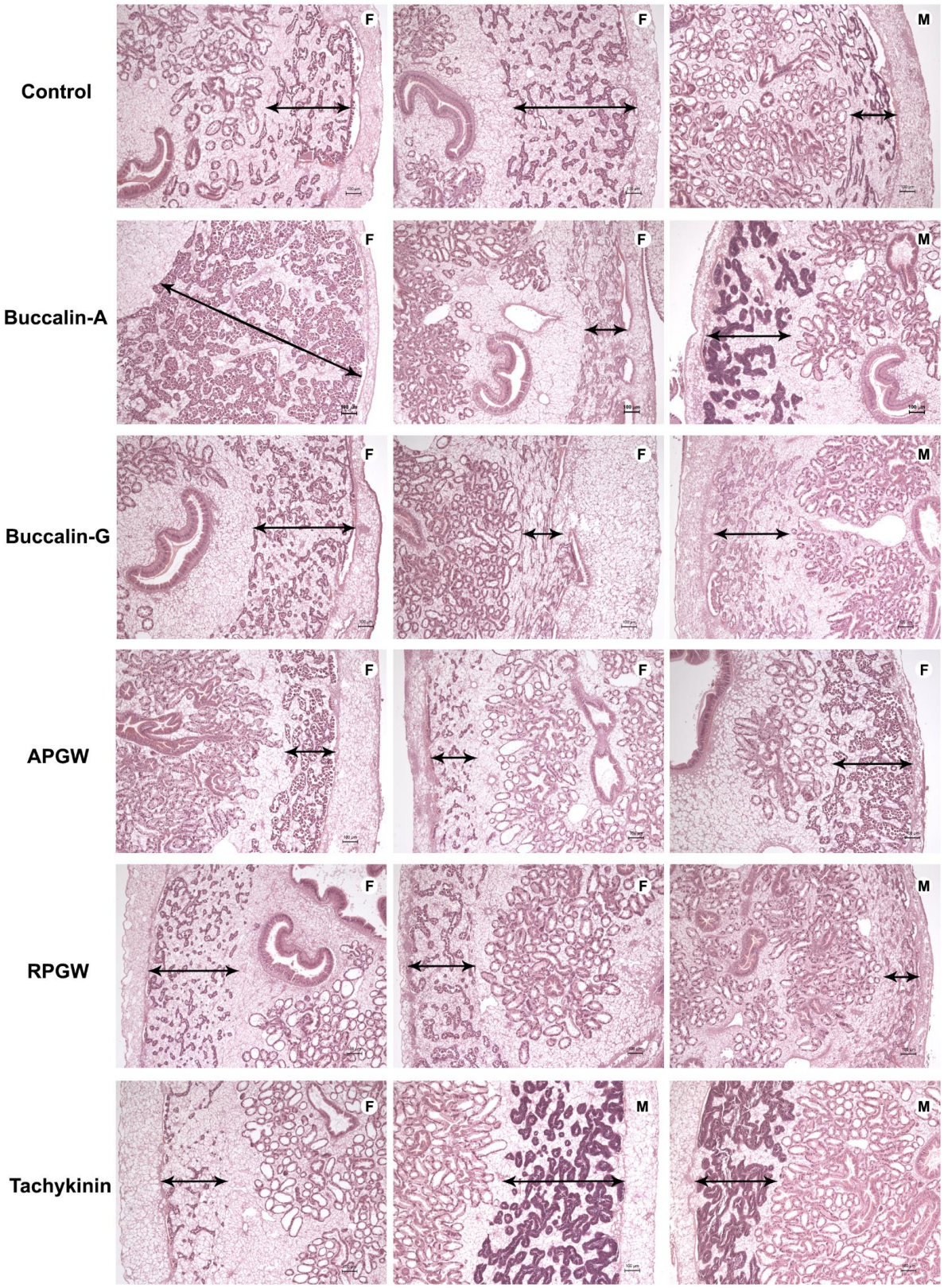


**Figure 2.16:** Histology of gonads at day 28 (post-treatment**).** Histology of gonads at day 28 post-treatment in the control, buccalin-A, buccalin-G, APGW, RPGW, and tachykinin treatments, respectively. Abbreviations: F, female; M, male. Arrows indicate gonad regions where gametes were present.

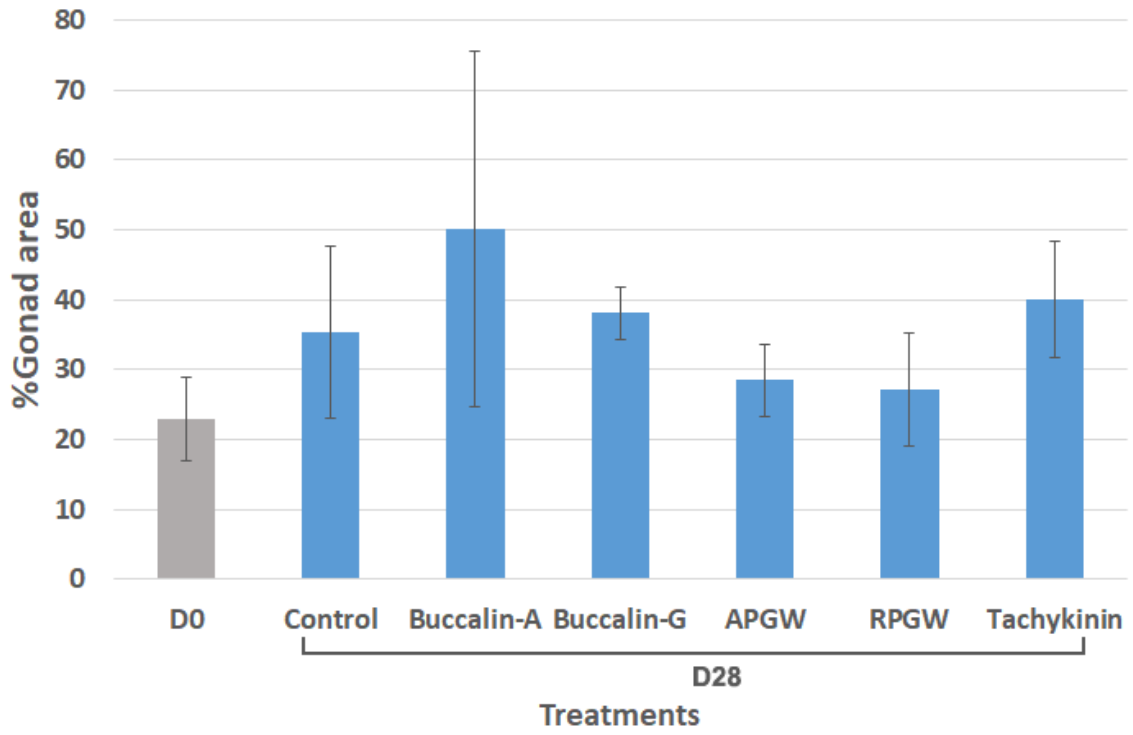


**Figure 2.17:** Sydney Rock Oyster gonad development at day 28 by quantitative histology.

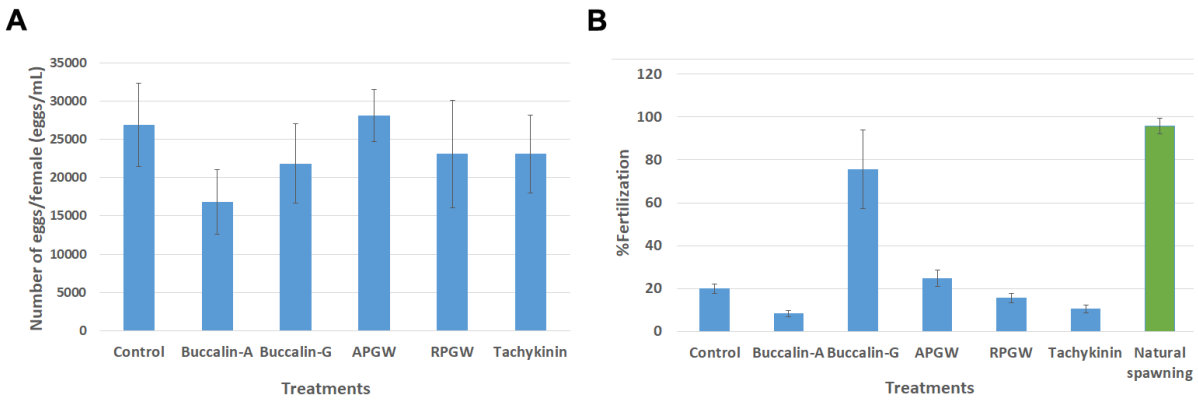


**Figure 2.18:** Number of eggs and rate of fertilisation. (A) Number of eggs per 1 mL of egg solution per female. (B) Percent fertilisation of eggs stripped from females (N=3 per group) in various treatment. Percent fertilisation in the natural spawning condition is also shown (green bar).

### *2.7.2 Transcriptome analysis of visceral ganglia after neuropeptide administration*

In Pacific Oysters, it has been shown that 2,482 genes in the gonads were differentially expressed between gametogenetic stages in both sexes (Dheilly et al. 2012). Since their expression was either increased or decreased following the advancement of gonad maturation, some of these genes could be used as gene markers to indicate the developmental stages of gonads. For example, the expression of vitellogenin (*Vg*), protein regulator of cytokinesis 1 (*Prc1*), bloom syndrome protein homolog (*Mus309*), and centromere protein F (*Cenpf*) have been shown to increase following the advancement

of gonad maturation, while the expression of *SoxH/Sox30/Sry* and the vasa-homolog (*VASA*) decreased following gonad maturation. Since Pacific Oysters and SROs are evolutionarily closely related (Powell et al. 2018), we assume that these gametogenesis-related genes should have similar functions, although further investigation on their expression in different stages of gonads is needed.

The relative expression of *Vg*, *Prc1*, *Mus309*, *Cenpf*, *SoxH*, and *VASA* in the gonad of SROs (both sexes) was investigated during gonad development, after neuropeptide treatments (buccalin-A, buccalin-G, APGWa, RPGWa, tachykinin). Despite no statistically significant differences between different treatments because of the variation among individuals, the expression of *Vg*, *Prc1*, *Mus309*, *Cenpf* and *VASA* were clearly distinguished between the neuropeptide-treated and control groups by day 14 post-treatment (Figure 2.19). We found that the relative expression levels of *VASA*, *Vg*, *Prc1*, *Mus309* and *Cenpf* in the gonads at day 14 post-treatment were increased following neuropeptide treatment, when compared with the control group.

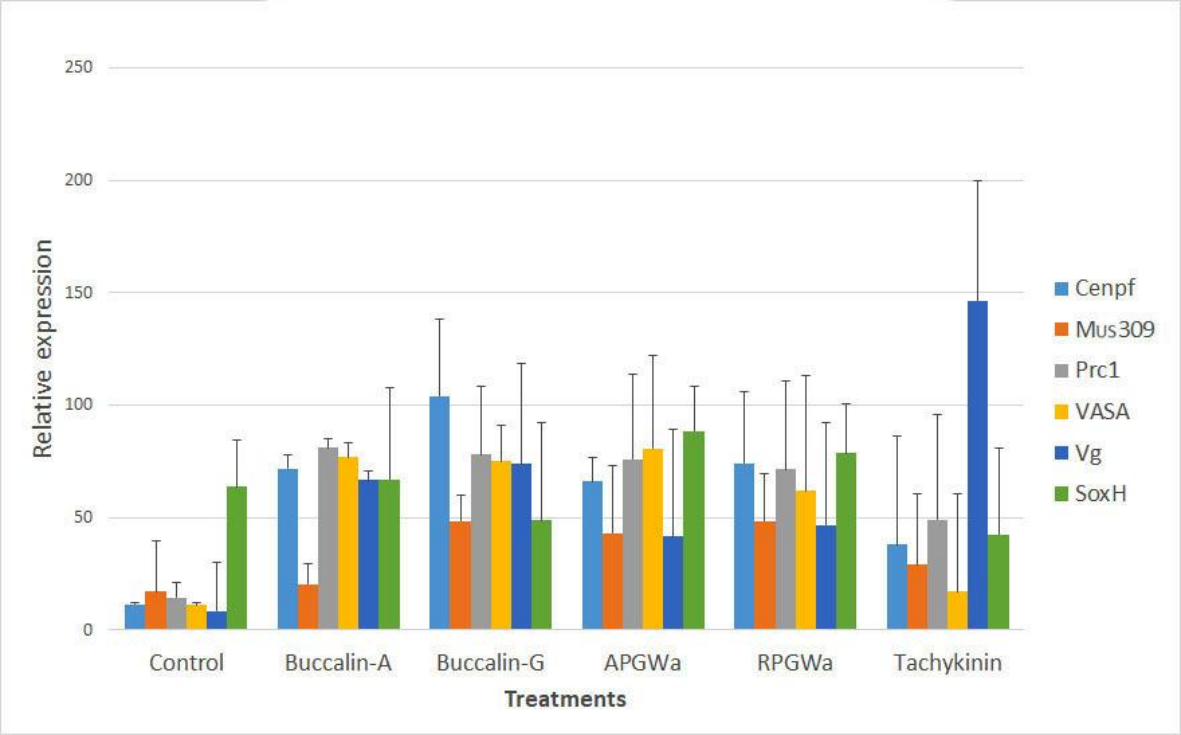


**Figure 2.19:** Gametogenesis-related gene expression by RT-PCR. Relative expression of gametogenesis-related genes, including *Cenpf*, *MUS309*, *Prc1*, *VASA*, *Vg* and *SoxH* relative to *18S* gene expression in the gonad tissue of the Sydney Rock Oysters at day 14 post-treatment (N=3; error bars indicate standard deviation).

Overall, these data suggest that injection of the selected neuropeptides could exert gonad development in SROs, in both males and females. In addition, gametogenesis-related gene expression was noticeably higher at day 14 post-treatment (after 2 times of neuropeptide injection), but not 7 days post-treatment (1 injection) (*data not shown*).

Neuropeptides, including GnRH, APGWamide and buccalin, are known to regulate reproductive processes in molluscs, and their effects on gonad conditioning and spawning were investigated in SROs (In et al. 2016). In the SRO, we found that most of key vertebrate reproductive neuropeptides/hormones are present. However, whetherthe SRO, as well as other molluscs, follows a

similar pattern of reproductive regulatory axis to that of vertebrates is not known. In the current study, transcriptome sequencing and differential gene expression analysis were therefore used to investigate target neuropeptide gene expression following various neuropeptide treatments (including buccalin, APGWa, GnRH and tachykinin). SRO (both sexes) were injected with different neuropeptides and their visceral ganglia were then collected and subsequently processed for transcriptome sequencing. The transcriptome sequence data was then analysed and differential gene expression (DGE) profiles assessed. The results indicate a number of genes are significantly up/down-regulated after neuropeptide injection (Figure 2.20). The 5HT-treated group (as a positive control group) had 499 total DGE, 212 were up-regulated and 287 were down-regulated. APGWa-treated group had 177 total DGE, of those 59 were up-regulated and 117 down-regulated. The buccalin-treated group had 192 total DGE, 72 up-regulated and 125 down-regulated genes. The GnRH-treated group had 146 total DGE, 58 up-regulated and 88 down-regulated genes. The tachykinin-treated group had 202 total DEG, 73 up- and 129 down-regulated genes (Figure 2.20).

The DGE was analysed by Venn diagram to identify unique genes in each group. To understand high-level functions and utilities of the biological system of DGE genes, unique genes that were differentially expressed in each treatment were analysed by KEGG pathway. APGWa-treated group had up-regulation of the protocadherin-16-like isoform X2 gene, which is involved in cell signalling including phospholipase D signaling pathway, PI3K-Akt signaling pathway, AMPK signaling pathway, mTOR signaling pathway and insulin signaling pathway. Additionally, up-regulation of the serine protease inhibitor dipetalogastin-like gene, which is involved in lipid and purine metabolism pathways, was observed. The atrial natriuretic peptide receptor 1-like, which is involved in cAMP signaling pathway, cGMP-PKG signaling pathway and cytokine-cytokine receptor interaction, was down-regulated in APGWa-treated group.

Interestingly, the interferon-induced protein 44 gene, which is involved in steroid hormone biosynthesis, was down-regulated as well. The buccalin-treated group had up-regulation of a gene that is involved in glycosaminoglycan biosynthesis, the heparin/heparin sulfate, and up-regulation of an uncharacterised gene involved in membrane trafficking. However, no unique genes related to reproduction and no KEGG hits for down-regulated unique genes were found in the buccalin-treated group. In the GnRH-treated group, there was up-regulation of 2 genes: a matrilin-2-like that is responsible for glycosphingolipid biosynthesis and lysosome pathway and a caveolin-1-like which is involved in endocytosis and focal adhesion. No unique genes related to reproduction and no KEGG hits for down-regulated unique genes were observed in the GnRH-treated group. Tachykinin had up-regulation of genes involved in carbohydrate metabolism and 2 genes involved in meiosis and DNA repair: the meiotic recombination protein DMC1/LIM15 homolog and the meiotic recombination protein SPO11. No KEGG hits for down-regulated unique genes were observed. The 5-HT-treated group had up-regulation of a glycine-rich cell wall structural protein 1-like isoform X2 that is involved in sulphur metabolism, and a synaptic vesicular amine transporter-like that is involved in RNA transport. This group had down-regulation of carbonic anhydrase 2-like gene that is part of the nitrogen metabolism and β-1,4-galactosyltransferase-1 that is involved in ribosome translation. No genes unique to this group directly related to reproduction were found.

Expression of target SRO neuropeptide genes (APGWa, buccalin, GnRH, and tachykinin) was investigated in each group and we found that there was no statistically significant association between neuropeptide injection and the expression of target neuropeptide genes at 24 h post-injection, although we found that injection of APGW led to a reduction of APGW expression in the CNS ($p<0.05$; fold-change =2.23), which implied its negative feedback. Therefore, we concluded that the expression of buccalin, APGWa, GnRH, and tachykinin in the visceral ganglia at 24 h post-neuropeptide injection appears to be independent. This raises further questions whether these neuropeptides synergistically regulate SRO reproduction and what are the key molecules that orchestrate the activity of these neuropeptides in order to successfully control the whole process of reproduction in this oyster. The DEG and KEGG analyses indicated that there were other aspects of biological processes that were influenced by neuropeptide injections. Whether those biological processes are associated with

reproductive process, either directly or indirectly, needs to be further analyzed and interpreted. Meanwhile, these findings also provide additional information of additional functions of each neuropeptide investigated, which might be helpful for the other studies related to different biological aspects in SRO, including metabolism, stress, and immune system.

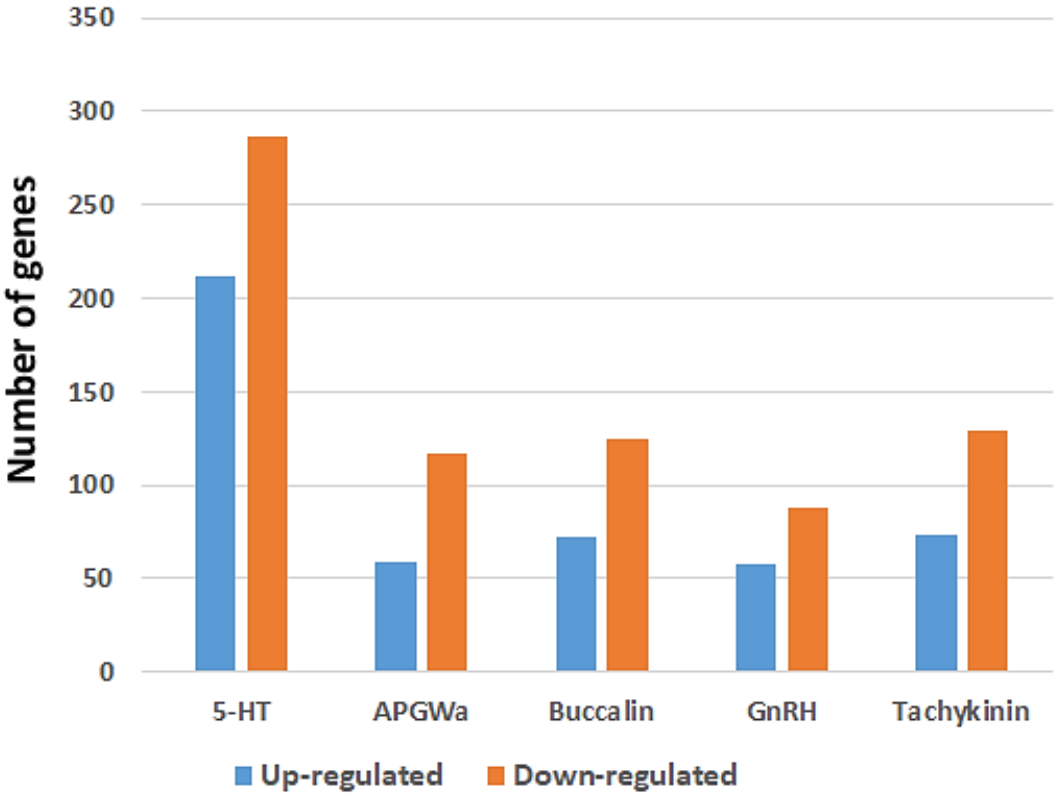


**Figure 2.20:** Differential gene expression (DEG) analysis in visceral ganglion transcriptomes after neuropeptide injections. Histogram shows the number of genes that were significantly up- and down-regulated in their expression at 24 h post-neuropeptide injection as compared with the control group ($p<0.05$). DEG in the 5-HT-treated group was used as a positive control.

## 2.8 Conclusion

APGWamide and buccalin have been shown to have stimulatory effects on gonad conditioning in SRO. In natural conditions, these peptides are produced and function in a cocktail form – a mixture of different bioactive peptides. According to results obtained from three independent trials, we found that administering the individual forms of buccalin and APGWamide showed a stimulation of gonad conditioning in SRO. Comparing the stimulatory effect of two different forms of APGWamide, APGWa and RPGWa, we found that the APGWa form was more potent than the RPGWa form. For buccalin, the buccalin-G form showed a better performance than the buccalin-A form in most of reproductive activities assessed. Considering the stimulatory effects of APGWa and buccalin-G on SRO conditioning, we found that their stimulatory effects appear to be comparable. Hence, we propose that either APGWa or buccalin-G can be applied to large-scale breeding programs for SRO. Considering the method of peptide delivery, we found that the use of cocoa butter to allow a slow release of peptide was not appropriate as it caused high mortality in SRO. Injection of peptides is

therefore preferable and multiple injections are expected to help in maintaining the level of the peptides in the oyster's circulatory system in order to successfully control gonad conditioning. Yet, delivery of peptide by injection is relatively difficult and uncontrollable since different individual oysters could receive a different amount of the neuropeptide per injection. To overcome this problem, delivery using other techniques, such as oral delivery by using peptide-encapsulated algae, should be considered and tested in the future.

# 3. Spawning induction in the Sydney Rock Oyster

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## 3.1 Introduction

Oysters are broadcast spawners, that is, eggs and sperm are released into the water where fertilisation occurs externally (Evans and Sherman 2013). SROs have a seasonal reproductive pattern where oysters are generally highly fecund and serially spawn though the months of November to April throughout most of their growing range (Dove and O'Connor 2012). Several factors are known to be involved with spawning activity in SROs, including environmental factors (e.g., water temperature, tides and currents) and pheromones (Bernard et al. 2016). Recently, a spawning inducing factor/pheromone has been established to be a proteinaceous substance present in the sperm solution which successfully induces spawning activity in the pearl oyster, *Pinctada maxima* (Taylor et al. 2018).

Commercial production of SROs in hatcheries uses two spawning techniques: the first, natural spawning where oysters are placed in tanks and temperature and salinity is manipulated to induce gamete release; and the second, strip-spawning, where oysters are sacrificed to physically remove gametes from the gonad using a scalpel or pipette. Fertilisation using naturally spawned SRO gametes result in high development rates (>90%) and low levels of larval deformities which increase the chance of successful rearing through the subsequent stages of larval development. On the other hand, strip-spawning SROs results in fertilisation deficiency in particular crosses, low rates of success when the fertilisation does occur and poor levels of larval development in the period immediately after fertilisation. Strip-spawning is necessary for the SRO BP to manage matings between a single male and female to create families however only 27% of the matings performed produced a family for ongoing assessment (Dove et al. 2020).

In this study, we aim to identify the spawning inducing pheromone(s)/factors in the sperm of the SRO. This is of particular interest to the SRO BP to:

- reduce the number of valuable broodstock sacrificed during breeding runs;
- increase the success and yields of each cross through using naturally released gametes; and
- use high performing individual oysters in the breeding program repeatedly over subsequent generations.

## 3.2 Method

The workflow for the spawning induction assays using sperm fractions is shown in Figure 3.1. In brief, various types of proteins are extracted and purified from the sperm using various methods, following the previous work (Taylor et al. 2018). The protein extracts are then tested in the spawning

induction bioassay to examine the spawning induction bioactivity. Finally, the protein extract showing spawning induction bioactivity is analysed by mass spectrometry for protein identification.

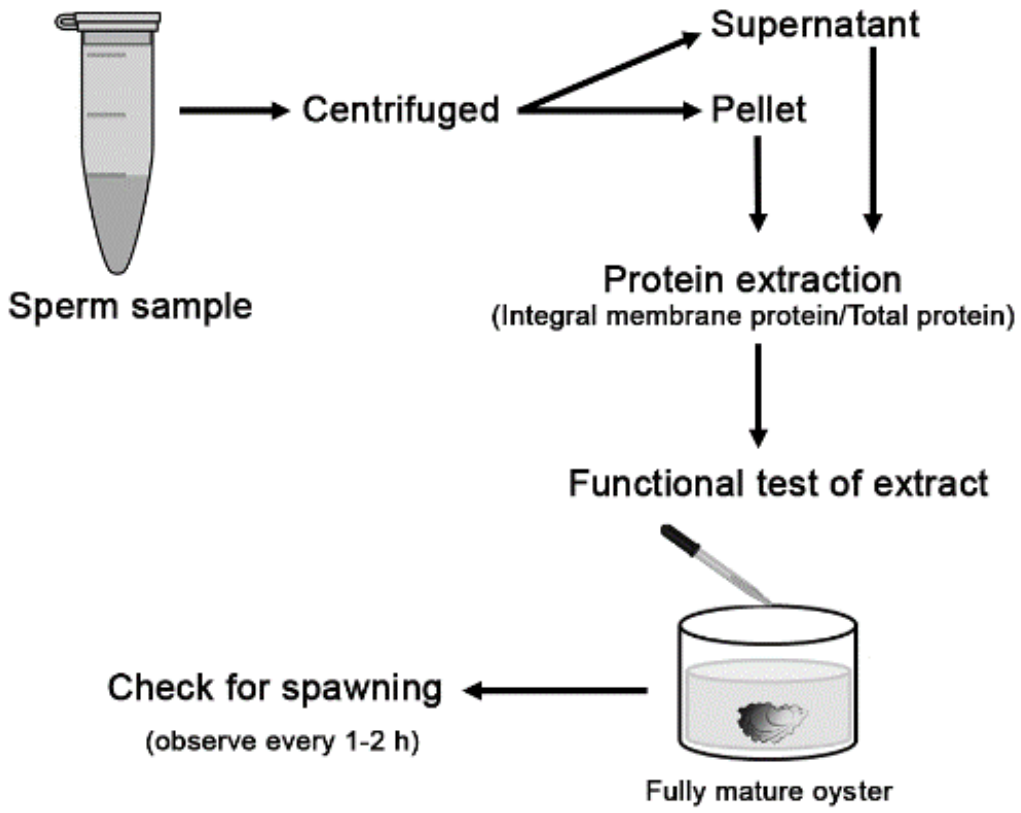


**Figure 3.1:** Overview of the identification of spawning inducing pheromone(s) in the Sydney Rock Oyster.

### *3.2.1 Part I: Extraction of sperm proteins*

Various types of proteins, for example, cytoplasmic proteins, intrinsic membrane proteins and extrinsic membrane proteins, were extracted from the sperm collected from mature male SROs. In brief, mature male oysters were sacrificed and approximately 15 mL of sperm were stripped and transferred into a sterile tube. Stripped sperm samples were kept at -80 °C until used for protein extraction. Further details of protein extraction are provided in Figure 3.2.

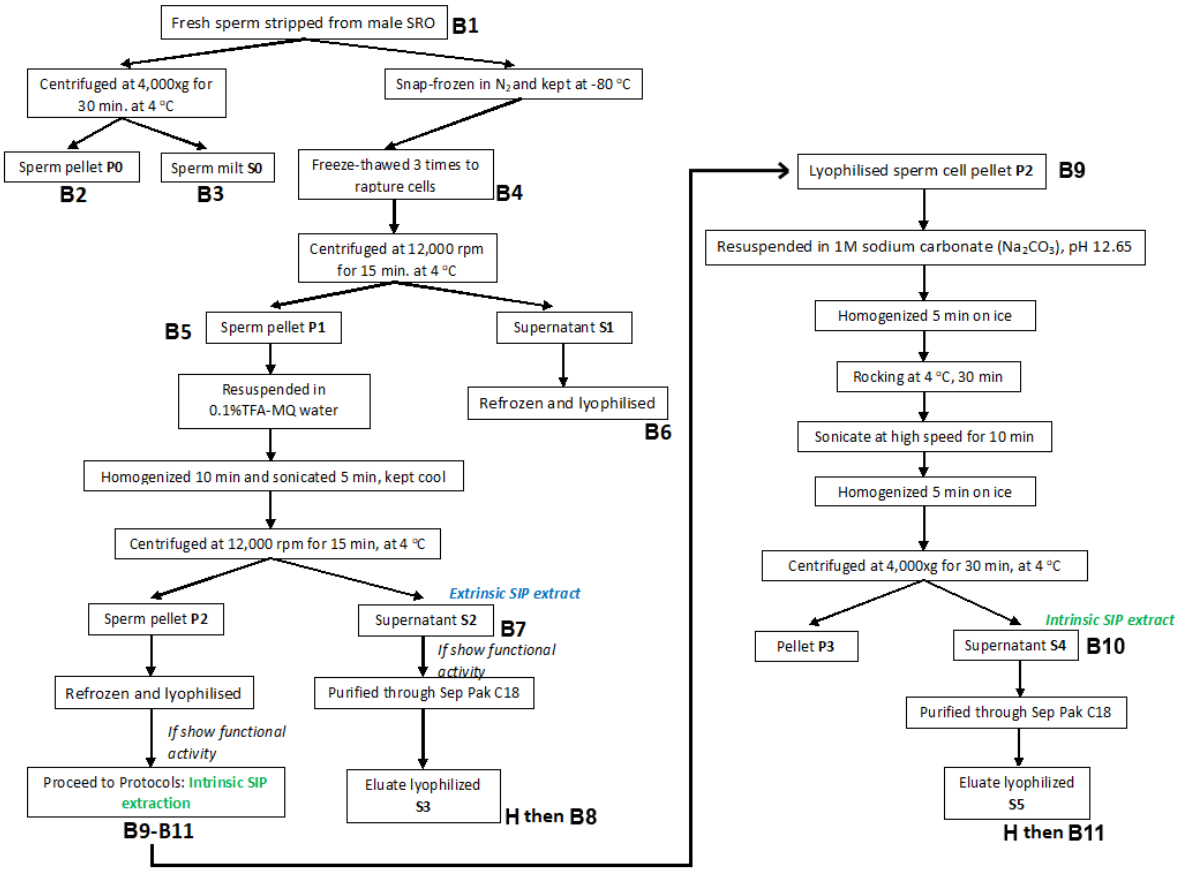


**Figure 3.2:** Workflow of sperm fractionation and designated inducers (B1-B11) used in the spawning induction bioassay. Abbreviations: SIP, spawning inducing pheromone; H, fractionation with RP-HPLC.

### *3.2.2 Part II: Purification of instrinsic (S3) and extrinsic (S5) sperm membrane proteins by RP-HPLC*

In brief, S3 and S5 extracts (Figure 3.2) that contained intrinsic and extrinsic membrane proteins, respectively, were purified through the C18 Sep-Pak column and eluted with 60%ACN containing 0.1%TFA. The eluent was lyophilised. Dried semi-purified S3 and S5 samples were then resuspened in 200 µL 0.1%TFA water and the concentration of the total protein measured using NanoDrop-2500 (at 280 nm). Then, 100 µL of semi-purified S3 and S5 were purified through HPLC analytical C18 column with mobile phase gradient setup as follows:

| Time (min) | %Solution A (0.1%TFA H2O) | %Solution B (0.1%TFA ACN) |
|---|---|---|
| 0 | 100 | 0 |
| 60 | 40 | 60 |

The fractions were collected into 15 mL tubes every 5 min. The fractions were then lyophilised and kept at -20 ºC until use in the spawning induction bioassay.

### *3.2.3 Part III: Preparation of fractions for spawning induction bioassay*

To prepare the fractions for bioassays, lyophilised fractions were resuspended in sterile seawater to the original volume of the stripped sperm. Based on the amount of extract used for HPLC purification, which is equivalent to 0.625 mL stripped sperm, the dried fraction was resuspended in 625 µL of sterile water and vigorously mixed by vortexing. The total protein concentration was then measured. For example, the total protein concentration of S3 fraction Min 41-45 was 0.035 µg $\mu L^{-1}$ (this concentration approximately equals to 1/2200 part of the S3#2 concentration prior to HPLC purification).

For spawning induction, fraction solution was diluted in seawater at the dilution of 1:100. Two-hundred microliter of the diluted fraction was then delivered into the oyster cavity. This means that 2 µL (0.07 µg total proteins) of fraction was used per 1 oyster. The oysters were placed out of the water for 1 h after fraction delivery to allow the physiological induction of spawning activity by the sperm fraction. After that, oysters were placed into the spawning tank and the time of spawning induction was recorded. For further details of spawning induction bioassay, see the following section.

### *3.2.4 Part IV: Spawning setup, sperm fraction delivery and spawning activity observation*

Spawning induction was performed in the spawning tanks containing 7-L seawater. The seawater salinity was adjusted to 22 ppt and the temperature to 27-28 ºC. Spawning induction was performed (10 oysters / treatment / tank). The seawater in the spawning tank was maintained at 27-28 ºC using a water heater and the water was circulated in the tank by a 1000 Watts submersible water pump (Figure 3.3). For sperm fraction delivery, oysters that have been kept out of water for 12-48 h were transferred into the spawning tank and acclimatized for at least 20 min or until their valves opened. A micropipette tip was inserted in between the valves to allow the fraction delivery into the shell cavity. Two-hundred microliters of the sperm fraction (at a concentration equivalent to 20X of the natural sperm concentration normally used for spawning induction [at dilution of 1/250,000 v/v for sperm: seawater]) was delivered into the shell cavity through the gap between the oyster valves, and the inserted micropipette subsequently removed. The oysters were left out of the water for 1 h (to allow the sperm fraction exposure), before being placed into the spawning tank at which time the observation of spawning activity began (as time 0; T0) (Figure 3.3). The observation was performed over a period of 2 h and the spawning induction time, which is the time taken from T0 until the first oyster begins to spawn, was recorded. During observations, the cycle of water current was controlled by turning the water pump on/off (15 min of ‘pump on’ and 5 min of ‘pump off’) to facilitate the observation of spawning. After the first oyster began to spawn, the spawning of other oysters within the tank was also observed and oyster sex recorded. The oysters were removed to a new tank filled with fresh seawater when spawning was completed. Oysters that did not respond (spawning) to the sperm fraction over the period of 2 h, they were recorded as ‘no response’. The spawning inducers, or sperm fractions, were tested following the flowchart in Figure 3.4. The test of sub-fraction was performed when their original/combined fraction was found to induce spawning activity.

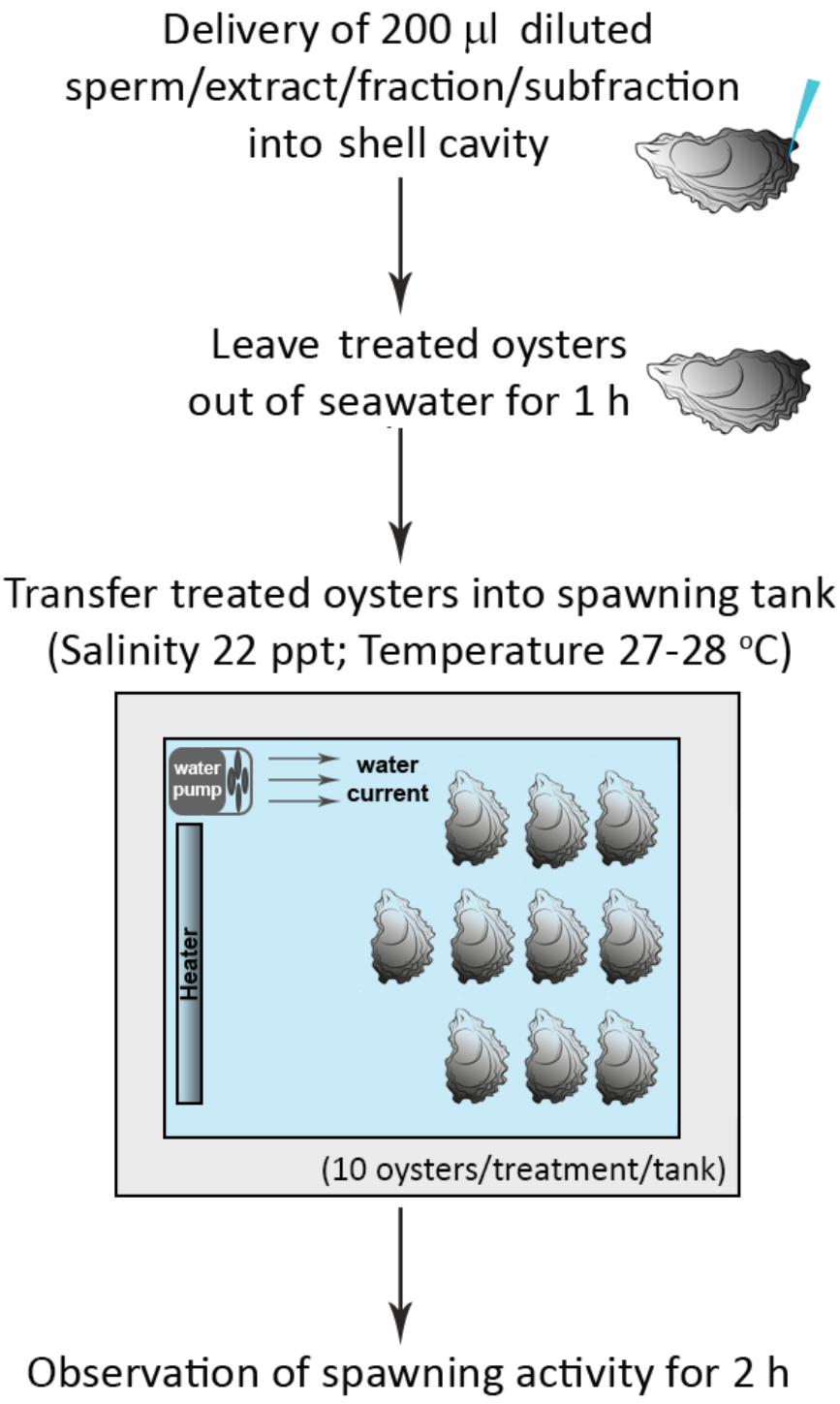


**Figure 3.3:** Spawning induction by sperm fraction exposure workflow.

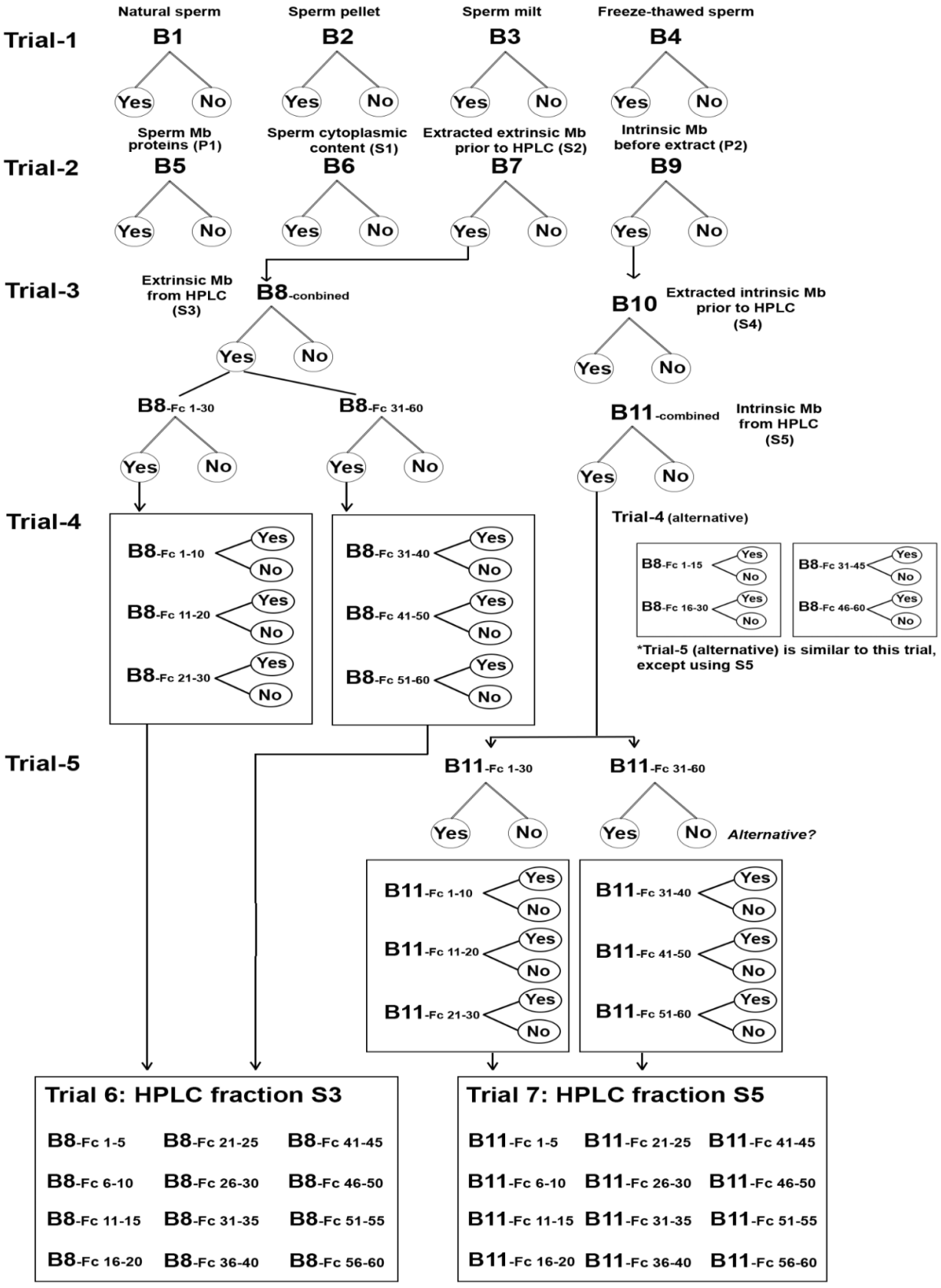


**Figure 3.4:** Spawning inducers tested in the spawning bioassay. The sub-fractions were tested when the original/combined fractions were found to induce the spawning activity (as 'Yes' in the flowchart).

### *3.2.5 Part V: Additional purification of instrinsic sperm membrane proteins in the fraction S3 at min 41-45 by RP-HPLC*

In brief, sperm fraction S3 (40 µL which equals to 0.24 mL sperm) was purified through HPLC analytical C18 column with mobile phase gradient setup as follows:

| Time (min) | %Solution A (0.1%TFA H2O) | %Solution B (0.1%TFA ACN) |
|---|---|---|
| 0 | 100 | 0 |
| 40 | 60 | 40 |
| 55 | 55 | 45 |
| 70 | 40 | 60 |

The gradient changes during the HPLC purification are shown in Figure 3.5. The fractions from mins 0-40 and 56-70 were collected into 15-mL tubes at every 5 min, while the fractions from min 41-55 (as a finer purification of fraction S3 min 41-45) were collected into 2 mL tubes at every 1 min. The fractions were then lyophilised and kept at -20 °C until use. To test the activity of the sub-fractions in spawning induction, sub-fractions were pooled at every 5 min of elution time, including sub-fraction 1-5, 6-10, and 11-15. The pooled sub-fractions were tested in the spawning induction bioassay (as described in the following section).

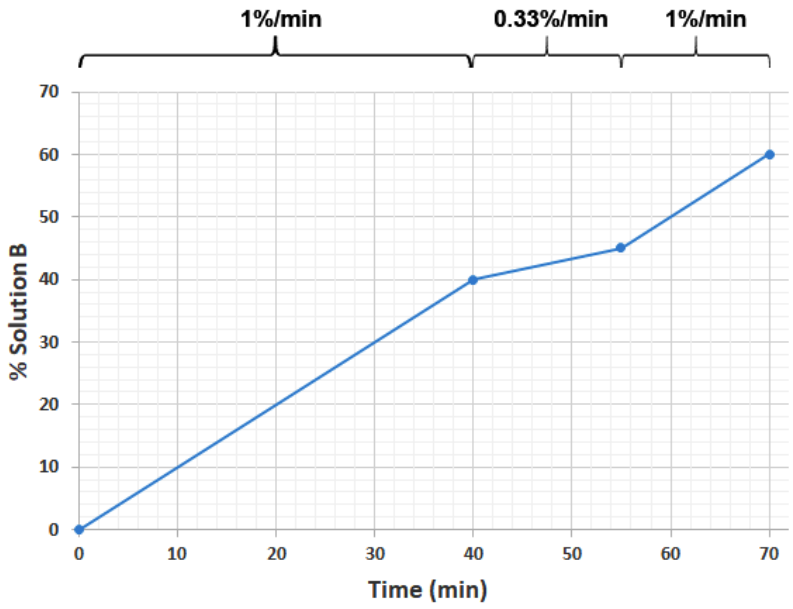


**Figure 3.5:** HPLC gradient setup for further purification of fraction S3 at min 41-45.

### *3.2.6 Part VI: Identification of proteins in active sub-fractions by mass spectrometry (MS) analysis*

Proteins within the pooled sub-fractions 6-10 and 11-15 were enzymatically digested into peptides prior to MS analysis. Then, the digested products were analysed by using the 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. Fragmentation data was analysed by PEAKS version 7.0 (BSI, Canada) software. Sequences of peptides were determined manually and/or by comparing the fragmentation patterns with those predicted from the SRO transcriptomes.

## 3.3 Results and Discussion

Purification of intrinsic (S3) and extrinsic (S5) membrane proteins from the SRO sperm was done by RP-HPLC and showed the basic protein profile at UV absorbance 210 and 280 nm (Figures 3.6 and 3.7), which revealed a number of proteins.

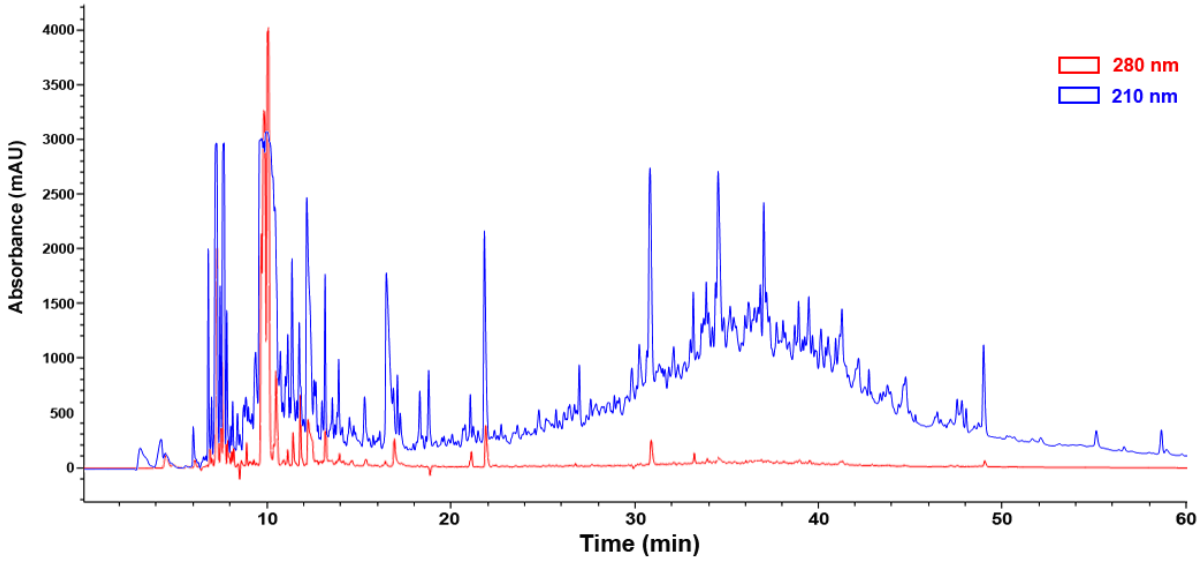


**Figure 3.6:** HPLC profile of the intrinsic sperm membrane proteins (S3). The protein absorbance was measured at UV wavelength 210 and 280 nm.

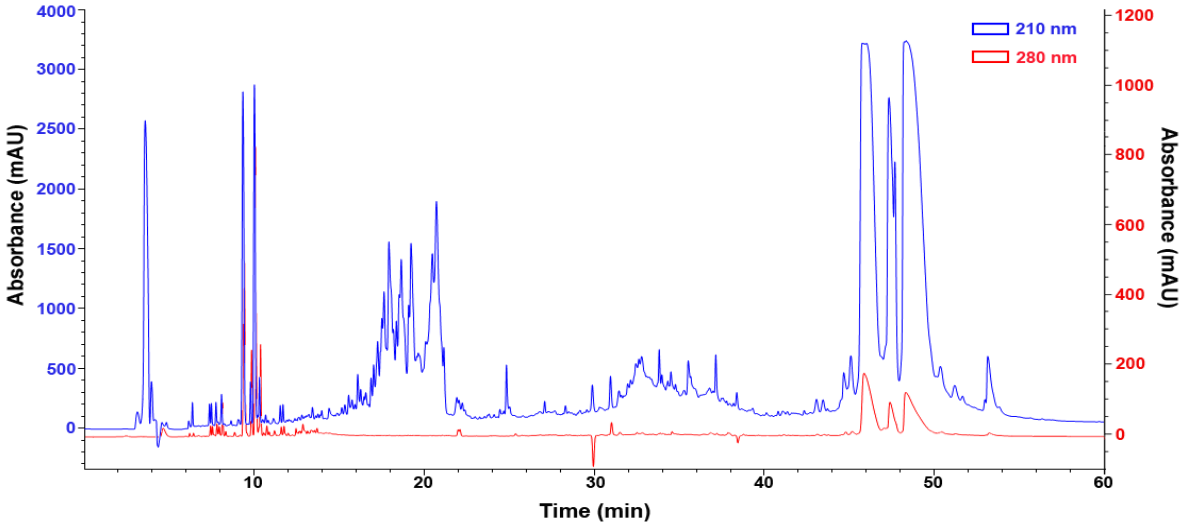


**Figure 3.7:** HPLC profile of the extrinsic sperm membrane proteins (S5). The protein absorbance was measured at UV wavelength 210 and 280 nm.

The results from the spawning induction of all fractions are summarized in Table 3.1. We found that the inducers obtained prior to the purification by HPLC, including the natural sperm, sperm cells, sperm membrane proteins and sperm cytoplasmic contents, could induce spawning in SROs. This suggests that the sperm membrane proteins are involved in the spawning induction. Hence, further spawning inductions were performed on the purified sperm membrane proteins, including the extrinsic- and intrinsic-type membrane proteins. We found that the extrinsic sperm membrane proteins, but not the intrinsic sperm membrane proteins, could effectively induce the spawning activity.

Spawning induction tests using the HPLC fractions of extrinsic sperm membrane proteins showed that the extrinsic sperm membrane proteins eluted at min 41-45 could induce spawning activity, suggesting the presence of spawning inducer(s). Further purification of fraction S3 at min 41-45 resulted in 15 sub-fractions, which corresponded to the elution time min 40 to 55 in the re-purification process (Figure 3.8).

We found that the pooled sub-fraction 1-5 did not induce spawning activity, while the pooled sub-fractions 6-10 and 11-15 could successfully induce the spawning (Table 3.2). Therefore, the pooled sub-fractions 6-10 and 11-15 were then analysed for their peptide contents by using mass spectrometry analysis.

**Table 3.1:** Summary of results from spawning induction using various sperm fractions.

| **Inducer** | **Spawning induction** | | **Spawning induction time and sex ratio (male : female)** | | **Remarks** |
|---|---|---|---|---|---|
| | **Yes** | **No** | **Replicate-1** | **Replicate-2** | |
| Natural sperm-B1 | | | 1 h 45 min | 20 min (USC) (1:9) | Performed at NSW DPI |
| Sperm pellet-B2 | | | 23 min | 64 min (USC) (2:8) | Performed at NSW DPI |
| Sperm milt-B3 | | | - | - (USC) (2:8) | Performed at NSW DPI |
| Freeze-thawed sperm-B4 | | | - | - | Performed at NSW DPI |
| Sperm membrane proteins (P1)-B5 | | | 45 min | 24 min (USC) | Performed at NSW DPI |
| Sperm cytoplasmic contents (S1)-B6 | | | 25 min | 29 min (USC) | Performed at NSW DPI |
| Extracted extrinsic membrane proteins (S2)-B7 | | | *Not required* | *Not required* | - |
| Intrinsic membrane proteins before extraction (P2)-B9 | | | *Not required* | *Not required* | - |
| Extrinsic membrane protein from HPLC (S3)-B8-combined | | | 1 h (3:7) | 98 min (3:7) | Performed at USC, QLD |
| Extrinsic membrane protein from HPLC (S3)-B8-Fc 1-30 | | | - | - | Performed at USC, QLD |
| Extrinsic membrane protein from HPLC (S3)-B8-Fc 31-60 | | | 33 min (3:7) | 27 min (2:8) | Performed at USC, QLD |
| Extrinsic membrane protein from HPLC (S3)-B8-Fc 31-40 | | | - | - | Performed at USC, QLD |
| Extrinsic membrane protein from HPLC (S3)-B8-Fc 41-50 | | | 54 min (5:5) | 30 min (4:6) | Performed at USC, QLD |
| Extrinsic membrane protein from HPLC (S3)-B8-Fc 51-60 | | | - | - | Performed at USC, QLD |
| Extrinsic membrane protein from HPLC (S3)-B8-Fc 41-45 | | | 55 min (2:8) | 28 min (5:5) | Performed at USC, QLD |
| Extracted intrinsic membrane proteins (S4)-B10 | | | *Not required* | *Not required* | Performed at USC, QLD |
| Intrinsic membrane protein from HPLC (S5)-B11-combined | | | - | - | Performed at USC, QLD |

#No spawning occurred in the negative controls

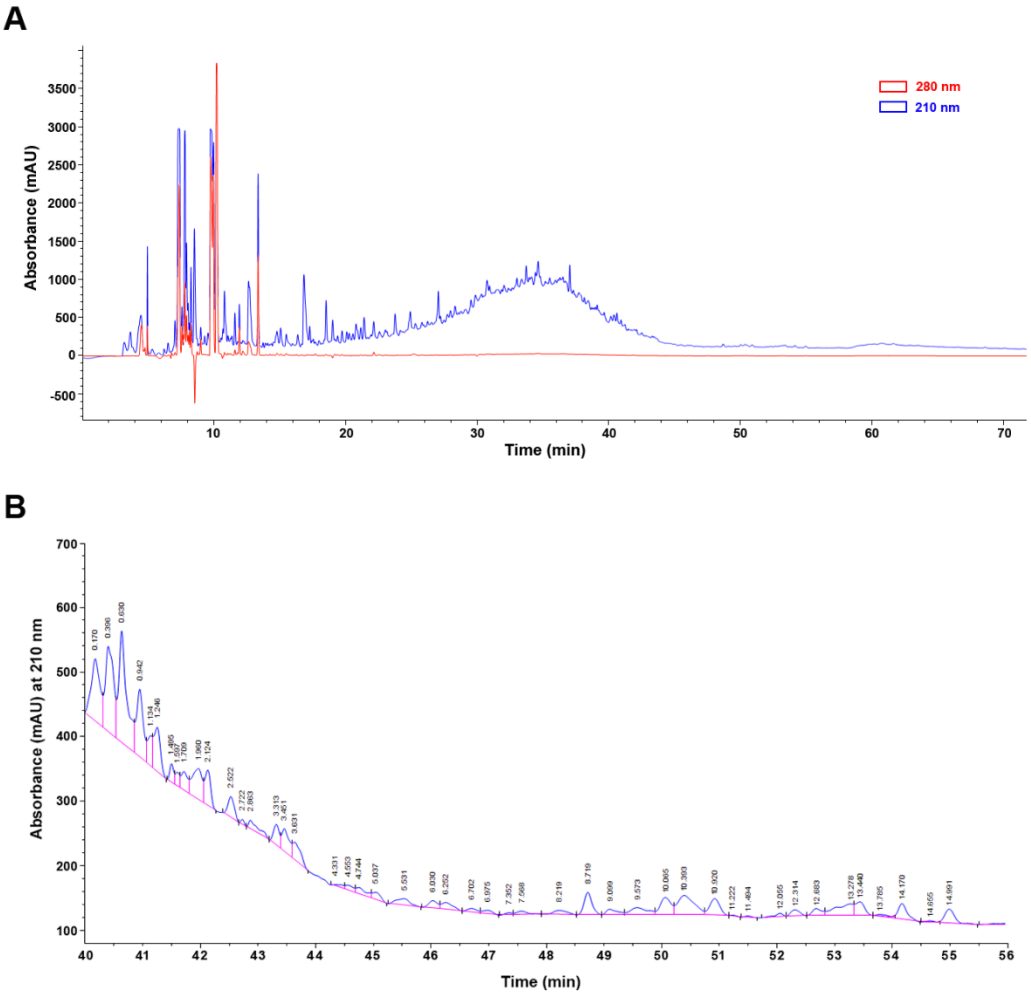


**Figure 3.8:** Re-purification of extrinsic membrane protein from HPLC (S3). (A) Purification of sample S3#2. The fraction S3#2 min 41-45 is further purified by using an increase of %solution B at 0.33% per min (from min 40-55). The protein absorbance was measured at UV wavelength 210 and 280 nm. (B) HPLC profile at UV wavelength 210 of the fraction S3 min 41-45 at higher magnification.

**Table 3.2:** Summary of results from spawning induction using re-purified fraction S3#2 min 41-45, all performed at USC.

| **Inducer** | **Spawning induction** | | **Spawning induction time and sex ratio (male : female)** | |
|---|---|---|---|---|
| | **Yes** | **No** | **Replicate-1** | **Replicate-2** |
| Re-purified fraction S3 min 41-45 by extending gradient time to 15 min (15 sub-fractions in total) | | | | |
| S3 min 41-45 sub-fraction 1-5 | | | - | - |
| S3 min 41-45 sub-fraction 6-10 | | | 31 min (5:5) | 60 min (2:8) |
| S3 min 41-45 sub-fraction 11-15 | | | 92 min (1:9) | 25 min (3:7) |

The peptides identified from the MS analysis are presented in Table 3.3. We found the aminopeptidase N was present in both pooled sub-fractions indicating it may play a role in the stimulation of spawning. However, other proteins such as the calmodulin, which has been known to associate with the fertilisation process in the invertebrates, may also be a potential spawning inducer. A single protein identified in sub-fraction 11-16 was found to be a novel protein, which requires a further investigation into its structure. In conclusion, the result from MS analysis provided the list of spawning inducer candidates that can be selected and subsequently tested for their spawning induction activity in the future study.

**Table 3.3:** Peptides identified in re-purified fraction S3#2 min 41-45 by MS analysis.

| Sub-fraction | Protein hit |
|---|---|
| **S3 min 41-45 sub-fraction 6-10** | |
| Sgl024998 | Calmodulin-beta-like isoform X1 |
| Sgl016337 | Aminopeptidase N |
| Sgl012981 | 60 kDa SS-A/Ro ribonucleoprotein isoform X1 |
| Sgl010680 | Helicase with zinc finger domain 2-like isoform X1 |
| Sgl018296 | Protein bark beetle-like isoform X1 |
| Sgl017740 | Failed axon connections homolog isoform X1 |
| **S3 min 41-45 sub-fraction 11-15** | |
| Sgl002208 | Nascent polypeptide-associated complex subunit alpha, muscle-specific form isoform X2 |
| Sgl016337 | Aminopeptidase N |
| Sgl002883 | Uncharacterized protein LOC111118744 |

## 3.4 Conclusion

In the current study, the spawning inducing factor/pheromone was found to be present in the sperm of the SRO. The sperm was isolated and proteins semi-purified, giving two major extract groups - the intrinsic and extrinsic sperm membrane proteins. Further purification was performed using RP-HPLC, resulting in multiple fractions of sperm membrane proteins. Crude extracts and RP-HPLC fractions were tested in a spawning induction bioassay, in which fully mature oysters were treated with the crude extract or fraction, prior to observation of spawning activity over the period of 2 h. We found that proteins extracted from the extrinsic sperm membrane, but not the intrinsic sperm membrane, could successfully induce spawning in the SRO. Further purification of positive extrinsic membrane fraction S3 min 41-45 led to sub-fractions that were also tested, resulting in two positive sub-fractions (at 6-10 min and 11-15 min). The MS analysis of each revealed 8 proteins, including: aminopeptidase N; calmodulin; 60 kDa SS-A/Ro ribonucleoprotein; protein bark beetle-like; helicase; failed axon connections homolog; nascent polypeptide-associated complex subunit alpha; and, a novel protein. We propose that one, or more of these proteins may play a critical role in stimulating spawning of the SRO.

# 4. Sydney Rock Oyster gamete storage protocols

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## 4.1 Introduction

Commercial hatchery production of SROs is more difficult compared to other species such as Pacific oysters and Eastern Oysters. More challenges are encountered when producing single pair mated SRO families using strip-spawned gametes due to fertilisation deficiency in certain crosses, low rates of success when fertilisation occurs and low levels of larval development in the 24 h period post fertilisation. This compares poorly with very high fertilisation and development rates in the initial 24 h period when natural SRO gametes are used.

A related Future Oysters CRC-P study aimed to accelerate genetic gains for key traits in the SRO BP. One strategy employed by this project to increase gains was to produce more families in each annual breeding run. This project was very successful in doubling the numbers of SRO families that can be produced in a single breeding run. However, to achieve more families, large numbers of valuable broodstock were sacrificed. This is due to only 27% of single pair mated crosses successfully producing a family that can be incorporated into the SRO BP (Dove et al. 2020). Additionally, broodstock available for a breeding run is limited due to all oysters used requiring hatchery conditioning because breeding runs are performed out-of-season. The ability to store gametes for a couple of days means that fertilisations that fail in the early stages of development (< 24 h) can be quickly repeated without sacrificing additional broodstock.

Although holding periods for male gametes (sperm) after manual stripping are potentially in the order of many days during which the gametes may retain fertilising capacity, the holding period for female gametes (ova) is short. Ova removed by manual stripping and held in storage media rapidly lose viability and fertilising capacity over a period of 6-9 h.

The objective of this project related to a straightforward method for benchtop storage of SRO gametes is to develop an understanding of the causes of oocyte degradation and to develop improved storage protocols for oocytes that extend the holding period in vitro after manual stripping. Specifically, the objective reported on here was to evaluate up to 3 methods for the improved benchtop storage of SRO gametes.

## 4.2 Method

### *4.2.1 Sydney Rock Oysters*

SROs were sourced from Port Stephens. All oysters were from non-selected farmed stocks and were at least a year old. Specimens were brought back to the laboratory facilities and maintained in tanks containing InstantOcean® (Product No. SS15-10; prepared as per manufacturer's instructions) at 23°C and used within 7 days. Sex was determined by observation of a gonad sample under a light microscope. Gametes were obtained by strip-spawning using the techniques described in Dove et al. (2019). Quality of gametes was assessed based on shape (oocytes) and activity (sperm).

### 4.2.2 Assessment of the suitability of Eosin Y to assess oocyte viability

Eosin Y is a live/dead stain that works based on stain exclusion from live cells to determine membrane integrity without the need for fluorescence microscopy. It was chosen as the candidate stain to determine cell integrity because of the simplicity of use and the potential to deploy it more widely in studies with limited access to advanced fluorescence microscopy facilities. Oocytes were strip-spawned and then stained using Eosin Y (0.5% w/v in InstantOcean®) at 0, 1, 5, 18 and 24 h by combining in a 1:1 ratio of sample and stain. Using a light microscope, the oocytes were scored as stained (dead) or unstained (alive). Results were displayed as a mean percentage alive over time.

### 4.2.3 Determining fertilisation success of oocytes at varying concentrations of artificial seawater media

The effect of the media at different concentrations on the fertilisation capacity of the gametes was investigated. A fertilisation experiment was conducted where oocytes were water hardened and held in different media ($dH_2O$, 30%, 60%, 100%, and 125% InstantOcean®, and an artificial seawater recipe) after strip-spawning, artificial fertilisation and observation after 3 h. Embryos were assessed for developmental stages after initial cleavage divisions. Fertilisation data were recorded as a percentage fertilised over total ova counted.

### 4.2.4 Determining osmolality and pH differences

Measurements were undertaken to determine whether artificial media properties were comparable to the *in situ* conditions within the gonad. The osmolality and pH of the SRO gonad was also determined as a potential source of modifications to incubation and storage media parameters. Osmolality of InstantOcean®, artificial seawater, homogenised whole SRO gonad, and filtered SRO gonad was determined using an osmometer. The pH of InstantOcean®, homogenised whole SRO gonad, and filtered SRO gonad were also determined using a pH probe.

### 4.2.5 Comparison of media

Pools of oocytes were obtained from female oysters by strip-spawning (Dove et al. 2019). Oocytes were divided among 14 treatments that were combinations of base media (InstantOcean® or artificial seawater) tested with either physiological changes (pH or osmolality) or media supplementation (antibiotics or PVP). Viability was determined over 24 h through determining membrane integrity with Eosin Y staining. Results were expressed as a percentage of oocytes with intact membrane over total number of cells counted.

## 4.3 Results

### 4.3.1 Testing Eosin Y for oocyte viability

Eosin Y was used successfully as a live cell stain for determining vitality (live/dead) of oocytes on the basis of membrane integrity. When used on SRO oocytes, integrity could be determined through penetration of the stain across the membrane wall into the cytoplasm of non-viable oocytes (Figure 4.1). This is the same mechanism for which the stain is used to assess sperm membrane integrity (and thus viability). Upon staining, cells with intact membranes remained clear, however stain entered cells with compromised membranes (Figure 4.1). Furthermore, observations over time indicated that membrane integrity decreased (Figure 4.2) which was expected for vitality over time for SRO oocytes.

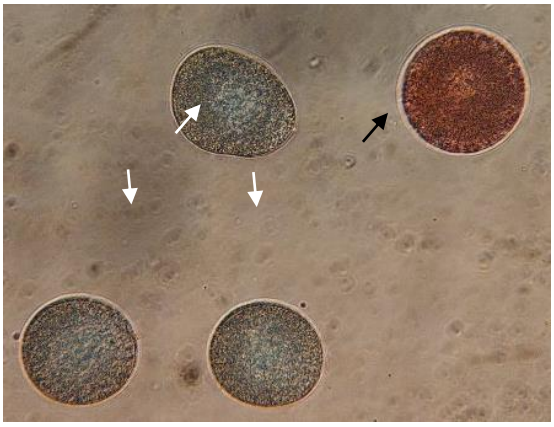

**Figure 4.1:** Comparison between live and dead Sydney Rock Oyster oocytes using Eosin Y stain. Note that the stain did not pass through the membrane of the live oocyte (unstained; denoted by the white arrows) but the stained oocyte (denoted by the black arrow) indicates that the membrane integrity of the oocyte has been compromised.

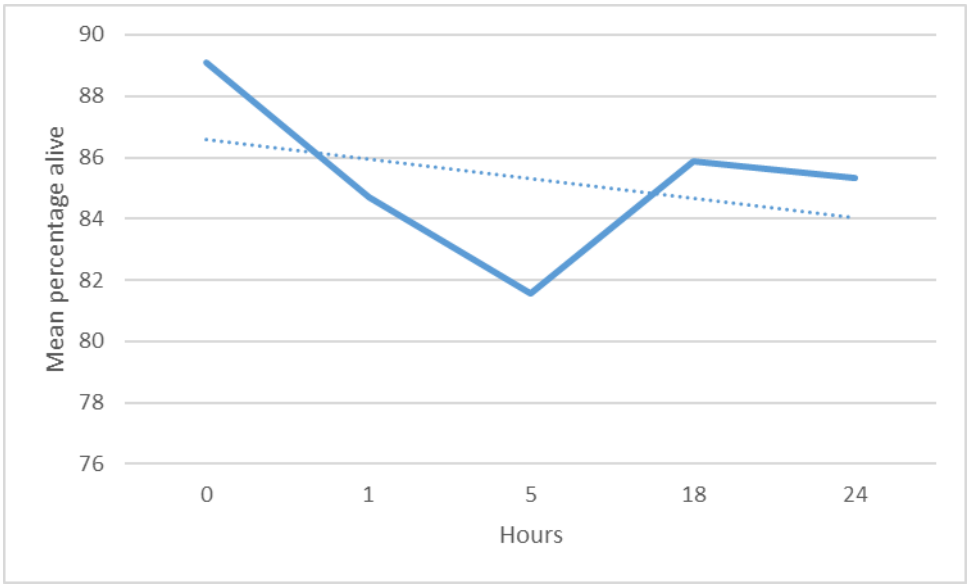


**Figure 4.2:** Mean percentage of Sydney Rock Oyster oocytes determined to be alive through Eosin Y staining over 24 h.

### *4.3.2 Testing fertilisation of oocytes at varying concentrations:*

Fertilisation in different media ($dH_2O$, 30%, 60%, 100%, and 125% InstantOcean®, and an artificial seawater recipe) indicated that fertilisation was highest in 100% InstantOcean® (16.7%), followed by the artificial seawater (6.4%) as seen through embryos undergoing early stages of cleavage division (Figure 4.3). No fertilisation was found in the $dH_2O$ treatment (negative control).

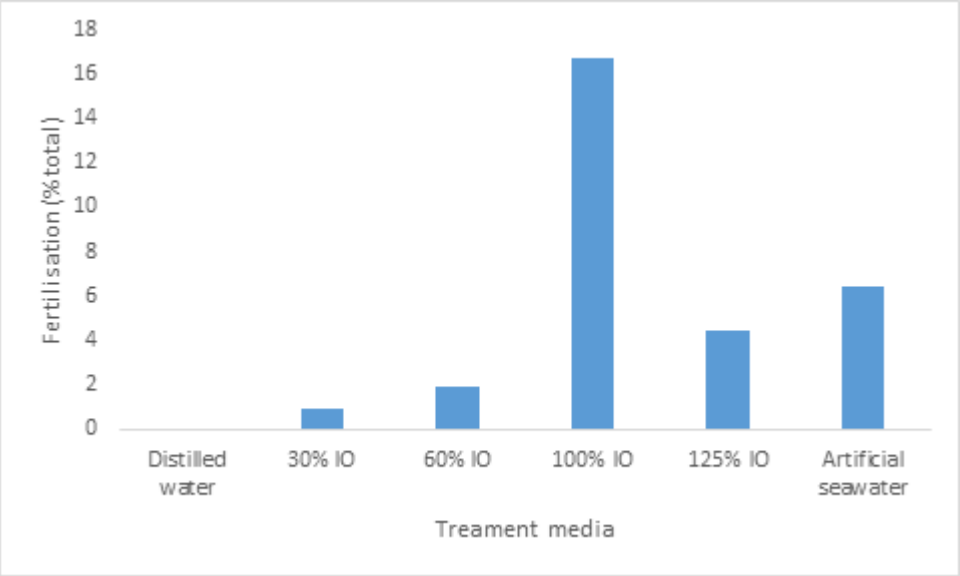


**Figure 4.3:** Fertilisation percentage of oocytes held in various media. IO = InstantOcean®

### *4.3.3 Determining osmolality and pH differences:*

It was considered likely that the physical properties of the media may be strongly influencing the viability of oocytes and embryos post-fertilisation. A micro-probe indicated that the pH of the gonad was around 6.4 whereas, InstantOcean® had a pH of 7.5. This is a difference of 1.1 pH units. A comparison of the osmolality between 100% InstantOcean®, artificial seawater, a homogenized sample of SRO gonad, and filtered gonad tissue found that osmolality was highest in the homogenized SRO gonad tissue (1432 mOsm $kg^{-1}$), then in the filtered gonad tissue (1412 mOsm $kg^{-1}$), artificial seawater (988 mOsm $kg^{-1}$), and lowest in 100% InstantOcean® (821 mOsm $kg^{-1}$) (Figure 4.4).

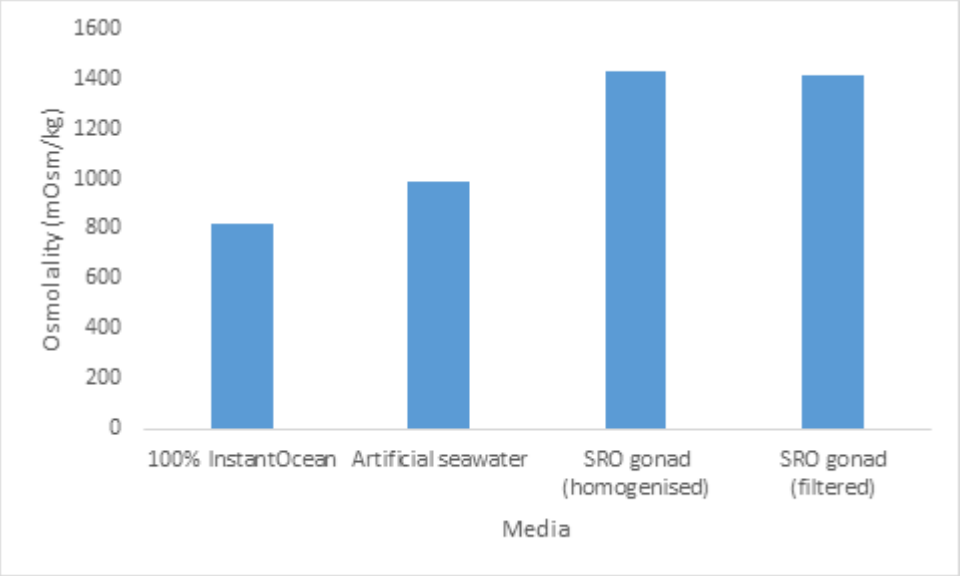


**Figure 4.4:** Differences in osmolality of Sydney Rock Oyster gonad and different media.

### *4.3.4 Comparison of media*

Based on measurements of the conditions within the gonad *in situ* determined previously, a series of 14 different media were developed to test stored oocyte viability over time after stripping to determine the effect of varying the media on prolonging cell viability and holding time.

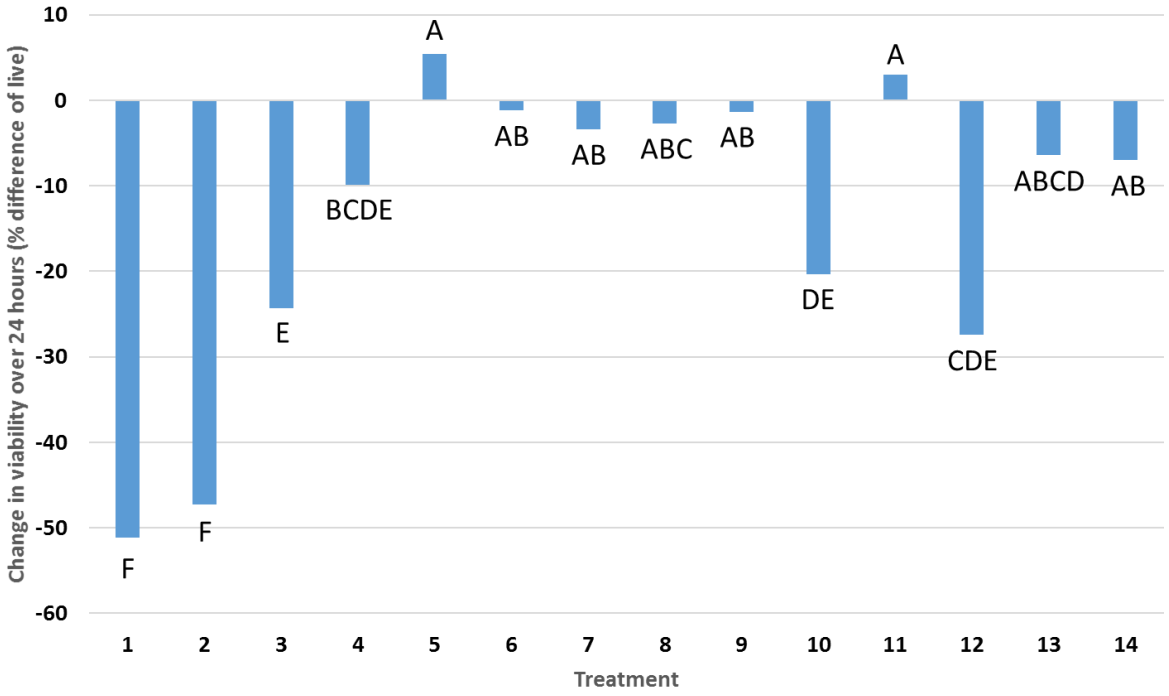


**Figure 4.5:** Differences in the percentage of viable oocytes with intact membranes after 24 h following various changes to incubation media. Bars indicate the change in the "vitality" from initial counts. A Tukey HSD All Pairwise Comparison was conducted between treatments using JMP Pro 14. Different letters indicate significant differences. Treatments are represented as numbers where even numbers do not use InstantOcean® as base media, even numbers use artificial seawater, then 1 and 2 are media without additives, 3 and 4 are pH adjusted, 5 and 6 have increased osmolality using mannitol, 7 and 8 have increased osmolality using mannitol and sucrose, 9 and 10 have 1% penicillin/streptomycin, 11 and 12 have 0.5% penicillin/streptomycin, and 13 and 14 have 3mg $mL^{-1}$ PVP added.

In Figure 4.5, it can be seen that oocytes in base media without alteration showed the greatest decline in oocyte viability (down 51.1% and 47.3% for treatment 1 and 2, respectively) whereas all treatments added some degree of increased viability. The treatments where osmolality (5, 6, 7, and 8) and PVP (13 and 14) were added or increased generally performed the best (71.5, 67.5, 63.1, 61.6, 61.1, and 68.8%, respectively) as did the penicillin/streptomycin when in combination with InstantOcean® (9 and 11 showing 64.4 and 69.5% viability respectively). Membrane integrity was also improved when pH was changed (3 and 4 showing 40.01 and 49.8% viability, respectively) and when penicillin/streptomycin was used in combination with artificial seawater (10 and 12 showing 40.6 and 42.0% viability, respectively).

## 4.4 Discussion

Determination of cell viability through exclusion dyes has been extensively used in the literature for a variety of different cell types (Phillips 1973; Strober 1997, Riss et al. 2016;). Eosin Y has been utilised widely in gamete research (Ahmadi and Ng 1997; Kumar et al. 1997; Ondrizek et al. 1999), however, it has not been used to stain oyster oocytes. Results from the present study indicated that vital (live) cells are able to be differentiated from compromised (dead) cells through dye exclusion. However, further analysis is required to determine whether the results from dye exclusion correlate with fertilisation rates of oocytes (i.e. are all live cells fertilisable).

*In situ*, SRO breeding is triggered by environmental changes such as salinity (Ingle 1951), or temperature changes (Nelson 1928; Ingle 1951), which initiates a synchronised release of male and female gametes into the external environment. It is assumed that the release into the environment initiates activation of the gametes – i.e. sperm to become motile, and oocytes to water harden. Initial testing of the fertilisation differences when oocytes were stored in different media aimed to determine whether the contents of the environment also contributed to lowered fertilisation. We compared InstantOcean® (a commercial salt mix targeted to mimicking seawater) at varying concentrations (30, 60, 100, and 125%). Results indicated that fertilisation was the most successful in the 100% InstantOcean®.

The physiological properties of the gonad were then determined to compare against the properties of InstantOcean®. It was assumed that if the characteristics of the gonad were different to the storage media, then these differences could be adopted in a modified media for extended holding of cells. It was shown that both the pH and the osmolality were different between gonad and tested media (InstantOcean® and artificial seawater). These values were used as a threshold for alterations to the media as an attempt to maintain osmotic homeostasis. The regulation of the cell's internal environment is essential to cell survival (Danziger and Zeidel 2015). Changes in the external environment may trigger cell death pathways within the cell (such as metabolism or apoptosis).

By adopting these changes in the holding media and testing their effects on incubated oocytes, results suggested that the alterations to the media lead to increased vitality determined after a period of 24 h. After 24 h, the treatments had at least a 50% increase in live cells compared to media without any alterations. This is an improvement where the oocytes typically decrease in vitality at an exponential rate in unaltered media.

Despite this result, further tests are required on larger sample sizes to determine whether this trend is generally reproducible and to determine the variability of the response between oysters across lines. As well as this, tests to determine changes in fertility of the oocytes in the holding media will be important in validating and further modifying the technique.

The goal of this project was to determine modifications to current storage media at room temperature for ova collected by manual stripping that can provide changes to be incorporated into the SRO BP Manual (Dove et al., 2019). This section represents the initial steps and the findings of preliminary experiments towards this goal to extend vitality of SRO oocytes at room temperature whilst families are created during breeding runs. Progress in this area opens up further opportunities to develop more efficient fertilisation and breeding protocols in which oocytes can be held for extended periods while the success of initial fertilisations are determined over the initial 48 h of development.

Longer holding periods for ova after removal from the gonad reduces the total number of broodstock sacrificed during a breeding run. This is due to the ability to store oocytes from female broodstock when searching for a male to create a specific cross. As well, if the quality of the pre-fertilisation gametes are also improved, the proportional survival of the larvae will be expected to increase, resulting in larger numbers of larvae at the beginning of a breeding run. Increased larval numbers at the start of a breeding run translates to more spat for each family that can be used by commercial hatcheries for multiplication of key families for industry.

By altering the physical properties of the media, the vitality of SRO oocytes was extended compared to media without changes. Four media properties were assessed. The results of this study showed that increases in the vitality and longer storage intervals could be achieved by: (i) changes in the osmolality and pH towards values closer to those within the natural conditions in the gonad, (ii) supplementation through anti-microbials to decrease contamination, and (iii) the addition of PVP to reduce cell conglomerating.

## 4.5 Conclusion

Fertilisation rates from manually stripped SROs is lower than that of SROs that spawn naturally. The SRO BP currently has a 27% success rate of obtaining oysters from a single pair mated cross. If naturally released gametes were used to create single pair mated families this could increase three fold resulting in less complications for rearing larvae and higher yields of oysters in each family for assessment and available to hatcheries for industry. Typically, SRO oocytes show the best fertilisation rates if fertilised within 24 h of stripping; afterwards, the success decreases exponentially. This project aimed to increase the knowledge of SRO oocyte biophysical characteristics through mimicking physical properties of the gonad, such as pH and osmolality, in storage media to extend the life cycle of the cell *in vitro*. The results obtained suggest that vitality can be improved if these characteristics are altered in the media, and when additives are included (such as antibiotics and PVP). This is a significant step in extending the life of a cell for breeding purposes, effectively extending the window in which quality testing and fertilisations can be conducted. Further approaches to oyster gamete storage are planned to continue at no additional cost to the Future Oysters CRC-P as part of a continuing PhD project at The Univeristy of Newcastle.

# Conclusion

The four aims of this project were to:

1. develop optimised protocols for tetraploidy SRO production and produce a batch of tetraploids for commercial production of triploid SROs.

2. reduce the overall hatchery conditioning period for SROs using neuropeptides.

3. investigate methods to induce natural release of gametes for SROs to increase fertilisation success rates.

4. develop benchtop storage media to hold SRO gametes during breeding run spawnings so that broodstock aren't wasted and important single pair crosses can be redone in the case that the initial attempt was not successful.

Tetraploid inductions were attempted on eleven occasions but none produced a batch of tetraploid SROs to enable hatchery production of triploids using the technique where a tetraploid male is crossed with a triploid female oyster. Eleven attempts were made and the major challenges encountered were low egg numbers in triploid SROs, asynchronous embryonic development in strip-spawned oocytes after fertilisation, poor development and poor larval survival. A very small number of spat were successfully settled from 2 trials however no tetraploids were found in these batches when oysters had reached a size large enough to assay tissue. Further tetraploid inductions are planned by Southern Cross Shellfish in the 2019/2020 reproductive season. Even though no tetraploids were produced, chemical inductions were successful in producing commercial numbers of SRO spat from QX disease reistant broodstock with a triploidy level of in excess of 90% for distribution to industry. NSW DPI evaluated QX disease resistance and oyster growth performance of a subset of these triploids in the Hawkesbury River. Although no QX disease occurred during the field evaluation triploid SROs had significantly faster growth rates compared to a group of selected YC2017 families and non-selected oysters.

Administering the individual forms of buccalin and APGWamide stimulated gonad conditioning in SROs. When the stimulatory effect of two different forms of APGWamide (APGWa and RPGWa) were compared, the APGWa form was more potent than the RPGWa form. For buccalin, the buccalin-G form was more effective than the buccalin-A form in most of reproductive activities assessed. The stimulatory effects of APGWa and buccalin-G on SRO conditioning was comparable. Hence, we propose that either APGWa or buccalin-G can be applied to enough SRO broodstock for a standard breeding or commercial run (40 to 400 individual oysters). Using cocoa butter to deliver a slow release of the peptide caused high mortalities in treated oysters. Direct injection of peptides is therefore preferable and multiple injections are required to maintain the level of the peptides in the oyster's circulatory system in order to successfully control gonad conditioning. Yet, delivery of peptide by injection is relatively difficult and uncontrollable since different individual oysters could receive different doses of the neuropeptide per injection. To overcome this problem, delivery using other techniques, such as oral delivery by using peptide-encapsulated algae, should be considered and tested in the future.

The spawning inducing factor/pheromone was identified in SRO sperm. SRO sperm was isolated and proteins semi-purified, giving two major extract groups - the intrinsic and extrinsic sperm membrane proteins. Further purification was performed using RP-HPLC, resulting in multiple fractions of sperm membrane proteins. Crude extracts and RP-HPLC fractions were tested in a spawning induction bioassay, where reproductively mature oysters were treated with the crude extract or fraction, prior to observation of spawning activity over the period of 2 h. Proteins extracted from the extrinsic sperm membrane, but not the intrinsic sperm membrane, could successfully induce spawning in SROs.

Further purification of positive extrinsic membrane fraction S3 minute 41-45 led to sub-fractions that were also tested, resulting in two positive sub-fractions (at 6-10 min and 11-15 min). The MS analysis of each revealed 8 proteins, including: aminopeptidase N; calmodulin; 60 kDa SS-A/Ro ribonucleoprotein; protein bark beetle-like; helicase; failed axon connections homolog; nascent polypeptide-associated complex subunit alpha; and, a novel protein. It is likely that one, or more of these proteins may play a critical role in stimulating spawning of SROs.

Another objective of this project related to a straightforward method for benchtop storage of SRO gametes. This required an understanding of the causes of oocyte degradation and development of improved storage protocols for oocytes that extend the holding period in vitro after strip-spawning gametes. Three methods for the improved benchtop storage of SRO gametes were assessed which altered the physical properties of the media. Increases in vitality and longer storage intervals of oocytes were achieved by:

- changes to the osmolality and pH towards values closer to those within the natural conditions in the gonad;
- supplementation through anti-microbials to decrease contamination; and
- the addition of PVP to reduce cell conglomerating lead to increases in vitality and longer storage intervals.

Typically, SRO oocytes show the best fertilisation rates if fertilised within 24 h of stripping; afterwards, the success decreases exponentially. The results achieved here are a significant step in extending the life of a cell for breeding purposes, effectively extending the window in which quality testing and fertilisations can be conducted. These findings can be used to increase single-pair fertilisations and be applied directly to the SRO breeding runs. Ongoing research is recommended to evaluate further approaches to gamete storage and this is being undertaken as part of a continuing PhD project at UoN.

Research is ongoing for tetraploidy induction techniques (SCS), neuropeptide delivery to broodstock (USC), spawn inducing factors (USC) and oocyte storage for SROs (UoN). Further research is required to incorporate all findings from this research into routine operations of the SRO BP. However, outcomes from this work are already providing benefits for this breeding program, notably technical improvements to the fertilisation process. This has increased family production success from 27% to 45%. This outcome is relevant for the SRO BP and commercial hatcheries as it reduces the time it takes to create families and the numbers of valuable broodstock required for a breeding or commercial hatchery run.

# Implications

Significant information related to increasing the success of SRO single pair matings was obtained from this project. Tetraploid inductions, neuropeptide broodstock conditioning and oocyte viability experiments all utilised fertilisation assays which led to a number of technical improvements to this process. New techniques were incorporated into the SRO BP manual and then used in the latest breeding run in October 2019. Family production success increased from an average of 27% for the 2015, 2016, 2017 and 2018 year classes to 45% for the 2019 year class. This outcome is relevant for the SRO BP and commercial hatcheries and will reduce the time it takes to create families and the numbers of valuable broodstock required for a breeding or commercial hatchery run.

Work to develop storage techniques for SRO gametes has both increased our understanding of gamete viability and increased the flexibility of procedures used for controlled mating of oysters. This has supported the improvement in SRO fertilisation success and provided new opportunities for mating plan designs not previously contemplated.

The achievements from this Future Oysters CRC-P project are liklely to be transferable to other oyster species. This research will be published to enable hatchery operators in Australia and around the globe to test gamete storage media, neuropeptides to increase broodstock conditioning rates and spawn inducing factors on Pacific Oysters as well as other commercially important bivalve species.

The knowledge gained and the improvements to SRO breeding achieved within the Future Oysters CRC-P have been synthesised into a new plan for SRO research that aims to reduce the annual cost of breeding by one third. For the SRO BP, annual breeding and progeny testing costs $350,000 and evaluations have started in order to lower this operational cost to below $250,000. This reduced operational cost is expected to accelerate full adoption of the SRO BP by industry.

Overall, hatcheries are now estimated to be providing up to 20% of the stock used by the SRO industry, which equates to a farm gate value for hatchery stock of approximately $10 million per annum. The proportion of this stock derived from selective breeding is increasing and is expected to form the majority of hatchery seed supply in forthcoming seasons.

# Recommendations and Further Development

Research continues on tetraploidy induction techniques, neuropeptide delivery to broodstock, spawning induction and oocyte storage for SROs. Further research is required to incorporate all findings from this Future Oysters CRC-P project into routine operations of the SRO BP. However, outcomes from this work are already providing benefits for this breeding program. Notably, the increase in family production success from 27% to 45%.

More tetraploid inductions are recommended to improve success of triploid female and diploid male crosses that are subjected to CB treatments. Focus will be placed on increasing fertilisation rates and improving the synchronicity of development after fertilisation. Using naturally released gametes will be one way to achieve this.

A more effective delivery method for neuropeptides to broodstock is likely to reduce the broodstock conditioning time period and reduce variability in results. Injection of the peptide multiple times throughout conditioning meant that additional stress was placed on the broodstock and it was difficult to ensure injection was directly into the muscle. This method was also time consuming when 400 oysters required treatment. Research into delivery using other techniques, such as oral delivery by using peptide-encapsulated algae, should be considered for future investigation.

Further research on testing the sperm fractions as a SRO spawning inducer is underway at USC. Ongoing research is recommended to evaluate further approaches to gamete storage and this is being undertaken as part of a continuing PhD project at UoN.

# Extension and Adoption

The outcomes from this research extend directly to the SRO BP and hatcheries producing SROs. Outcomes related to conditioning and strip-spawning that improve these processes have been incorporated into the SRO BP Manual (Dove et al. 2019). Improvements to conditioning and strip-spawning were used in the most recent SRO breeding run to produce the 2019 year class. The success rate of single pair mated crosses in this run was 45% which meant that fewer broodstock were sacrificed and only oysters that were selected from within families for superior growth were used – a first for the SRO BP.

Project outcomes have directly contributed to:

- increased reliability in SRO breeding;
- delivery of improved oysters to industry that have superior QX disease resistance, growth and marketability characteristics; and
- enhanced methods for SRO breeding documented in the SRO BP manual.

Publication of this research in international scientific journals is anticipated. The authors have extended research outcomes from this project at conferences, oyster industry seminars and sharing knowledge with growers during field work. Extension of outcomes from this project was provided through the following channels:

The references provided below are in chronological order with the most recent listed first.

**Conference presentations**

Dove, M., Kube, P., Lind, C., Cumbo, V., O'Connor, W., Saowaros, M., Elizur, A., Seeto, R., Gibb, Z., Abramov, T., Raftos, D., Wilkie, E., The Select Oyster Company and Southern Cross Shellfish (2019). New technologies to improve Sydney Rock Oyster breeding and production. The South Australian Oyster Industry 2019 Seminar, 21-23 August 2019, Streaky Bay, South Australia.

Dove, M., Kube, P., Lind, C., Cumbo, V., O'Connor, W., Saowaros, M., Elizur, A., Seeto, R., Gibb, Z., Abramov, T., Raftos, D., Wilkie, E., The Select Oyster Company and Southern Cross Shellfish (2019). The Sydney Rock Oyster Breeding Program – update and lessons for the Australian oyster industry. Shellfish Futures 2019, 16-17 August 2019, Orford, Tasmania.

Dove, M., Kube, P., Lind, C., O'Connor, W. and The Select Oyster Company (2019). Sydney Rock Oyster Breeding Program – traits, trials and achievements. 2019 NSW Oyster Conference, 6-8 August 2019, Wallis Lake, New South Wales.

Dove, M., Kube, P., Lind, C., Cumbo, V., O'Connor, W., Saowaros, M., Elizur, A., Seeto, R., Gibb, Z., Abramov, T., Raftos, D., Wilkie, E., The Select Oyster Company and Southern Cross Shellfish (2019). Advancement in the Sydney Rock Oyster Breeding Program – families and new technologies. 2019 NSW Oyster Conference, 6-8 August 2019, Wallis Lake. New South Wales.

Dove, M. and O'Connor, W. (2019). Refining Sydney Rock Oyster breeding. 3rd Australia New Zealand Marine Biotechnology Conference, 20-22 May 2019, The University of New South Wales, Sydney Australia.

Dove, M. and O'Connor, W. (2018). History, status and future of oyster culture in Australia. Rushan International Oyster Forum, 21-23 April, China.

McOrrie, S., Dove, M. and O'Connor, W.A. (2017). Breeding a "better" disease-resistant oyster – the trade-offs. 7th International Oyster Symposium, 11-14 September 2017, Bangor, UK.

Wilkie, E. (2017). Boosting Sydney Rock Oyster production through an industry owned breeding program. Seafood Directions, 27 September 2017, Sydney, Australia.

Dove, M., Kube, P., O'Connor, W., Johnston, K., Archer, B. and Wilkie, E. (2017). Breeding better oysters: traits and trade-offs. NSW Oyster Conference, 22-24 August, Merimbula, NSW.

Dove, M., Kube, P. and O'Connor, W. (2017). Breeding disease-resistant Sydney Rock Oysters for the future. NSW Oyster Conference, 22-24 August, Merimbula, NSW.

**Non-scientific communications**

Regular project updates were provided by Dove and O'Connor to the NSW Aquaculture Research Advisory committee.

Dove, M. (2019). Sydney Rock Oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 24, December 2019.

Dove, M. (2018). CRC-P News: Smart Strategies for Sydney Rocks. Oysters Australia website https://www.oystersaustralia.org/blog/smart-strategies-for-sydney-rocks

Dove, M. (2018). Sydney Rock Oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 23, December 2018.

Dove, M. (2018). Sydney Rock Oyster breeding program – Update from Mike Dove. OceanWatch Australia for Oyster News (December edition).

Dove, M. (2018). Sydney Rock Oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 22, May 2018.

Dove, M. (2018). 2016-803 - New Technologies to Improve Sydney Rock Oyster Breeding and Production. Presentation at the Oyster Australia R&D Meeting, Sydney Fish Market, Sydney, NSW, 9 April 2018.

Dove, M. (2017). Sydney Rock Oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 21, June 2017.

Dove, M.C. and O'Connor, W.A. (2017). NSW DPI breeding and research update. Oceanwatch and Local Land Services Travelling Workshops for the South Coast Oyster Industry, 30 May – 2 June: Tanja, Pambula, Batemans Bay and Greenwell Point, NSW.

Dove, M.C. and O'Connor, W.A. (2017). Sydney Rock Oyster breeding: current progress and future directions. FRDC Hatchery Hub Workshop, 8 February. Port Stephens Fisheries Institute, Taylors Beach, NSW.

Dove, M. (2016). Sydney Rock Oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 20, December 2016.

## Project coverage

Information related to this project and the Future Oysters CRC-P was provided to the following ABC programs:

| | |
|---|---|
| 7 August 2019 | ABC Mid and North Coast Rural Report (Reporter: Michael Cavanagh). Interview at Barclays Oyster Shed at the NSW Oyster Conference 6:15 am. https://www.abc.net.au/radio/midnorthcoast/programs/mid-and-north-coast-rural-report/mid-and-north-coast-rural-report/11372472 |
| 3 October 2018 | ABC Landline Interview with Sean Murphy at Port Stephens Fisheries Institute 'Rock Steady: Sydney rock oysters enjoy a resurgence in price and popularity' https://www.abc.net.au/news/2018-11-03/rock-steady:-sydney-rock-oysters-enjoy-a/10463346 |
| 23 February 2017 | ABC Rural Report: (Reporter: Bronwyn Herbert) Oyster growers hopeful new genetics boost quality. http://www.abc.net.au/news/rural/2017-02-23/growers-access-to-oyster-genetics/8296764 |

# Project materials developed

Sydney Rock Oyster Breeding Manual updates

# Appendices

## List of researchers and project staff

Mr Tomer Abramov, The University of the Sunshine Coast

Mr Brandt Archer, NSW Department of Primary Industries

Assoc. Prof. John Clulow, The University of Newcastle

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## Intellectual Property

### Prior intellectual property that project partners brought to this project:

NSW DPI's multi-generational data set on traits of Sydney Rock Oyster breeding and selection.

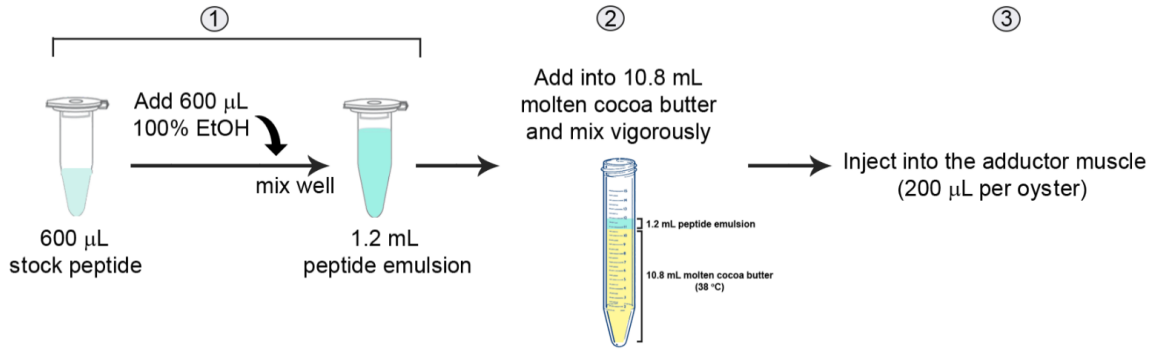
NSW DPI's selected family lines of Sydney Rock Oysters including those supplied by NSW DPI or held/supplied by third parties.

Oyster Selective Breeding database, owned by CSIRO.

### Intellectual property arising from this project:

Breeding population animals, the pedigree records and the performance data relating to those animals from the 2016, 2017 and 2018 year classes generated during the life of this project is owned by NSW DPI.

## Preparation of Peptides for Injection (Section 2)




1. Prepare peptide emulsion by mixing **600 µL stock peptide** (concentration of 2 mg $mL^{-1}$) with **600 µL 100% EtOH**, mix well and ***keep on ice***. This volume will be enough for injecting 50 oysters (extra volume included).

2. Just before the injection*****, transfer **1.2 mL of peptide emulsion** into 15mL tube containing **10.8 mL molten cocoa butter**[#], mix by vortexing or shaking vigorously. The total volume was 12 mL.

3. Load 1 mL of peptide-cocoa butter mixture into 1 mL syringe (avoiding air bubbles; can use 16G needle to assist peptide loading). Keep the remaining peptide-cocoa butter mixture in the water bath (38 °C). For injection, insert the needle (size 28G) into the middle area of adductor muscle. Inject **200 µL of peptide-cocoa butter mixture** slowly into the muscle. Remove the needle and leave the injected oyster out of the water for at least 20 min before placing it in the culture tank.

***** Since peptides are not stable in warm cocoa butter, the peptide-cocoa butter mixture must be used immediately after preparation.

[#] Cocoa butter can be melted faster in hot water and subsequently kept in warm water (38 °C) to maintain it in liquid form. Before mixing the peptide with cocoa butter, the temperature of cocoa butter should be about 38 °C.

## References


Ahmadi, A. and Ng, S.C. (1997). Fertilization and development of mouse oocytes injected with membrane-damaged spermatozoa. *Human Reproduction*, 12:2797-2801.

Allen Jr, S.K. (1988). Triploid oysters ensure year-round supply. *Oceanus*, 31:58-63.

Allen Jr, S.K., Downing, S.L. and Chew, K.K. (1989). Hatchery manual for producing triploid oysters. University of Washington Press. Seattle, WA, USA.

Allen Jr, S.K. (2012). Enhancement of tetraploid and triploid production in the Australian Pacific Oyster Industry. Final Report for the Australian Seafood Cooperative Research Centre Project No. 2012/728. 9 pp.

Askew, G.G. (1978). A generalised growth and mortality model for assessing the economics of bivalve culture. *Aquaculture*, 14:91-94.

Benzie J.A.H., Grove-Jones R.P. and Adlard R.D. (2002) Review of hatchery production technology and breeding program for Sydney rock oysters. FRDC Project No. 2001/213

Bernard, I., Massabuau, J.C., Ciret, P., Sow, M., Sottolichio, A., Pouvreau, S. and Tran, D. (2016). *In situ* spawning in a marine broadcast spawner, the Pacific oyster *Crassostrea gigas*: Timing and environmental triggers. *Limnology and Oceanography*, 61:635-647.

Burbach, J. P. (2011). What are neuropeptides? *Methods Mol. Biol.*, 789:1-36.

Byrne, J.H., Cristo, C.D. and Koene, J.M. (2017). *Neurobiology of Reproduction in Mollusks: Mechanisms and Evolution*: Oxford University Press.

Cosmo, A. D., and Polese, G. (2013). Chapter 39 - Molluscan Bioactive Peptides A2 - Kastin, Abba J *Handbook of Biologically Active Peptides (Second Edition)* (pp. 276-286). Boston: Academic Press.

Danziger, J. and Zeidel, M.L. (2015). Osmotic homeostasis. *Clin. J. Am. Soc. Nephrol.*, 10:852-62.

Desrosiers, R., Gerard, A., Peignon, J.M., Naciri, Y., Degresne, L., Morasse, J., Ledu, C., Phelipot, P., Guerrier, P. and Dubé, F. (1993). A novel method to produce triploids in bivalve molluscs by the use of 6 dimethylaminopurine. *J. Exp. Mar. Biol. Ecol.*, 170: 29-43.

Dheilly, N.M., Lelong, C., Huvet, A., Kellner, K., Dubos, M.-P., Riviere, G. and Favrel, P. (2012). Gametogenesis in the Pacific oyster *Crassostrea gigas*: a microarrays-based analysis identifies sex and stage specific genes. *PLOS ONE, 7*(5), e36353-e36353.

Dinamani, P. (1974) Reproductive cycle and gonadial changes in the New Zealand rock oyster *Crassostrea glomerata*. *New Zealand Journal of Marine and Freshwater Research*, 8:39-65.

Dove, M.C. and O'Connor, W.A. (2012) Reproductive cycle of Sydney rock oysters, *Saccostrea glomerata* (Gould 1850) selectively bred for faster growth. Aquaculture, 324:218-225.

Dove, M., Kube, P., Lind, C., Cumbo, V., Raftos, D. and O'Connor, W. (2020). Accelerated Sydney Rock Oyster (SRO) Breeding Research. Future Oysters CRC-P Project 2016-802, 41 pp. NSW Department of Primary Industries, November 2019.

Dove M., Kube P., O'Connor S., Lind C., Johnston K. Archer B. and Select Oyster Company (2019) The Sydney rock oyster family based breeding program manual. NSW Department of Primary Industries, Taylors Beach, NSW Australia.

Eudeline, B., Allen, Jr., S.K. and Guo, X. (2000). Optimization of tetraploid induction in Pacific oysters, *Crassostrea gigas*, using 1st polar body as a natural indicator. *Aquaculture*, 187:73-84.

Evans, J.P. and Sherman, C.D. (2013). Sexual selection and the evolution of egg-sperm interactions in broadcast-spawning invertebrates. *Biol. Bull.*, 224:166-183.

Geraerts, W.P.M., Smit, A.B., Li, K.W., Vreugdenhil, E. and van Heerikhuizen, H. (1991). Neuropeptide gene families that control reproductive behaviour and growth in molluscs. In N. N. Osborne (Ed.), *Current Aspects of the Neurosciences: Volume 3* (pp. 255-304). London: Macmillan Education UK.

Guo, X., DeBrosse, G.A. and Allen Jr, S.K., (1996). All-triploid oyster (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. *Aquaculture*, 142:149-161.

Hand, R.E., Nell, J.A. and Maguire, G.B. (1998a). Studies on triploid oysters in Australia. X. Growth and mortality of diploid and triploid Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). *Journal of Shellfish Research*, 17:1115-1127.

Hand, R.E., Nell, J.A. and Thompson, P.A. (2004). Studies on triploid oysters in Australia. XIII. Performance of diploid and triploid Sydney rock oyster, *Saccostrea glomerata* (Gould, 1850), progeny from a third generation breeding line. *Aquaculture*, 233:93-107.

Hand, R.E., Nell, J.A., Smith, I.R. and Maguire, G.B. (1998b). Studies on triploid oysters in Australia. XI. Survival of diploid and triploid Sydney rock oysters (*Saccostrea commercialis* (Iredale and Roughley) through outbreaks of winter mortality caused by *Mikrocytos roughleyi* infestation. *Journal of Shellfish Research*, 17:1129-1135.

In, V. V., Ntalamagka, N., O'Connor, W., Wang, T., Powell, D., Cummins, S. F., and Elizur, A. (2016). Reproductive neuropeptides that stimulate spawning in the Sydney Rock Oyster (*Saccostrea glomerata*). *Peptides*, 82:109-119.

Ingle, R.M. (1951). Spawning and setting of oysters in relation to seasonal environmental changes. *Bulletin of Marine Science*, 1:111-135.

Kumar, T.R., Wang, Y., Lu, N. and Matzuk, M.M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature Genetics*, 15:201-204.

Lawrence, D.R., Scott, G.I., 1982. The determination and use of condition index in oysters. Estuaries 5, 23-27.

McCombie, H., Ledu, C., Pheliot, P., Lapegue, S., Boudry, P. and Gerard, A. (2005). A complementary method for the production of tetraploid *Crassostrea gigas* using crosses between diploids and tetraploids with cytochalasin B treatments. *Marine Biotechnology*, 7:318-330.

Morishita, F., Furukawa, Y., Matsushima, O. and Minakata, H. (2010). Regulatory actions of neuropeptides and peptide hormones on the reproduction of molluscs. *Canadian Journal of Zoology*, 88:825-845.

Nagasawa, K., Oouchi, H., Itoh, N., Takahashi, K.G. and Osada, M. (2015). *In vivo* administration of scallop GnRH-like peptide influences on gonad development in the Yesso scallop, *Patinopecten yessoensis*. *PLOS ONE*, 10(6), e0129571. doi:10.1371/journal.pone.0129571

Nässel, D. R. and Larhammar, D. (2013). Neuropeptides and Peptide Hormones. In G. C. Galizia and P.-M. Lledo (Eds.), *Neurosciences - From Molecule to Behavior: a university textbook* (pp. 213-237). Berlin, Heidelberg: Springer Berlin Heidelberg.

Nell, J.A. (2006). Manual for selection of Sydney rock oysters for fast growth and disease resistance. NSW Department of Primary Industries. Cronulla, NSW, Australia. 52 pp.

Nell, J.A., Hand, R.E., Goard, L.J, McAdam, S.P. and Maguire, G.B. (1996). Studies on triploid oysters in Australia: Evaluation of cytochalasin B and 6-dimethylaminopurine for triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). *Aquaculture Research*, 27:689-698.

Nell, J.A., McMahon, G.A. and Hand, R.E. (1998). Tetraploidy induction in Sydney rock oysters. Aquaculture CRC Project D.4.2. NSW Fisheries, Port Stephens Research Centre, Taylors Beach, NSW, Australia. 26 pp.

Nelson, T. C. (1928). Relation of spawning of the oyster to temperature. *Ecology*, 9:145-154.

O'Connor W.A., Dove M.C., Finn, B. and O'Connor, S.J. (2008). Manual for Hatchery Production of Sydney Rock Oysters (*Saccostrea glomerata*). Final Report to Fisheries Research and Development Corporation, Deakin, ACT, Australia. NSW Department of Primary Industries-Fisheries Research Report Series Nº 20, 53 pp.

Ondrizek, R.R., Chan, P.J., Patton, W.C. and King, A. (1999). An alternative medicine study of herbal effects on the penetration of zona-free hamster oocytes and the integrity of sperm deoxyribonucleic acid. *Fertility and Sterility*, 71:517-522.

Pantelouris, E.M. (1967). Chapter 35 - Asexual and sexual reproduction. *Introduction to Animal Physiology and Physiological Genetics* (pp. 367-377): Pergamon.

Peachy, B.L. and Allen Jr, S.K. (2016). Evaluation of cytochalasin B and 6-dimethylaminopurine for tetraploid induction in the eastern oyster, *Crassostrea virginica*. *Aquaculture*, 450:199-205.

Phillips, H.J. (1973). Dye exclusion tests for cell viability. In *Tissue culture*, pp. 406-408: Elsevier.
Powell, D., Subramanian, S., Suwansa-ard, S., Zhao, M., O'Connor, W., Raftos, D. and Elizur, A. (2018). The genome of the oyster *Saccostrea* offers insight into the environmental resilience of bivalves. *DNA Research*, 25:655-665. doi:10.1093/dnares/dsy032
Riss, T.L., Moravec, R.A., Niles, A.L., Duellman, S., Benink, H.A., Worzella, T.J. and Minor, L. (2016). Cell viability assays. In *Assay Guidance Manual [Internet]*: Eli Lilly and Company and the National Center for Advancing Translational Sciences.
Royer, J., Seguineau, C., Park, K.-I., Pouvreau, S., Choi, K.S. and Costil, K. (2008). Gametogenetic cycle and reproductive effort assessed by two methods in 3 age classes of Pacific oysters, *Crassostrea gigas*, reared in Normandy. *Aquaculture*, 277:313–320.
Rye M. (2012) Review of Australian breeding programs for Pacific Oysters, Sydney Rock Oyster, Barramundi and Prawns. Akvaforsk Genetics Centre A/S Norway. Confidential report for the Seafood CRC.
Select Oyster Company, Dove M., Kube P. and Lind C. (2019) Genetic services for the multi-trait, single pair mated Sydney Rock Oyster breeding program. FRDC Project No. 2015/230.
Stanley, J.G., Hidu, H. and Allen Jr, S.K. (1984). Growth of American oysters increased by polyploidy induced by blocking meiosis I but not meiosis II. *Aquaculture*, 37:147-155.
Strober, W. (1997). Trypan blue exclusion test of cell viability. *Current Protocols in Immunology*, 21:A. 3B. 1-A. 3B. 2.
Taylor, A., Mills, D., Wang, T., Ntalamagka, N., Cummins, S. F. and Elizur, A. (2018). A sperm spawn-inducing pheromone in the silver lip pearl oyster (*Pinctada maxima*). *Mar. Biotechnol.*, 20:531-541. doi:10.1007/s10126-018-9824-6
Wadsworth, P., Wilson, A.E. and Walton, W.C. (2019). A meta-analysis of the growth rate in diploid and triploid oysters. *Aquaculture*, 499:9-16.