JAMES COOK UNIVERSITY
College of Science and Engineering

Broodstock conditioning techniques, filtration rate and bioremediatory ability of the tropical Blacklip rock oyster *(Saccostrea echinata)*

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Statement of the Contribution of Others

- Financial support was received from The Cooperative Research Centre for Developing Northern Australia (CRCNA) and Northern Territory Government Department of Industry, Tourism and Trade as part of the 'Northern Australian Tropical Rock Oyster research and development project'.
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ABSTRACT

The tropical Blacklip rock oyster, *Saccostrea echinata* (Quoy and Gaimard 1835) is an emerging aquaculture species which displays great potential and will likely play an important role in expanding Australia's well established temperate oyster sector into the tropics. Due to limited spat supply, this species has not yet reached large-scale commercial production, however, current research and development is seeking to overcome this bottleneck. Effective broodstock conditioning methods are a first step towards accomplishing reliable, year-round spat production.

Present day prawn culture typically results in elevated levels of nutrients, suspended inorganic solids and chlorophyll-A in effluent water, resulting from pond silt, shrimp waste and undigested feed. The ecological consequences surrounding effluent discharge have prompted interest into evaluating biological filtration methods as a cost-effective alternative to current waste water treatment methods. Bivalves have long been recognised for their role in maintaining healthy waterways and improving water quality through filtration.

Chapter 2 was a pilot study to record the effect of elevated temperature on *S. echinata* broodstock reproductive condition, over a 12 week period. Tissue samples were collected at weeks 1, 5, 8, 11 and 12 to measure progress of gonad development. Biometric data was used to calculate a mean condition index (CI) and reproductive condition was assessed histologically to classify the developmental stage of individuals and generate a mean gonad index (GI). Mean CI remained relatively stable within the control treatment, while the heated treatment displayed a gradual pattern of decline. There was no significant difference in mean CI between tank or treatment ($p > 0.05$) and no linear relationship present between mean CI and temperature (R = 0.18). Histology assessment highlighted a large proportion (87%) of sampled broodstock remained in the regressive (R) stage and were gender indeterminant. Mean GI within the control treatment remained stable throughout the experiment period, whilst the heated treatment experienced a minor increase in week 3. Treatments were not significantly different and a moderate positive relationship was present between GI and temperature (R = 0.35). This study demonstrates that temperature manipulation alone is not an effective treatment to condition *S. echinata* broodstock outside of their natural reproductive season.

Following from Chapter 2, which focused on the effects of temperature, Chapter 3 involved a study to investigate the effect of salinity (20, 25, 30 or 36 ppt) on *S. echinata* broodstock conditioning, over a period of 12 weeks. Broodstock tissue samples were collected at the commencement of the trial and in weeks 2, 4, 6, 7, 8, 9, 10, 11 and 12. As per Chapter 2, biometric data was used to calculate a mean CI and reproductive condition was assessed histologically to classify the development stage of individuals and generate a mean GI. Mean CI remained stable throughout the conditioning period and did not differ significantly between treatments (F = 0.609; *df 1/357*; $p < 0.05$). In contrast, mean GI displayed greater variability, and differed significantly between treatments (F = 6,455; *df 1/35*; $p <$

0.05). A Pearson's Correlation coefficient of R = -0.397 detected a moderate negative relationship between GI and salinity. Analyses of gonad stage frequency revealed that a significantly greater proportion of oysters had reached ripe condition within the lower salinity treatments of 20 ppt (P = 0.007) and 25 ppt (P = 0.001) over the duration of the trial. Investigation of histology slides also revealed unintended spawning occurred during the conditioning trial. At completion of the conditioning trial 11 broodstock were successfully induced to spawn, with the majority of spawning individuals from the two lowest salinity treatments (20 and 25 ppt). This study documents successful broodstock conditioning of *S. echinata*, for the first time, with results confirming that gonad maturation is influenced by salinity.

Chapter 4, investigated the bioremediatory potential of Blacklip rock oysters. Part A of this Chapter determined the influence of temperature (20, 24, 28, 32 °C) on the filtration rate of *S. echinata.* While, Part B investigated the bioremediatory ability of *S. echinata* to reduce total nitrogen (TN), total phosphorus (TP), total suspended solids (TSS) and chlorophyll-A within effluent from a prawn farm. Results demonstrated that higher water temperatures promote a faster filtration rate and identified an optimal performance range of 24 – 32 °C for a filtration rate of 12.68 – 15.20 L/hr/g. A maximum filtration rate of 15.20 L/hr/g was achieved at 24 °C. Following this, Part B results demonstrated the high density (0.66 oysters / L) of stocked oysters resulted in significant reduction of all parameters, with TN reduced by 21%, TP reduced by 27%, TSS reduced by 99% and chlorophyll-A reduced by 39% when compared to the original effluent. Tissue analysis of 10 oysters with a mean whole weight of 75.4 g, revealed a mean of 0.09g of nitrogen per oyster. Scaling these values suggests that 1.20 kg of nitrogen is removed per tonne of harvested oysters. This study is the first to investigate the bioremediatory potential of *S. echinata* and demonstrates their potential to improve aquaculture wastewater treatment practices and bioremediation. The findings of these several studies have a direct application to this emerging industry and will hopefully assist in the future commercialisation of the species.

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List of Abbreviations

DVM: Dorso-ventral measurement

APM: Anterior-posterior measurement

GI: Gonad index

CI: Condition index

MARFU: Marine and Aquaculture Research Facility

TSS: Total suspended solids

TN: Total nitrogen

TP: Total phosphorus

FR: Filtration rate

GBRMP: Great Barrier Reef Marine Park

IMTA: Integrated multi-trophic aquaculture

List of equations:

Total daily algal feed: Number of oysters X algal concentration per oyster per day Eq. 1

Mean Gonad Index: $M = \frac{\Sigma(ns)}{N}$, Eq. 2

Mean Condition Index: $CI = \frac{v}{w-s}$ X 100, Eq. 3

Filtration Rate $FR = (V/nt)\ln(C0/Ct)/w$ Eq. 4

1 CHAPTER 1. GENERAL INTRODUCTION

1.1 Background

Increasing marine food production is essential to sustain the protein demand of our rising global population (Wijsman et al., 2018). Edible bivalves, such as oysters, clams, mussels and scallops, are one of the most widely consumed marine products, providing a rich source of omega-3 fatty acids, iron, selenium and zinc for human nutrition to local communities (Glude 1984; Wright et al., 2018). Edible marine bivalves account for 14% of total marine production worldwide, which amounted to 16 million tonnes with a net value of 26 million US dollars, in 2015 (Wijsman et al., 2018). Aquaculture is the primary contributor to global bivalve production and has risen steadily from 1995 to 2005 with an average increase of 6.8% annually (FAO Globefish, 2019). In contrast to aquaculture sector growth, the contribution of wild harvest to the bivalve production sector is in decline, contributing only 11% in 2014 (Wijsman et al., 2018). Market demand for sustainably sourced bivalves continues to be strong, with reports suggesting that the associated health benefits of improved cardiac function, reduced cholesterol and blood pressure, and their environmentally friendly, green image are among the primary reasons driving consumer interest (FAO Globefish, 2019; Venugopal & Gopakumar, 2017).

1.2 The global picture

The cultivation of bivalves is one of the earliest documented aquaculture practises, with records showing that the Indigenous Peoples of British Columbia harvested shellfish from man-made clam gardens over 3500 years ago (Smith et al., 2019). Since its early origin, a number of countries now produce commercial quantities of edible oysters, encompassing a wide variety of species, spat supply methods and grow out techniques (Duthie, 2012; Pawiro, 2010; Wijsman et al., 2018). The global production and value of edible oysters has increased steadily since 2004, with both temperate and tropical regions having experienced substantial growth in the last decade (FAO FishStatJ, 2019) (Figure 1.1). Global production is dominated by the Pacific cupped oyster (*Crassostrea gigas*) which accounts for approximately 80% of total production, followed by the American cupped (*Crassostrea virginica*) and Slipper cupped (*Crassostrea bilineata*) oysters, which contribute 14% and 3% respectively. The remaining 3% is representative of nine additional species, all of which contribute marginally to overall sector yields (FAO FishStatJ, 2019). China is currently the leading producer of edible oysters, responsible for approximately 85% of the global aggregate (Table 1.1)(FAO FishStatJ, 2019; Statistica, 2019). Despite a comparatively low gross domestic product (GDP) ranking of 12, South Korea is the second largest producer in the sector contributing 5.5%, whilst Japan and the United States each supply 3% and 2.5% respectively (FAO FishStatJ, 2019; Statistica, 2019)(Table 1.1). Despite the majority of commercial scale edible oyster culture taking place in temperate regions, subsistence and small-scale culture is prevalent in tropical countries. These community focused oyster

farms often culture multiple species and typically use spat sourced directly from the wild (Glude, 1984; Nowland et al., 2019). In contrast, commercial farms from temperate regions tend to employ a single species culture approach and source spat from hatcheries (Helm et al., 2004).

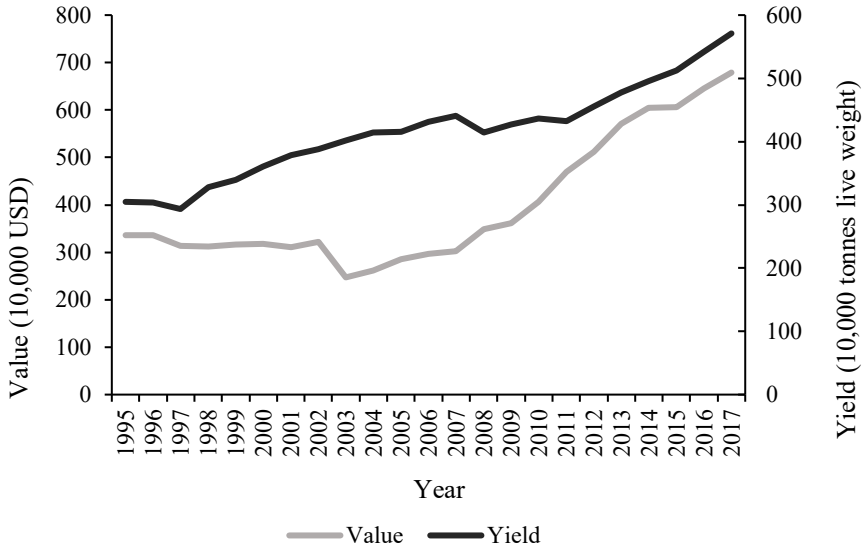


Figure 1.1. The combined global value and yield of edible oyster aquaculture from 1995 – 2017 (FAO FishStatJ, 2019).

Table 1.1. Global yield (tonnes – live weight) and value (USD $1000) of oysters grouped by Country and Gross Domestic Product ranking (GDP) in 2017 (FAO FishStatJ, 2019; Statistica, 2019).

Country	**GDP Rank 2017**	**GDP 2017**	**2017 Yield**	**2017 Value**
United States of America	1	19.49 trillion	140,892	186,664
China	2	12.06 trillion	4,879,422	5,255,137
Japan	3	4.86 trillion	173,800	362,194
Germany	4	3.70 trillion	80	720
India	5	2.65 trillion	4,000 F	39,923
United Kingdom	6	2.64 trillion	2,317	9,987
France	7	2.58 trillion	64,910	373,238
Brazil	8	2.05 trillion	2,700 F	3,384
Italy	9	1.93 trillion	145 F	751
Canada	10	1.65 trillion	13,800	35,118
Russian Federation	11	1.57 trillion	531	690
South Korea	12	1.53 trillion	315,255	189,779
Australia	13	1.32 trillion	11,929	85,667
Spain	14	1.31 trillion	1,093 F	4,529
Mexico	15	1.15 trillion	14,892	17,973

" F " = FAO estimate from available sources of information. Source: Statistica 2019 and FAO. 2019. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2017 (FishstatJ). In: *FAO Fisheries and Aquaculture Department* [online]. Rome. Updated 2019

1.3 Tropical oyster culture

The prospect of future aquaculture developments in tropical regions is attracting new interest, with attention directed towards low impact species (Willer & Aldridge, 2020). Particularly in high risk, fragile environments such as the estuaries on the west coast of USA, the culture of bivalve species, such as oysters and mussels, are favourable in place of other species with a greater ecological footprint, such as intensive shrimp and fish farming (Dumbauld et al., 2009). Due to their filter feeding nature and minimal required infrastructure, oyster culture has very few inputs and in some cases can be beneficial to the processes of nutrient cycling and water quality of the systems in which they are grown (Dewey et al., 2011; Pollack et al., 2013). Despite several failed attempts to culture temperate oyster species in the tropics, the prospect of culturing oyster species native to such areas has been identified as having great potential and requiring further investigation (Nowland et al., 2019). Tropical rock oysters display a range of promising attributes as a culture species including fast growth rates and large size, capable of driving a globally significant aquaculture industry (Nowland et al., 2019).

Accurate production statistics regarding tropical oyster culture are difficult to acquire, as many of the region's economies are still developing (Sachs, 2001). Nonetheless, commercial-scale production in excess of 100 tonnes per annum occurs in a number of countries including Mexico, Thailand, Senegal, Indonesia, the Philippines, India, northern Chile, Cuba, Brazil, southern China and Vietnam (Table 1.3). In comparison, small-scale commercial, subsistence and experimental farming is more commonly practiced and often incorporates multiple culture species within a single region. For example, the farming of the Cupped Oyster *(Crassostrea iredalei)* in the Philippines takes place in conjunction with the Hooded Oyster *(Crassostrea malabonensis),* where they are equally successful and highly regarded (Erazo-Pagador, 2010). While the value of such small-scale operations may seem limited in economist's views, the role they play as a food source and economic boost to remote communities, should not be underestimated. In addition to a form of income, oyster farming in the remote areas of north Vietnam has been a source of pride and motivation within the community, and provides an appreciation of the importance of water quality (Pierce & O'Connor, 2014).

1.4 Australian oyster industry

Commercial scale production in Australia originated with the Sydney rock oyster (*Saccostrea glomerata*) and originally utilised wild spat collectors to source juveniles for grow out (Duthie, 2012). The successful culture of the introduced Pacific oyster (*C. gigas*) and the native Flat oyster (*Ostrea angasi*) were achieved in much the same way, until hatchery techniques were first developed and employed by the Tasmanian Pacific oyster sector, in order to overcome the challenge of wild caught spat decline (Nell, 2001). Commercial oyster culture presently occurs along much of Australia's

eastern and southern coastlines, stretching from southern Queensland, throughout New South Wales, extending into South Australia, Tasmania and Western Australia (Duthie, 2012). In 2017, the Australian edible oyster industry was valued at over 102 million AUD (ABARES, 2018). At present, there is no large scale commercial production of tropical oysters in Australia, however small scale commercial farms are operating in Bowen, north Queensland and Warruwi and Grooyte Eylandt in the Northern Territory (Nowland et al., 2019).

1.5 Challenges to oyster culture

Oyster culture has typically been a highly labour intensive business venture and is ready to be upscaled and modernised with efficient technology (Hennig & Jain, 2017). Climate change impacts, specifically increasing water temperatures and ocean acidification, presents challenges to farmers during the growout phase. Elevated water temperatures contain reduced dissolved oxygen, and oysters consequently must spend more effort to obtain sufficient oxygen to maintain metabolic function (Specht & Fuchs, 2018). Furthermore, marine calcifying organisms are amongst the most at risk species to ocean acidification (Kroeker et al., 2013; Wittmann & Pörtner, 2013). The ability for oysters to form and maintain stability of their calcified structures will likely be compromised by predicted changes to ocean carbon chemistry, with flow on effects to reproductive efficiency, immune responses and reduced survival rates also expected (Orr et al., 2005; Waldbusser et al., 2015). Deterioration in coastal water quality will likely be challenging to maintain food safety standards and optimal grow-out conditions. For example, high sediment loads impede the feeding efficiency of oysters while heavy metal pollutants can bioaccumulate within oyster tissue (Fleming et al., 2015; Lefebvre et al., 2000). Furthermore, a predicted increase in frequency of extreme weather events will present significant challenges to farmers and business owners through the damage of key infrastructure. Patterns suggest that the impact of extreme weather events are typically stronger in tropical locations, adding increased pressure to these developing regions (Coeroli et al., 1984; Hamman & Deane, 2018).

1.6 Bioremediation

One potential solution to predicted declines in water quality, as a result of climate change, is the use of oysters for bioremediation. Oysters play a vital role in healthy marine environments, with numerous connections established between improved water quality and a high biomass of bivalve species (Bricker et al., 2018; Higgins et al., 2011). Bioremediation refers to the process of removing contaminants or pollutant material from the environment through a biological means (Gifford et al., 2005). Bivalves are a preferred species for applied bioremediation, due to their tolerance to a wide range of environmental conditions and resilience to poor water quality (Martinez-Cordova & Martinez-Porchas, 2006). Several studies have quantified the ability of bivalves to reduce total nitrogen, total phosphorus, total suspended solids, chlorophyll-A and bacteria levels in water; both

within their natural ecosystem and commercial aquaculture settings (Jones et al., 2002; Petersen et al., 2014; 2016). During the process of bivalve filtration, ingested particles are sorted based on size, weight and nutritional value (Dumbauld et al., 2009). Organic particles such as those from faecal matter or undigested commercial feeds, are retained and used as a source of food for the bivalves. Fine inorganic particles such as sediment or clay, are filtered from the water column, flocculated together and ejected as pseudofaeces (Tenore & Dunstan, 1973). These pseudofaeces are typically heavier than the original particles and settle out of suspension faster. It is by both of these means that bivalves can assist in reducing turbidity and total suspended solids (Lefebvre et al., 2000). Previous studies on the bioremediatory ability of the Sydney rock oyster (*S. glomerata*) (Jones & Preston, 1999; Jones, et al., 2001; 2002), the Cortez oyster (*Crassostrea corteziensis*) (Peña-Messina et al., 2009) and the Pacific oyster (*C. gigas*) (Lefebvre et al., 2000), all returned varying degrees of success in improving water quality in commercial aquaculture effluent. The combination of oysters and macroalgae as a form of integrated multi-trophic aquaculture (IMTA) has also returned promising outcomes (Jones et al., 2002). In addition to the water quality improvements and reduced strain on mechanical filtration methods, both oysters and macroalgae are highly marketable secondary products that could provide economic benefit by production of a second cash crop.

1.7 Demand for consistent spat supply

In tropical regions, spat is predominately obtained from the wild, using spat collectors made from readily available materials such as bamboo, plastic sheeting and mangrove roots (Angell, 1986). Inadequate and inconsistent spat supply remains the greatest bottleneck to tropical oyster farmers, with wild collection methods in many areas unable to support a commercially viable operation (Gianasi, 2017). The importance of closing the life-cycle and advancing hatchery spat production, cannot be overstated. The commercial successes experienced by both the Australian Sydney rock oyster and the Pacific oyster sectors respectively, can be greatly attributed to the development of hatchery techniques, permitting captive spawning, hatching and settlement of larvae (Nell, 2001). An improved understanding of these species biological requirements and reproductive capacity has facilitated the progression of intensive production methods and strengthened supply to the ocean-based growout phase (Duthie, 2012; O'Connor et al., 2008). Ultimately, the key to large-scale commercialisation of tropical oysters is the development of successful hatchery production methods.

1.8 Phases within hatchery production

There are five primary components within hatchery production of oysters, each bringing with it a unique set of challenges.

i. Broodstock
ii. Larvae
iii. Settlement
iv. Nursery
v. Algal production

The hatchery process begins with broodstock and it is therefore essential to select healthy, fecund individuals that display desirable culture traits. Furthermore, the broodstock collection site is an important factor, as is understanding the risk of affecting the local genetic pool by introducing new individuals to an open culture environment (Duthie, 2012). Bivalve larvae are highly susceptible to infection, therefore screening for disease and appropriate physical and biological separation of broodstock is critical to prevent the spread of pathogens from broodstock to larvae (Duthie, 2012; O'Connor et al., 2008). Settlement refers to the process whereby oyster larvae metamorphose into spat and are no longer free swimming. Settlement success rates often vary between 40-70%, however the transition to using epinephrine to induce setting, has returned favourable success rates of between 60 to 78% across the species *S. glomerata, Crassostrea brasiliana* and *C. iredalei* (O'Connor et al., 2008; Silveira et al., 2011; Teh et al., 2012). Once a quantity of spat is secured, the nursery phase is used to protect recently settled spat from biofouling, turbulent ocean conditions and predation. Once spat reach an approximate size of between 5 to 10 mm, they are transferred from the nursery to grow-out farms (Helm et al., 2004). The culture of microalgae as source of feed is a critical component of the hatchery phase as it underpins the entire process. All stages, including both broodstock and larvae, rely on the successful and pathogen free culture of microalgae to ensure a nutritionally complete diet is available (Nell, 1993; O'Connor et al., 2008).

1.9 The Blacklip rock oyster

The Blacklip rock oyster, *Saccostrea echinata,* (Figure 1.2) inhabits inshore and coastal waters of the south western Pacific and is prominent throughout northern Australia. Its reported range extends throughout the tropical Indo-Pacific region; from Taketomi Island in Japan, to Noumea in New Caledonia, and across northern Australia; from Cone Bay in Western Australia, to Bowen in Queensland (McDougall, 2018; Nowland, 2019; Thomson, 1953). In addition to displaying fast growth rates and large sizes, *S. echinata* has exhibited promising suitability towards hatchery production and a strong market acceptance (Fleming, 2015; Glude, 1984; Nowland, 2019).

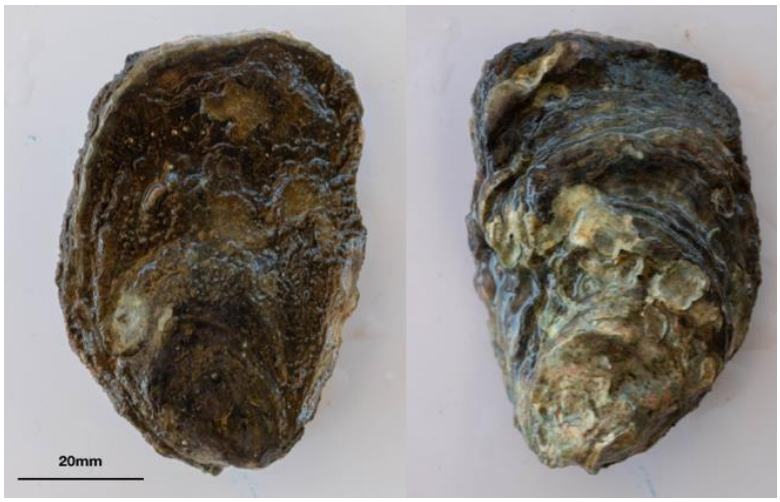


Figure 1.2. Mature broodstock specimens of the Blacklip rock oyster, *Saccostrea echinata*, collected from South Goulburn Island, NT, Australia (11'38'49"S 133'25'23"E). Scale bar = 20mm.

Several Pacific Island nations, including New Caledonia, have attempted to culture *S. echinata* utilising wild sourced spat; however low spat collection yields proved to be a limiting factor to success (Coeroli et al., 1984). In Australia, a small farm on Magnetic Island in Queensland was also closed due to the limitations imposed by unreliable and insufficient spat supply (White, 1991). Currently, only small-scale farms in northern Australia (Warruwi and Grooyte Eylandt), eastern Australia (Bowen), New Caledonia (Noumea) and Fiji (Mago Island), are culturing this species (Nowland et al., 2019). The Northern Territory Government in Australia is working alongside remote Indigenous communities to commercialise this species, due to local knowledge, cultural value and the potential for cost effective farming methods (Fleming, 2015; Nowland, 2019).

1.9.1 Key knowledge gaps and bottlenecks to the commercial progression of *S. echinata*

Despite the favourable culture traits of *S. echinata*, the inadequate and unreliable supply of wild spat remains the greatest impediment to its commercial development (Nowland 2019; Southgate & Lee, 1998). Furthermore, the inability to distinguish wild-caught Blacklip oyster spat from smaller and slower growing species, has hindered small-scale commercial efforts thus far. Captive spawning is currently restricted to their natural reproductive season, meaning that spawning can only take place for six months of the year and grow-out schedules cannot be tailored to meet seasonal demand. Due to a knowledge gap in broodstock physical and nutritional requirements, hatcheries have minimal control over broodstock maturation cycles (Nowland, 2019). At present, broodstock husbandry protocols for *S. echinata* are based on those for *S. glomerata* and fail to consider the differences in reproductive biology between tropical and temperate species (O'Connor et al., 2008).

1.10 Broodstock conditioning

The term broodstock conditioning refers to a series of endogenous and exogenous modifications employed by hatchery facilities to both prolong their season of production and reduce dependence on the brief period in which mature adults are reproducing in the wild (Helm et al., 2004). The effective maturation of broodstock is a critical step in the hatchery phase of production, leading to the successful closure of a species life-cycle (Wilson et al., 1996). Successful conditioning requires primary biological and reproductive characteristics to be identified and applied to provide an optimal holding environment that ensures oysters reach advanced stages of sexual maturation and undergo gametogenesis. The ultimate goal of a conditioning program is to promote maximal fecundity of broodstock, whilst ensuring they produce sufficient quality larvae for hatchery and growout phases (Utting & Millican, 1997).

There are several techniques used to evaluate the degree of success of bivalve broodstock conditioning practises. One of the most widely utilised procedures is histological analysis with the purpose of determining the gametogenic stage of individual broodstock. Gonadal stages are assessed based on morphological characteristics, presence or absence of gametes and their degree of development. Condition Index (CI) is a method of measuring overall oyster health and describes tissue mass relative to shell mass (Hopkins, 1949). While, Gonad index (GI) is a technique extensively used for describing and quantifying the reproductive cycle of aquaculture species (Sreedevi et al., 2014).

1.10.1 Temperature

Water temperature is widely regarded as one of the primary driving factors regulating the reproductive cycle of bivalve species (Chávez-Villalba et al., 2002). A clear correlation has been established between the manipulation of temperature and the extent of gonadal development for several species. Spawning cycles of bivalves are often highly seasonal, utilising external physical factors to synchronise their endogenous rhythms and identify the appropriate time to undergo spawning. Whilst tropical and temperate species respond to different temperature thresholds, there is a common trend across both regions of warmer summer temperatures resulting in increased gonad tissue maturation, whilst cooler winter temperatures initiate a dormant phase of recovery (Fabioux et al., 2005; Maneiro et al., 2016; Ramos et al., 2014).

Studies across several bivalve species have emphasised this common trend of increased temperatures initiating maturation, whilst colder temperatures promote dormancy and cell recovery. For example, research conducted on the temperate species *C. gigas* (Chávez-Villalba et al., 2002; Fabioux et al., 2005) and *O. edulis* (Maneiro et al., 2016), in addition to the tropical Green mussel (*Perna viridis)* (Sreedevi et al., 2014), and tropical oyster *Crassostrea gasar* (Ramos et al., 2014), all displayed a

comparable pattern of gonad development in response to an increase in temperature. Ramos et al., (2014) clearly demonstrated seasonal periodicity in the Mangrove oyster (*C. gasar),* held at three temperature variations (18, 22 and 26°C). The results suggest that during cooler wintering temperatures of 18°C or less, an accumulation of energy reserves takes place, while during accelerated summer temperatures gonad maturation occurs at temperatures greater than 22°C (Ramos et al., 2014). Specifically regarding the influence of temperature on *S. echinata,* both Nowland et al., (2019) and Lindsay (1994) recorded strong positive correlations between increased temperature and GI of wild oyster populations ($r = 0.783$ and $r = 0.894$ respectively).

1.10.2 Salinity

Estuarine and coastal environments often experience significant and prolonged changes in salinity. These changes are especially prominent in tropical regions that receive heavy freshwater inputs due to monsoons (Lindsay, 1994). Throughout northern Australia, the monsoon season can occur from October through to April (Holland, 1985). The reproductive seasonality of *S. echinata* has been closely linked to rainfall patterns, whereby they exhibit semi-continuous spawning throughout the monsoon season (October – April) and enter an extended resting phase throughout the dry season months (May – September) (Nowland et al., 2019). Furthermore, rapid reduction of salinity has been highlighted as a spawning trigger for several species of tropical rock oyster, including *Striostrea prismatica* in Costa Rica (Fournier, 1992) and *S. echinata* in northern Australia (Southgate & Lee, 1998). However, the effects of salinity on reproductive maturation are less apparent, as findings may reflect simultaneous variations in nutrient profiles. Nowland et al., (2019) identified a moderately positive correlation between wild populations of *S. echinata* GI and rainfall ($r = 0.496$). Which is similar to a study by Paixão et al., (2013) investigating the gonadal development of *C. gasar,* and its relationship to rainfall and salinity. Significant correlations were found between both of these factors and oyster GI, suggesting reproductive patterns are regulated by seasonal rainfall patterns in the Amazon region (Paixão et al., 2013).

1.11 Thesis overview

This project was undertaken in partnership with the Corporate Research Centre for Developing Northern Australia (CRCNA) tropical rock oyster research and development project. One of the goals of that project specifically relates to securing commercial quantitites of spat. As such, the aims of this project closely align with those set out within this sub project, resolving the issue of seed supply and using this knowledge to help benefit Indigenous run farms in the Northern Territory of Australia. In recent years, several studies have been conducted on this species focussing on larval rearing and mapping wild reproductive patterns (Nowland, 2019). The following research chapters aim to build on these findings to inform study design, and fill knowledge gaps required to enable hatchery

production. Focussing specifically on the exogenous factors of temperature and salinity, these studies intend to determine the optimal conditioning environment for *S. echinata* and enhance hatchery spawning. In order to promote the future expansion of this species throughout tropical regions and northern Australia, a filtration rate study aimed to establish the optimal temperature for maximal filtration and inform future site selection for the grow-out period. To further demonstrate the versatility of this species, additional studies into their suitability for aquaculture bioremediation aimed to determine their suitability for integrated multi-trophic aquaculture and quantify the environmental service they provide.

Therefore, the overall objectives of this thesis were to develop broodstock conditioning protocols for *S. echinata*, and to assess their bioremediation ability. This was achieved by addressing three primary aims, which were to:

i. Evaluate the influence of temperature manipulation on reproductive maturation of *S. echinata* broodstock
ii. Investigate the effect of salinity manipulation on reproductive maturation of *S. echinata* broodstock
iii. Assess the bioremediatory ability of *S. echinata* and their capacity to improve water quality of shrimp aquaculture effluent.

Each of these objectives are assessed and presented, across three separate data chapters within this thesis.

Chapter 2 was a pilot study investigating the effects of elevated holding temperature on the reproductive maturation of broodstock. This study was undertaken at the Darwin Aquaculture Centre, Northern Territory, Australia, and used broodstock sourced from Goulburn Island, Northern Territory. Tissue samples, in addition to biometric measurements were sampled at regular intervals throughout the 12 week experiment period. Using histology techniques to assess gonad development, a mean GI was generated and used as the primary evaluation method. The outcomes of this pilot study provided a basis for the experimental design in Chapter 3.

Chapter 3 was the first full-scale laboratory study to investigate the effect of salinity on broodstock conditioning of *S. echinata*. This trial employed the same sampling and histology analysis techniques outlined in Chapter 2. In addition to investigating the effect of salinity, the key point of differences in this study were; increased replication, the use of formulated algal paste to supplement the live algae diet and an attempt to spawn from experimental broodstock to assess reproductive condition at

experiment completion. Outcomes identified the optimal holding salinity for broodstock conditioning and informed future trials and spawning success with this species.

Chapter 4 aimed to assess the bioremediatory ability of *S. echinata* and their capacity to improve water quality within shrimp aquaculture effluent. This trial was separated into two parts; Part A investigated the effects of temperature on the filtration rate of *S. echinata,* by quantifying the rate at which microalgae cell concentration decreased with time, and Part B evaluated the bioremediatory ability of *S. echinata* and assessed their suitability at improving shrimp effluent water quality. This was achieved by a five hour biofiltration period using raw shrimp effluent and measuring how the parameters of total nitrogen, total phosphorus, total suspended solids and chlorophyll-A changed over time. The outcomes of this study identified the optimal temperature window for *S. echinata* filtration and highlighted its potential for future integration as a biofilter.

2 CHAPTER 2. THE EFFECT OF TEMPERATURE ON TROPICAL BLACKLIP ROCK OYSTER (*SACCOSTREA ECHINATA)*(QUOY AND GAIMAARD, 1835) BROODSTOCK CONDITIONING

2.1 Introduction

2.1.1 Tropical oysters

Tropical rock oyster species display the potential to drive a globally significant aquaculture sector (Nowland et al., 2019). Currently, they provide a valuable source of subsistence food and contribute to economies in developing regions (Nowland et al., 2019; Willer & Aldridge, 2020). Innovative and cost effective techniques, have enabled a variety of new oyster species to be successfully cultured throughout the tropics (Coeroli et al., 1984; Glude, 1984). Despite high marketability and favorable production features, such as rapid growth rates, oyster aquaculture in the tropics remains in its infancy compared to the temperate oyster industry (Joseph, 1998; Nowland et al., 2019; Willer & Aldridge, 2020). Success of the temperate oyster sector is largely due to well-established hatchery methods and selective breeding programs, enabling sufficient production of spat to meet growout demand (Helm et al., 2004; Nell 2001).

2.1.2 The Blacklip rock oyster

The Blacklip rock oyster, *Saccostrea echinata* (Quoy and Gaimaard, 1835), is a tropical oyster species with aquaculture potential. Blacklip rock oysters have been locally harvested for millennia, throughout the Moluccas, Papua New Guinea, Indonesia, Micronesia, the Philippines and northern Australia (Lindsay, 1994; Thomson, 1953). This species displays a number of favorable aquaculture traits including fast growth, high market acceptance and suitability to hatchery production (Glude, 1984; Nowland, 2019; Southgate & Lee, 1998). Tropical oyster culture, including that of *S. echinata*, is currently reliant on wild spat collection, which is typically insufficient and unreliable. At present, this species is not cultured on a large commercial scale, however, small-scale commercial and experimental farms are located in Goulburn Island and Groote Eylandt in northern Australia, Bowen in north-eastern Australia, Mago Island in Fiji, and Noumea in New Caledonia (Nowland, 2019). Preliminary farming trials conducted in northern Australia, have demonstrated the industry potential of Blacklip rock oyster culture using hatchery produced spat (Nowland, 2019).

The knowledge gap concerning broodstock requirements and seasonal spawning restrictions, is a significant impediment to the progression of *S. echinata* as a commercial culture species. This species has been highlighted as being adaptable to pre-existing hatchery techniques, however, low spat yields indicate that further research is required to guarantee sufficient spat for commercial production (Coeroli et al., 1984; Southgate & Lee, 1998). Recent studies have focused on describing embryonic,

larval and post larval development and optimising hatchery culture techniques (Nowland, 2019; Nowland et al., 2018; Southgate & Lee, 1998).

2.1.3 Reproduction

Oyster reproduction comprises several key stages including gamete growth and ripening, spawning, resting and gonad regeneration (Dinamani, 1974; Gosling, 2003). A comprehensive knowledge of seasonal gonad condition in oysters, in addition to the key regulatory factors that drive reproduction processes, is crucial for effective aquaculture production (Helm et al., 2004). Bivalve reproductive patterns are coordinated by endogenous factors, such as genotype and neuroendocrine cycles, and exogenous factors, such as water temperature, food type and availability, salinity and photoperiod (Gosling, 2003; Lucas, 2012). Without an understanding of these influences on reproductive development, broodstock conditioning within the hatchery cannot be achieved and sub-optimal gametes may result. Bivalve breeding programs require effective broodstock maturation, which is typically attained by simulating natural seasonal conditions under controlled settings (Arguella-Guevara et al., 2013).

Reproductive seasonality in the tropics has been demonstrated across several popular aquaculture species. For example, populations of the common tiger prawn (*Penaeus esculentus*) in northern Queensland, Australia, exhibit increased gonad and somatic growth throughout late spring and early summer (O'Connor, 1979). The Sandfish (*Holothuria scabra*) similarly displays phases of gonad growth that correlate with seasonal physical factors of temperature and light availability (Muthiga et al., 2009). Reproduction patterns in tropical oysters also appear to be seasonal (Lindsay, 1994; Nowland et al., 2019). A study conducted by Lindsay, (1994), described the spawning season for wild *S. echinata* in north Queensland, where gonad development commenced in mid spring (September and October), with the majority of oysters attaining ripe condition by early summer (November and December), which persisited until spawning occurred in late January to early February. It was later suggested that gametogenesis occurred following an increase in water temperature by 7°C and increased algae availability (Lindsay, 1994). A more recent study undertaken by Nowland et al., (2019), described a semicontinuous pattern of *S. echinata* reproduction in the Northern Territory, Australia. Spawning activity occurred throughout the monsoon season (October to April), with an extended resting phase throughout the dry season (May to September). Temperature appears to be a key regulatory factor within *S. echinata* reproduction, with both studies returning strong positive correlations between temperature and reproductive condition ($r = 0.783$ and $r = 0.894$, respectively) (Lindsay, 1994; Nowland et al., 2019).

2.1.4 Broodstock conditioning

Broodstock conditioning is a process of maximizing fecundity of parent animals whilst maintaining egg quality and larval viability. During this practice, both the physical environment and nutrition of broodstock are manipulated to stimulate gonad development and gametogenesis (Utting & Millican, 1997). A study conducted on the Mangrove oyster, *Crassostrea gasar,* demonstrated the importance of manipulating physical parameters to condition broodstock. The findings supported the concept that temperature can be an effective means of coordinating broodstock maturation patterns (Ramos et al., 2014). Water temperature is commonly accepted as the primary factor influencing reproductive maturation in temperate rock oyster species (Chávez-Villalba et al., 2002; Fabioux et al., 2005; Orton, 1920). Orton (1920) theorized that a species-specific temperature threshold must be exceeded before gametogenesis will commence. This concept of temperature as a key factor has been confirmed for several temperate oyster species including; *Crassostrea gigas* (Chávez-Villalba et al., 2002; Fabioux et al., 2005), *Saccostrea glomerata* (Dinamani, 1974; Dove & O'Connor, 2012)*, Ostrea edulis* (Maneiro et al., 2016) and *Crassostrea virginica* (O'Beirn et al., 1996) in addition to the tropical mangrove oyster, *C. gasar* (Ramos et al. 2014).

Temperature has further been recognised as a factor of influence on the reproductive cycles of some tropical oyster species. *Striostrea prismatica* is one such species, distributed along the southern coast of Ecuador, where an association between gonad maturation and surrounding water temperature has been reported; gonad index values were recorded at their highest during the summer months of January and February, which coincide with thermal cycles bringing warmer sea surface temperatures of between 28-30 °C (Loor & Sonnenholzner, 2016). The Mangrove oyster, *C. gasar,* experiences similar temperature variation, inhabiting central West Africa, French Guiana and Southern Brazil. Ramos et al (2014) assessed the influence of water temperature on gonad tissue maturation of *C. gasar* and discovered that at temperatures higher than 22 °C, gamete maturation occurred. Considering tropical bivalves more broadly, the Green mussel *, Perna viridis,* from the Indo-Pacific region, demonstrated a strong affinity to temperature conditioning methods with optimal gonad development occurring at 30-32 °C (Sreedevi et al., 2014). These studies suggest that the technique of temperature driven conditioning is not limited to temperate bivalve species and can be applied to tropical species.

To support the commercialisation of the Blacklip rock oyster a large volume of high quality, hatchery produced spat is required to meet growout demand. Identifying the key requirements and optimal holding environment for gonad development is the next step in developing a successful broodstock conditioning protocol. This would enable predictable spawning outcomes and year-round production. Therefore, the aim of this study was to investigate the effect of increased water temperature on broodstock conditioning of *S. echinata*.

2.2 Materials and methods

2.2.1 Experimental design

A total of 106 Blacklip oysters were sourced from wild intertidal stocks on Goulburn Island (11° 38' 49" S, 133° 25' 23" E) at low tide and held in earthen ocean filled ponds near the Northern Territory Government's, Darwin Aquaculture Centre, for use when required. Fouling organisms and sediments were removed from the shells before being introduced to an ambient holding system at 36 ppt. Recirculating systems (n=4) of 100 L cylindrical aquaria, each supplied by a 1000 L conical sump, were used. Both treatments utilised two replicate tanks for each seawater temperature treatment; ambient or heated. Due to the limited access of broodstock numbers, this pilot trial used two replicate tanks for each treatment. The heated treatment replicates were raised from ambient (24 °C) by 1 °C per day until the required temperature of 31±1 °C was attained (EHEIM thermocontrol 25, Germany) while control replicates were held at ambient temperatures to mimic the diurnal fluctuations in the intertidal environment. The temperature of 31°C was selected for the heated treatment as this was found to initiate the highest reproductive condition of Blacklip oysters in local wild populations (Nowland et al., 2019). Salinity was maintained at 36 ppt throughout the experimental period. All conditioning systems were cleaned using 200 ppm chlorine (Nowland & Hartley, 2021) and a full water exchange on alternate days.

Between 26 and 27 broodstock oysters were suspended in each plastic tray, with the dimensions of 60 x 25 x 10 cm (L x W x H). This was done for ease of cleaning and were transferred directly to a separate holding tank during cleaning to minimise stress. The holding tanks were of identical temperature and salinity to each of the respective treatments, in order to prevent shock induced spawning. Temperature, dissolved oxygen (DO), pH and salinity were measured and recorded each morning for all systems, in addition to visually checking for mortalities. One temperature data logger (HOBO, USA) was deployed in each aquarium for the duration of the conditioning trial.

2.2.2 Feed regime

The feeding regime was based on the Sydney Rock Oyster broodstock conditioning protocol detailed by O'Connor et al. (2008). This comprised of 50% diatoms (45% *Chaetoceros muelleri* (CS-176 CSIRO catalogue code) and 5% *Skeletonema pseudocostatum (CS-252* CSIRO catalogue code)) and 50% flagellated species (18% *Tisochrysis lutea (CS-177* CSIRO catalogue code), 16% *Pavlova* spp. (CS-50 CSIRO catalogue code), and *16% Tetraselmis chui (CS-26* CSIRO catalogue code)). Algae culture concentrations were counted daily, and harvested volumes were adjusted accordingly. The broodstock were initially fed 2 billion cells per oyster per day. This concentration was increased to 2.5 billion cells per oyster per day at commencement of week five. Live microalgae were drip fed into the

holding tanks via dosing pumps (Grundfos®) to ensure a continuous supply of microalgae. These tanks were cleaned and re-stocked every day with the tanks total daily feed (Eq.1).

Number of oysters X algal concentration per oyster per day = Total algal feed per day Eq. 1.1

2.2.3 Biometric data sampling and histology

Biometric data collection consisted of taking whole oyster weight, determined by an electronic balance with a precision of 0.1 g. Following this, the visceral mass was extracted, excess water removed by placing onto paper towel and wet weight was determined (to a precision of 0.001 g). Shell weight was also determined (to a precision of 0.1 g). Dorso-ventral measurement (DVM) and antero-posterior measurement (APM) were taken for each oyster using calipers and recorded to the nearest millimeter. Prior to the commencement of the trial, biometric measurements and cross sections for histological assessment were collected for six broodstock. Additional oysters (n=12) were sampled in weeks five, eight, eleven and twelve (Table 2.1). For each oyster sampled, a 6mm-thick cross section of the dorsal visceral mass was removed from the point where the labial palps meet the gills and parallel to the hinge line (Figure 2.1). The cross sections (A and B) were then placed into histological cassettes and immersed in 10% seawater buffered formalin for 48 hours before being processed and fixed in paraffin. Using standard histological procedures, 4 µm sections were prepared and stained with both hematoxylin and eosin for histological assessment (Luna 1968; Raphael et al 1976).

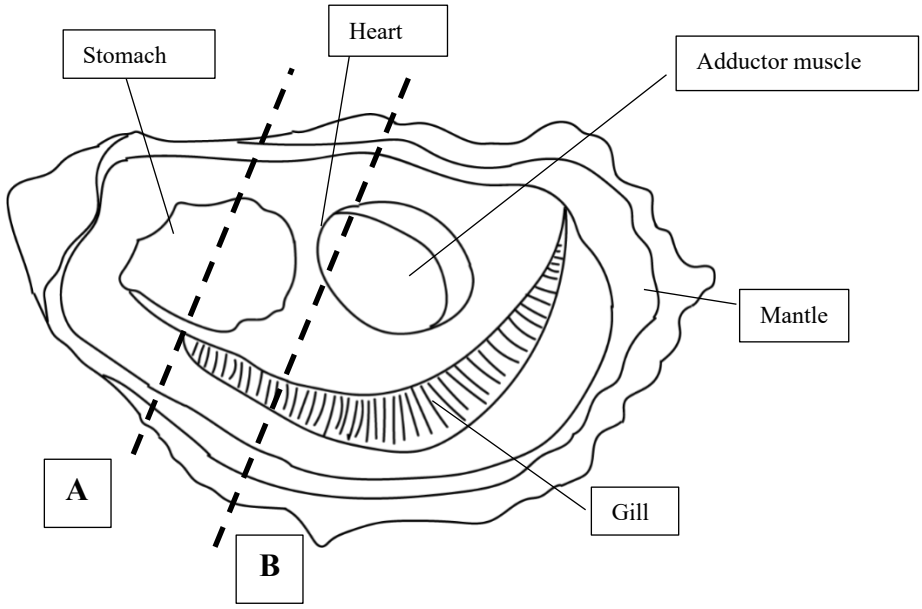


Figure 2.1. Anatomy of an oyster showing 6 mm histological cross-sections (A and B). Image adapted from (Thompson, 1954).

Histological slides were evaluated microscopically in accordance with the Dinamani (1974) classification system for *S. glomerata*, to determine gender and degree of reproductive condition. Based primarily on the factors of cell size and shape, nuclear features and staining characteristics, broodstock were allocated a rating of gamete development; resting (R), early development (G1–G2), late development (G3), ripe (G4), discharged (G5) and residual (Gx). In cases where two or more stages were present in the same sample, the dominant stage was allocated.

Table 2.1. Sampling frequency of broodstock oysters, *Saccostrea echinata*, including sample date, week, number of oysters, sampled quantity, and the number of remaining oysters.

Sampling date	Week	No. oysters	No. sampled	Remaining oysters
3/7/19	1	106	6	100
31/7/19	5	96	12	84
27/8/19	8	84	12	72
11/9/19	11	72	12	60
25/9/19	12	60	12	48

2.2.4 Mean gonad index

Mean gonad index (GI) was calculated for each of the sample events, following the formula described by Seed (1969);

$$M = \frac{\Sigma(ns)}{N}, \qquad \text{Eq. 1.2}$$

Where *n* is the number of oysters present is a given gametogenic stage, *s* is the numerical ranking of that particular stage, and *N* is the total number of oysters in the sample (Braley, 1982, 1984; Seed, 1969). Each gametogenic phase was allocated a numerical ranking, following the classification system outlined by Dinamani (1974): R = 0, G1 = 1, G2 = 2, G3 = 3, G4 = 4, G5 = 0, and Gx = 0. The gonad index can vary from 0, when all the individuals within a sample are in the resting (R) phase or have completed spawning (G5), to 4, when all broodstock are sexually mature (G4).

2.2.5 Mean condition index

The wet weight condition index (CI) was determined for each oyster sampled and calculated using the formula as described by Hopkins (1949);

$$CI = \frac{v}{w-s} \text{ X } 100, \qquad \text{Eq. 1.3}$$

where *v* is the visceral mass wet weight (g), *w* is the whole oyster weight and *s* is the shell weight (g).

2.2.6 Statistical analyses

Biometric data (DVM, APM, whole oyster weight, and CI), GI, water temperature, salinity, DO and pH data were tested for homogeneity of variance. All datasets were first analysed with a one-way ANOVA. The Pearson correlation coefficient was used to test for a linear relationship existing between GI or CI and temperature treatment. Following this, a *post-hoc* Tukey HSD test was completed to determine if there was a significant difference in CI between tanks and treatments. All analyses were completed and plots created using R (R Core Team 2019).

2.3 Results

2.3.1 Water quality data

Stable temperatures were successfully maintained in the heated treatment for the duration of the conditioning period. Once the temperature increase from 25 to 31°C was achieved, the mean temperature of the heated treatment remained stable (31± 1°C) (Figure 2.2). The ambient treatment water temperature fluctuated with the surrounding climate, however, no unexpected variations were detected and mean temperature ranged from to 24.4 to 27.6 °C (Figure 2.2). Across both treatments salinity remained ambient at 36±1 ppt, DO ranged from 79.8 – 108% and pH ranged from 8.04 – 8.21. None of these parameters differed significantly between treatments.

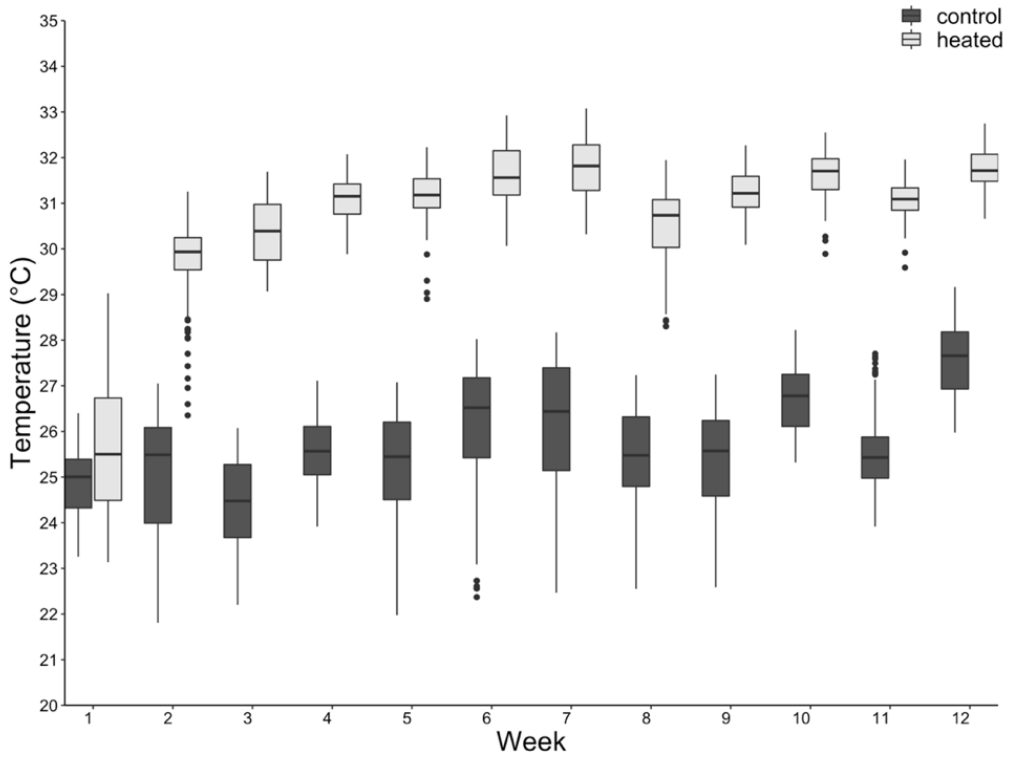


Figure 2.2. Box plot describing temperature of the control (ambient) and heated (31 ± 1°C) treatments.

2.3.2 Biometric data

A total of 54 oysters were sampled during the 12 week experiment. The mean whole weight of oysters stocked into the heated treatment was 202.80 ± 13.45 g with a mean DVM and APM of 102.79 ± 3.08 and 77.08 ± 2.84 mm, respectively. The mean whole weight of oysters in the control treatment was 227.97 ± 18.14 g with a mean DVM and APM of 108.16 ± 3.64 mm and 79.40 ± 2.40 mm, respectively. Whole oyster weights did not differ significantly between treatments (F = 1.221; *df* 4/49; P = 0.314). Similarly, no significant differences were recorded between treatments for DVM (F = 1.102; *df* 4/49; P = 0.366) and APM (F = 0.49; *df* 4/49; P = 0.743). Four mortalities were recorded throughout the duration of the trial (One individual from tank 2 during week 3, and three individuals from tank 3 between the weeks of 2 to 4).

2.3.3 Histology, gonad index and condition index

Histological slides depicting both female and male gonad developmental stages are shown in Figure 2.3. On evaluation, 87% of all broodstock sampled throughout the experiment period were classified as gender indeterminant. Within the control (ambient) treatment, one male (3.33%), one female (3.33%) and 28 indeterminate (93.33%) specimens were identified, and within the heated treatment three males (12.5%), two females (8.33%) and 19 indeterminate (79.16%) specimens.

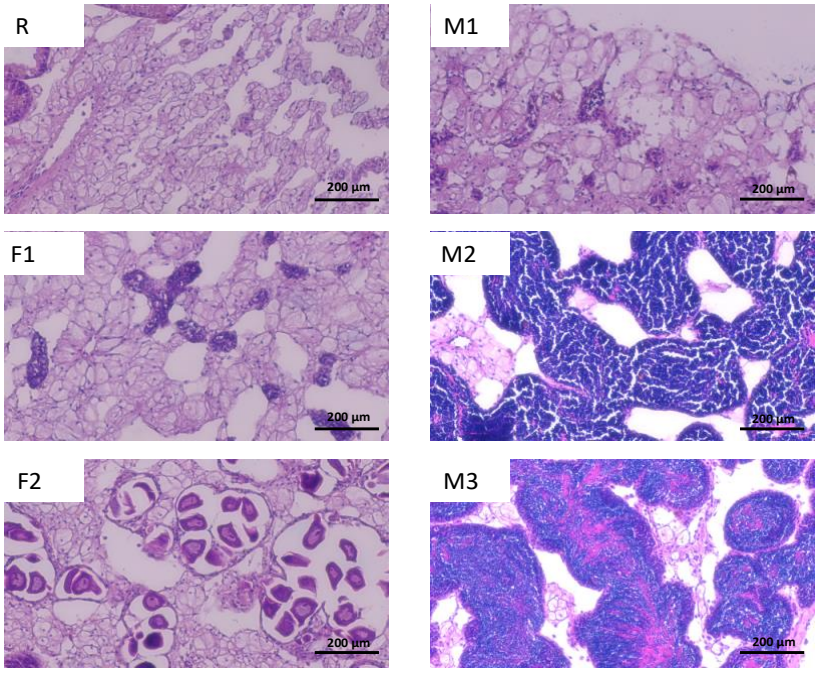


Figure 2.3. Photomicrographs of observed gonad stages in both female (F) and male (M) *Saccostrea echinata*. Scale bar = 200 µm. Resting (R); early development (1 – 2); late development (3).

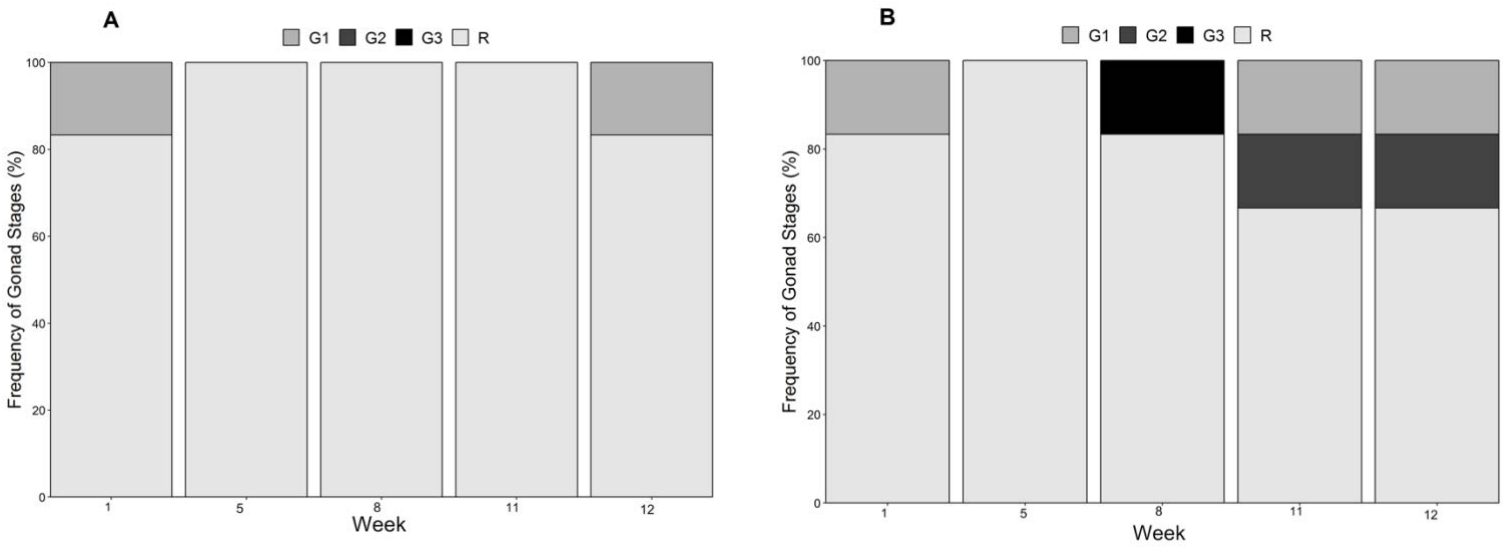


Figure 2.4. Frequency of *Saccostrea echinata* gonad stages (G1-G2, early development; G3, late development; and R, resting) for control (A) and heated (B) treatments, over 12 weeks.

Within the control treatment, the mean CI varied slightly between weeks, with a maximum CI of 65.46 recorded from week 12 and a minimum CI of 51.49 from week five (Figure 2.5). In contrast, the heated treatment displayed a minor but steady decline over the conditioning period. A maximum CI of 57.87 was recorded from week one and a minimum CI of 43.93 from week 12 (Figure 2.5). A *post hoc* Tukey HSD test demonstrated that there was no significant difference in CI between tank or treatment ($p > 0.5$). Furthermore, the Pearson's Correlation coefficient ($r = -0.48$) indicated that moderate negative correlation was present between mean CI and temperature.

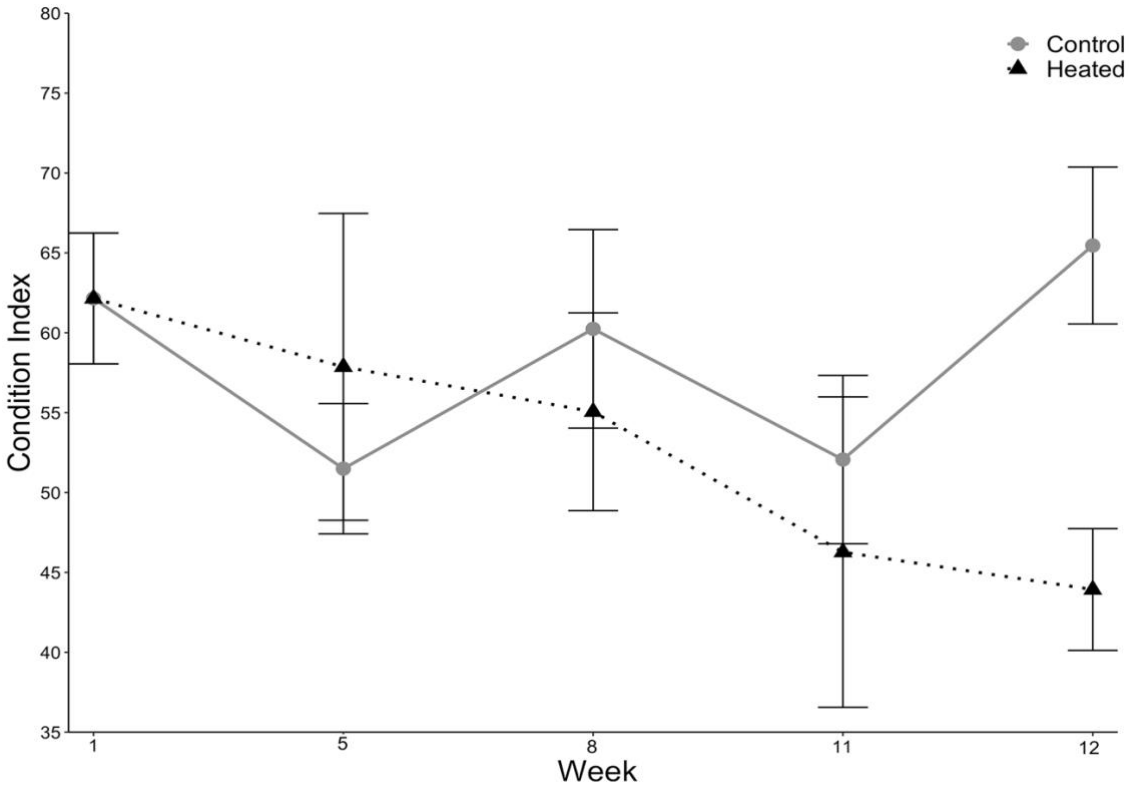


Figure 2.5. Changes in mean CI (± SE) of *Saccostrea echinata* broodstock from heated and control treatments, over 12 weeks.

Similarly, GI fluctuated slightly between weeks yet remained low throughout the experiment period. The control treatment remained stable throughout the conditioning period, whilst the heated treatment experienced a minor increase in GI between weeks five and eight, before plateauing and remaining constant (Figure 2.6). The GI values ranged between 0 and 0.17 in the control, and 0 and 0.5 in the heated treatment. Whilst the heated treatment appears to show an increase in GI in the later stages of the trial, the difference between the control and the heated treatment was not significant ($p > 0.05$). A Pearson's Correlation coefficient of $R = 0.35$ demonstrated a weak positive correlation was present between GI and temperature.

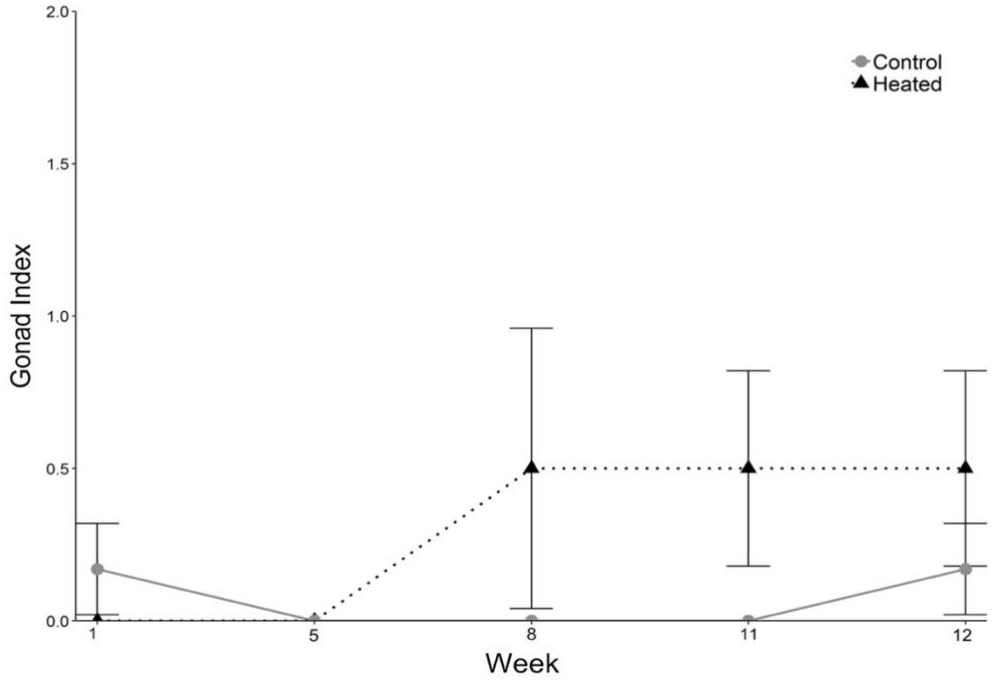


Figure 2.6. Changes in mean GI (± SE) of *Saccostrea echinata* broodstock from heated and control treatments over 12 weeks.

2.4 Discussion

This is the first investigation into the effect of temperature on *S. echinata* broodstock condition. The results obtained from this study indicate that water temperature, as a stand-alone exogenous factor, does not effectively initiate reproductive maturation of *S. echinata* broodstock. Broodstock exhibited an overall poor state of reproductive condition, with a large proportion remaining gender indeterminate and in the regressive stage of gonad development. This stands in contrast to temperature being the main environmental factor regulating gametogenesis in marine bivalves (Chávez-Villalba et al., 2002; Fabioux et al., 2005; Orton, 1920). It is likely that due to the reduction in annual water temperature variation in tropical locations, that temperature as a factor does not play a key role in driving reproductive maturation. While histological analysis can display variation, the limited number of broodstock available for this pilot trial determined that two replicate system were possible. The reduced sample size of broodstock is not believed to have detracted from the overall conclusions of the study.

Condition index (CI) is an effective technique for the macroscopic evaluation of oyster meat quality and yield. It is frequently used in such trials, due to its capacity to evaluate marketability (Mason & Nell, 1995). The CI values obtained in this study displayed minimal variation and were considerably

lower than those recorded by Nowland (2019) from wild populations of *S. echinata* from Melville Island, Goulburn Island and Mooroongga Island across the Northern Territory, Australia. The captive broodstock from the current study varied in CI values from 45 to 65, whilst the wild sampled oysters displayed CI values of 60–85 (Nowland, 2019). The perception that our recorded values were comparatively low, is further supported by the CI values of *S. glomerata* obtained by Dove & O'Connor (2012), where values of between approximately 45-90 were recorded from wild oyster populations across three estuary systems in New South Wales, Australia. A possible explanation for the comparatively lower CI values, is that the trial diet was not meeting the broodstock's nutritional demands and the reproductive tissue was remaining regressed and underdeveloped.

Gonad index (GI) values of 0 to 4 were recorded by Nowland et al., (2019) from wild *S. echinata* populations, and identified that oysters displayed a high GI of 3.5 to 4 during September to October indicating the spawning season. A similar period of high GI of 3.7 to 4 was identified by Lindsay (1994), between the months of October to November and described a yearly range of 0 to 4. This is in contrast to the present study, which achieved a maximum GI of 0.5, which relates to a gonad stage of between R (regressive) and G1 (early development), indicating that gametes are not mature enough for spawning.

The acclimation schedule of increasing temperature by 1°C per day until reaching the required temperature, was chosen based on past success in comparable studies by Chávez-Villalba et al., (2002) and Arguella-Guevara et al., (2013). Additionally, the treatment temperature of 31°C was selected based on local seawater temperature, which corresponds with maximum oyster condition in October/November. It is therefore considered unlikely that the chosen acclimation protocol and conditioning temperature was responsible for the observed poor reproductive condition obtained in the current study. Although, the influence of temperature on rock oyster reproductive conditioning is widely supported, seasonal shifts in water temperature typically display less variation in the tropics compared to temperate regions (Fournier, 1992; Loor & Sonnenholzner, 2016; Paixão et al., 2013). A long term study conducted in the temperate Clyde and Shoalhaven river systems of New South Wales, Australia, recorded temperature variations of 11.7 – 26.8°C and 13.8 – 24.7°C, respectively (Rubio, 2007). In comparison, water temperature data from the location of the present study (Darwin, Northern Territory) fluctuated between 25.7 – 31.4 °C over the course of the year (World Sea Temperature 2020). In summary, temperature appears not to be a key factor of influence due to its comparatively minor seasonal variation in tropical regions.

Due to the reproductive seasonality that many of these species exhibit, there is likely a varied degree of interaction between several exogenous factors. This enables oysters within close proximity of one another to synchronise their broadcast spawning and maximise the chances of successful fertilisation.

Due to the monsoon season, salinity is likely to be of key significance for tropical oyster reproduction (Nowland et al., 2019; Paixão et al., 2013). Correlative relationships between salinity and gonad maturation have been established in Brazilian populations of *C. gasar* (Paixão et al., 2013). A moderate link between rainfall and GI in northern Australian populations of *S. echinata,* has also been documented (r = 0.496), however, in this study seawater salinity was not directly measured, so it may play a more important role than these finding indicate (Nowland et al., 2019). Rapidly decreasing salinity has been linked to spawning induction in both populations of *S. prismatica* in Costa Rica (Fournier, 1992) and *S. echinata* in northern Australia, with salinity reduction also inducing spawning in the hatchery (Southgate & Lee, 1998). The specific effects of reduced salinity on gonadal maturation in *S. echinata* are unknown, therefore, it is recommended that salinity, coupled with elevated water temperature, be the focus of future broodstock conditioning studies.

Monsoonal events also have a coinciding impact upon nutrient profiles due to runoff and estuarine plumes, and this effect can vary greatly between locations (Fournier, 1992). Rising seawater temperatures coincide with the monsoonal season in September in northern Australia around the same time as widespread phytoplankton blooms (Munksgaard et al., 2017). These blooms, including *Trichodesmium*, cyanobacteria filaments and Bacillariophyceae, typically occur at the same time of year as GI increases in *S. echinata,* with the late stages of gametogenesis likely supported by the increased nutrient availability. Furthermore, it has been suggested that phytoplankton bloom events may act as a spawning signal, indicating that there is adequate food and favourable conditions for resulting larvae (Starr et al., 1990). The diet of live microalgae used throughout this study was based on the broodstock diet developed for *S. glomerata* by O'Connor et al., (2008). Whilst this diet has been very effective for temperate Australian species, it is possible that a key component specific to tropical species has not yet been identified and included. It is recommended that further studies be undertaken to investigate the diet of wild Blacklip oysters to further understand the dietary requirements of reproductively mature broodstock.

2.4.1 Conclusions

In summary, this study demonstrated that temperature is not an effective stand-alone method for the broodstock conditioning of *S. echinata.* Despite the previously established links between bivalve reproductive condition and temperature, increasing temperature does not achieve adequate maturation of *S. echinata* outside of its natural spawning season. Further research is required to better understand the effect of other exogenous factors, such as salinity and nutrition, on *S. echinata* broodstock conditioning.

3 CHAPTER 3. THE EFFECT OF SALINITY ON BROODSTOCK CONDITIONING OF TROPICAL BLACKLIP ROCK OYSTERS (*SACCOSTREA ECHINATA)*

3.1 Introduction

3.1.1 Tropical oysters

Recent innovations coupled with cost effective hatchery techniques, have enabled a variety of new bivalve species to gain traction within the tropical aquaculture industry (Nowland et al., 2019; Pawiro, 2010). Tropical rock oysters, in particular, have great potential to stimulate a globally important production sector (Nowland et al., 2019). At present, they provide a valuable source of protein and support economic growth in remote and developing regions (Nowland et al., 2019). Whilst tropical rock oysters display high marketability, consumer acceptance and favorable culture attributes, their production is currently vastly exceeded by their temperate counterparts (Nell, 2001; Nowland, 2019) For example, in Australia in 2017, small scale farms produced 1,000 dozen tropical oysters compared to 11,900 tonnes produced by the temperate sector (FAO Globefish, 2019; JR Collison pers. comm. 2018). One factor that has contributed to the widespread success of the temperate oyster sector is the establishment of reliable hatchery methods and subsequent production of spat (juvenile oysters), to satisfy growout demand (Helm et al., 2004).

3.1.2 The Blacklip rock oyster

The Blacklip rock oyster, *Saccostrea echinata* (Quoy and Gaimaard, 1835), is an emerging species of tropical oyster displaying strong aquaculture potential. They are a food source for many Indo-Pacific island nations and are harvested locally throughout Papua New Guinea, Indonesia, Micronesia, the Philippines and northern Australia (Lindsay, 1994; Thomson, 1953). Despite their rapid growth rate, high market acceptance, and potential for hatchery success, *S. echinata* is presently not cultured on a large commercial scale (Glude, 1984; Nowland, 2019; Southgate & Lee, 1998). Like most tropical oyster species, culture of *S. echinata* remains heavily reliant on wild spat supply, which is often unreliable and insufficient (Southgate & Lee, 1998). Currently, small-scale commercial farms are operational in Bowen in north-eastern Australia, Mago Island in Fiji, and Noumea in New Caledonia, while experimental farms are active in Goulburn Island and Groote Eylandt in northern Australia, (Nowland, 2019).

3.1.3 Reproduction

The reproductive maturation cycle of oysters comprises several key stages including gamete growth and ripening, spawning, resting and gonad regeneration (Dinamani, 1974; Helm et al., 2004). Reproductive patterns of bivalves are typically initiated and synchronized by endogenous factors such as genotype and neuroendocrine cycles and exogenous factors such as water temperature, food type and availability, salinity and photoperiod (Lindsay, 1994; Utting & Millican, 1997). These key factors

of influence show great variability between tropical and temperate locations and are often species-specific (Utting & Millican, 1997). For aquaculture production to be successful, a detailed understanding of the endogenous and exogenous factors driving broodstock maturation is crucial so that these factors can be manipulated in a controlled environment to induce reliable maturation and spawning.

3.1.4 Broodstock conditioning

Broodstock conditioning is a process which aims to maximise the fecundity of parent animals (broodstock) whilst maintaining egg quality and larval viability. During this practice, exogenous factors, typically the physical environment and nutrition, are manipulated to stimulate gonad development and gametogenesis (Utting & Millican, 1997). The impact of temperature has been investigated and confirmed across several temperate species including; *Crassostrea gigas* (Fabioux et al., 2005), *Saccostrea glomerata* (Dove & O'Connor, 2012), *Ostrea edulis* (Maneiro et al., 2016) and *Crassostrea virginica* (O'Beirn et al., 1996) in addition to the tropical oysters *Crassostrea gasar* (Ramos et al. 2014) and *Striostrea prismatica* (Arguella-Guevara et al., 2013). However, findings from Chapter two indicate increased water temperatures alone, had no positive effect on gonad maturation. Furthermore, studies undertaken on wild tropical Blacklip oyster populations have demonstrated a reproductive peak in the wet season in both the Northern Territory and north Queensland, Australia (Lindsay, 1994; Nowland et al., 2019). Natural breeding patterns were recorded by Nowland et al (2019), and include a semi-continous spawning phase thoughout the monsoon season (October to April) and an extended resting phase during the dry season (May to September). Due to the seasonal fluctations of coastal salinity, coupled with the relatively stable seawater temperatures in the tropics it is hypothesised that rainfall events and subsequent reduction in salinity play a role in regulating tropical oyster maturation (Nowland et al., 2019). For example, this has been confirmed for *C. gasar*, a tropical species native to the Amazon region, where increased precipitation in the wet season and subsequent salinity reduction, initiated reproductive maturation in wild stocks (Paixão et al., 2013). Futhermore, an additional study into the reproductive behaviour of *S. prismatica* in Costa Rica, highlighted spawning to occur during the wet season, indicating a synergistic effect between temperature, salinity and gonad maturation is likely present (Fournier, 1992).

For the tropical Blacklip rock oyster to become a commercially viable aquaculture species, a large volume of high quality, hatchery produced spat is required to meet current growout demand. Identifying the factors regulating broodstock conditioning will enable year-round hatchery production to meet current demands. Therefore, the aim of this study was to investigate the effect of salinity on broodstock conditioning of Blacklip rock oysters.

3.2 Materials and methods

3.2.1 Experimental system

A total of 440 Blacklip broodstock oysters were obtained from the Bowen Fresh Oyster lease, located in a tidal creek system north of Bowen, Queensland, Australia. This trial was conducted utilising the facilities at the Marine and Aquaculture Research Facility (MARFU), James Cook University, Townsville, Australia. The conditioning trial ran for a total of 12 weeks, from July through to October. A total of eight recirculating systems were used, each consisting of five 35 L holding aquaria, which were each supplied with seawater and food (microalgae) by a 290 L sump. At the commencement of the trial, each system housed 50 oysters with 10 oysters per aquaria. Plastic mesh suspended within each aquarium, provided additional substrate for oysters, enabling adequate spacing and water circulation in each aquarium (i.e. two levels in each aquarium with five oysters on each level). Two replicate systems were utilised for each salinity treatment (20, 25, 30 or 36 ppt) and temperature was maintained constant at 29 ±1 °C. The limited space and facilities available for this trial determined that two replicate systems per salinity were possible. The temperature of 29 °C was selected based on the results from the temperature pilot study conducted in Chapter 2. Additionally, this temperature also falls with the typical water temperatures of the Townsville region during the months of November to February (27 – 30°C), which corresponded with natural oyster reproduction in local populations off Orpheus Island (Lindsay, 1994) just north of Townsville. The control salinity of 36 ppt was selected, as oceanic salinity remains relatively stable at 36 ppt in north Queensland, outside peak monsoon activity. The treatment salinities of 20, 25 and 30 ppt were selected to include a likely range of variation experienced in coastal areas. All broodstock underwent an acclimation period of seven days upon arrival at the MARFU laboratory, whereby salinity and temperature were gradually adjusted from farm conditions (32 ppt and 24 °C) to meet the required experimental conditions (Table 3.1). Temperature acclimation was achieved by increasing temperature 1 °C per day, for seven days until reaching 29°C (Arguella-Guevara et al., 2013; Chávez-Villalba et al., 2002). Salinity acclimation was achieved by adjusting the salinity by 1-2 ppt per day until the required level was met.

To maintain water quality, all broodstock were transferred to a clean holding system twice weekly. This allowed holding tanks and sumps to be cleaned and a 60% water exchange to take place. The original sump water was used to refill the clean holding tanks, thereby minimising time oysters spent out of water and reducing the likelihood of stress induced spawning. Faecal matter was removed by siphoning on alternate days. Temperature and salinity were recorded daily, while ammonia, nitrate and nitrite were recorded at the start of the trial to ensure the system design and water exchange rates were adequate. Dissolved oxygen (DO) and pH levels were measured three times per week using hand held probes (Laqua, Japan). Two temperature data loggers (HOBO, USA) were deployed as a secondary temperature recording system, and these recorded temperature hourly.

Table 3.1. Salinity and temperature acclimation schedule for broodstock *Saccostrea echinata* from farm to experimental conditions. Experiments commenced on day 8.

	Salinity acclimation (ppt) for each treatment				Temperature acclimation (°C) (all systems)
	20	25	30	36 (Control)	
Day 1	32	32	32	32	24
Day 2	30	32	32	32	24
Day 3	28	30	30	34	25
Day 4	26	28	30	36	26
Day 5	24	26	30	36	27
Day 6	22	25	30	36	28
Day 7	20	25	30	36	29

3.2.2 Feeding regime

Oysters were primarily fed formulated algal paste (Shellfish Diet 1800, Pro Aqua, Australia), supplemented with live algae species *Chaetoceros muelleri* (CS-176 CSIRO catalogue code), *Tisochrysis lutea (CS-177* CSIRO catalogue code) and *Pavlova* sp. (CS-50 CSIRO catalogue code) when available (see Appendix 2 for food schedule). Broodstock were initially fed at a rate of 2 billion cells per oyster per day. This feed rate was increased to 2.5 billion cells per oyster per day at week 5, 3 billion cells per oyster per day at week 7 and 3.5 billion at week 9. The respective quantities of algal paste and live algae were batch fed twice daily at approximately 0700 and 1700 hours.

Number of oysters X algal concentration per oyster per day = Total algal feed per day Eq. 3.1

3.2.3 Biometric data collection and histology

Oysters (n = 10 per treatment) were sampled at regular intervals (bi-weekly to weekly) to assess reproductive condition (Table 3.2). At the conclusion of the experiment (week 12), ten oysters from the Bowen farm were collected and included in sampling to provide a point of reference to wild stocks. Prior to the commencement of the acclimation period, biometric measurements and histological cross sections A and B were collected for 40 broodstock oysters (Figure 3.1). During all sampling periods, whole oyster weight was determined by an electronic balance with a precision of 0.1 g. Dorso-ventral measurement (DVM) and antero-posterior measurement (APM) were taken for each oyster using a ruler, recorded to the nearest millimeter. Following this, the visceral mass was extracted and excess water removed by placing onto paper towel for approximately ten seconds, before the visceral mass wet weight and shell weight were determined to a precision of 0.1 g.

Table 3.2. The sampling frequency of broodstock *Saccostrea echinata*.

Weeks of conditioning	No. sampled	No. sampled per treatment	Total Remaining oysters
0	40	-	400
2	40	10	360
4	40	10	320
6	40	10	280
7	40	10	240
8	40	10	200
9	40	10	160
10	40	10	120
11	40	10	80
12	40	10	40

A 6 mm thick cross-section of the dorsal-visceral mass was obtained from the region where the labial palps meet the gills, and parallel with the hinge (Figure 3.1). The sections (A and B) were then placed into histological cassettes, fixed in 10% seawater formalin for 48 h and then processed and embedded in paraffin. Cross sections 4 µm thick were mounted and stained with hematoxylin and eosin at the JCU Veterinary diagnostic pathology laboratory using standard methods (Luna 1968; Raphael et al. 1976). Once processed, the slides were evaluated microscopically following the Dinamani (1974) classification system for *S. glomerata*, to determine gender and reproductive condition. All slides underwent a re-assessment at the completion of the experiment to ensure grading consistency. Based on the factors of cell size and shape, nuclear features and staining characteristics, broodstock were allocated a rating of gamete development; resting (R), early development (G1-G2), late development (G3), ripe (G4), discharged (G5) and residual (Gx) (Dinamani, 1974).

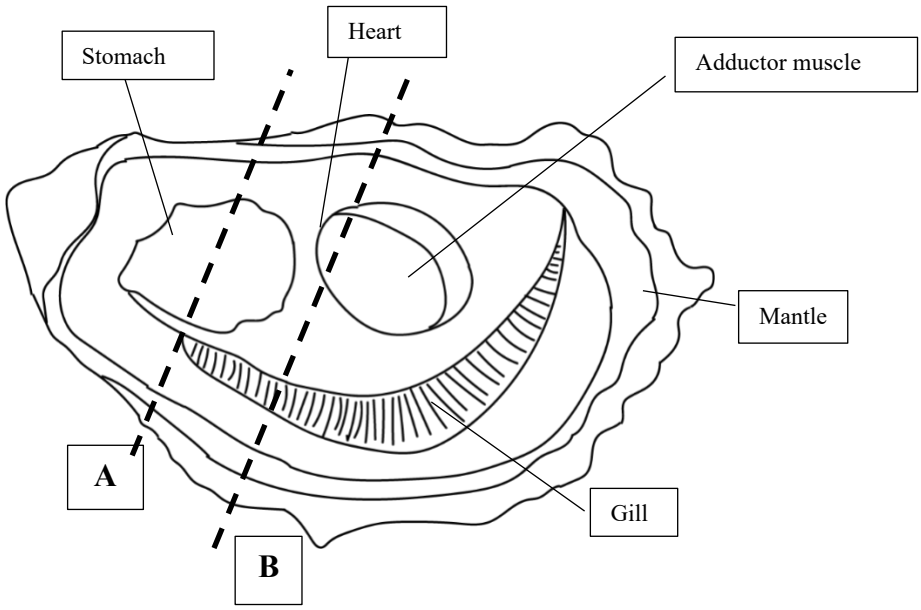


Figure 3.1. Anatomy of an oyster showing 6 mm histological cross-sections (A and B). Image adapted from Thompson (1954).

3.2.4 Mean gonad index

Gonad index (GI) is a widely used microscopic assessment technique in broodstock conditioning, as it classifies the reproductive maturation stage that has been attained. Mean GI was calculated for each of the sampling periods, following the formula described by Seed (1969);

$$M = \frac{\Sigma(ns)}{N}, \qquad \text{Eq. 3.2}$$

Where *n* is the number of oysters present in a given gametogenic stage, *s* is the numerical ranking of that particular stage, and *N* is the total number of oysters in the sample (Braley, 1982, 1984; Seed, 1969). Each gametogenic phase was allocated a numerical ranking, following the classification system outlined by Dinamani (1974); R = 0, G1 = 1, G2 = 2, G3 = 3, G4 = 4, G5 = 0, and Gx = 0. The gonad index can vary from 0, when all the individuals within a sample are in the resting (R) phase or have completely spawned (G5), to 4 when all broodstock are sexually mature (G4).

3.2.5 Mean condition index

Condition index (CI) is a technique for the macroscopic evaluation of oyster meat quality and yield (Mason & Nell, 1995). The wet weight condition index was determined for each oyster sampled and calculated using the formula as described by Hopkins (1949);

$$CI = \frac{v}{w-s} \text{ X } 100, \qquad \text{Eq. 3.3}$$

where *v* is the visceral mass wet weight (g), *w* is the whole oyster weight (g) and *s* is the shell weight (g).

3.2.6 Spawning and fecundity assessment

Following completion of the experiment, spawning was induced in order to non-destructively assess reproductive maturity of remaining broodstock. Spawning was induced via a reduction in salinity and addition of sperm as described by Nowland et al., (2021). Briefly, 40 broodstock were labelled according to their salinity treatment (n=10) and randomly arranged on a 160 L spawning table (246 cm x 51 cm x 13 cm), where they were left to air dry overnight (17 h). The spawning system contained a 290 L sump with a pump to re-circulate seawater.

The following morning, the spawning table was filled with 36 ppt seawater from the sump and broodstock were observed until they had opened and began to filter the surrounding water (approximately 45 min). Freshwater was then added to the sump to rapidly lower the salinity from 36 to 20 ppt, before pumping through the table to induce spawning. As spawning commenced, males were recorded and left to spawn on the table to induce other oysters to spawn. When a spawning female was identified, it was removed from the table and placed in an individual labeled 2 L aquarium, to allow spawning completion. The 2 L aquaria containing seawater and eggs were transferred to a clean bucket and filled to 10 L with seawater. After stirring the bucket to achieve an even distribution of eggs, three replicate 100 µL samples were taken to determine fecundity and a 1 mL sample was collected from each bucket and stored in a 3 mL tube with 10% seawater formalin, to determine average egg size using an eyepiece graticule. Sperm from each of the males was collected and the time was noted as the sperm lasts for approximately 20 minutes. The eggs were then fertilised as soon as practically possible. Enough sperm was supplied so that at least one sperm is seen at the periphery of each egg (approximately 5 mL of pooled sperm).

Using a compound microscope at 10 x magnification, eggs were inspected. Once a polar body could be viewed on the periphery of 60% of eggs, they were gently washed using filtered saltwater (28 °C and 36 ppt) through a pair of 15 µm nylon mesh nets, before being transferred into a clean bucket with 10 L of seawater. Once the majority of eggs were fertilised, cleaned, and starting to divide, the water volume in the bucket was made up to 15 L and air stones were added for gentle aeration. The following day, at 15 h post fertilization, the number of D-stage veliger larvae in each bucket were counted to determine hatch rate. The process of spawning via reduction in salinity and addition of sperm, was repeated twice daily for two consecutive days, after which time it was assumed that remaining oysters that had not spawned were not in adequate reproductive condition.

3.2.7 Statistical analyses

Water quality parameters were analysed using a one-way ANOVA. Biometric data (DVM, APM, whole weight and CI) and GI were tested for normality and homogeneity of variance. A one-way ANOVA, followed by a post hoc Tukey honest significant difference (HSD) test was used to investigate differences in whole weight, DVM and APM of stocked oysters and variation of CI between treatments. The application of a Chi-squared test was used to determine significant differences in gender frequencies between the treatment groups. A two-way ANOVA including treatment and week as fixed effects was used to investigate their effect upon GI. To assess the prevalence of ripe (G3-G4) stage between treatments, a general linear model with binomial response was used. Following this, a pairwise multi-comparison test was also performed using a Tukey HSD test. The Pearson correlation coefficient was used to test for a linear relationship between GI and salinity. All analyses were completed and plots created using R (R Core Team 2020).

3.3 Results

3.3.1 Water quality parameters

Mean temperature variation was not significantly different across the four treatments and ranged from 28.9 – 29.4 °C. Dissolved oxygen and pH mean variation was also not significant and ranged between 5.52 – 7.88 mg/L, (87.6 – 101.2 % saturation) and 8.04 – 8.25 respectively across the treatments (Table 3.3).

Table 3.3. Salinity, temperature, dissolved oxygen, pH, ammonia, nitrite and nitrate recordings within treatments.

Treatment	Salinity (ppt)	Temperature (°C)	DO (mg/L)	DO %	pH	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)
20	19 – 22	28.8 – 29.2	5.52 – 7.88	87.6 – 101.2	8.06 – 8.2	0 – 0.25	0 – 0.5	0 - 20
25	24 – 26	28.8 – 29.2	5.87 – 7.68	92.7 – 100.1	8.04 – 8.2	0 – 0.5	0 – 1.0	0 - 20
30	29 – 31	28.2 – 29.4	6.02 – 7.72	94.7 - 100	8.08 – 8.18	0 – 0.25	0 – 0.5	0 - 20
36	33 – 36	28.9 – 29.3	6.01 – 7.66	94.8 - 100	8.09 – 8.25	0 – 1.0	0 – 0.25	0 - 20

3.3.2 Biometric data

A total of 400 oysters were sampled during the 12 week conditioning experiment. No significant difference in mean whole weight, DVM or APM of stocked oysters was detected between treatments, except for those between 20 and 36 ppt. Oysters stocked into the 20 ppt treatment were larger and had a longer mean DVM and APM. However, the range of whole weight, DVM and APM was; 290.4 to

69.3 g, 130 to 68 mm and 85 to 43 mm respectively, for 20 ppt and 234.1 to 80.3 g, 106 to 68 mm and 81 to 49 mm respectively for 36 ppt (Table 3.4). This difference was therefore not expected to influence the experimental results.

Table 3.4. Mean whole weight (g), DVM (mm) and APM (mm) of stocked oysters, *Saccostrea echinata*.

Salinity treatment (ppt)	Mean whole weight ± SE (g)	Mean DVM ± SE (mm)	Mean APM ± SE (mm)
20 ppt	177.39 ± 6.13	95.18 ± 1.59	67.51 ± 1.01
25 ppt	161.49 ± 4.37	91.01 ± 1.21	66.24 ± 0.75
30 ppt	160.46 ± 3.68	91.49 ± 1.03	66.20 ± 0.75
36 ppt	156.08 ± 3.67	88.14 ± 0.88	64.90 ± 0.74

3.3.3 Histology, condition index and gonad index

Assessment of gender indicated that 30.0% of all broodstock sampled were gender indeterminant. Of those remaining, 31.6% were male and 38.3% female. The lower salinity treatments of 20, 25 and 30 ppt resulted in a proportionately larger percentage of females than males. This difference however, was not significant (Figure 3.2). The 25 and 30 ppt treatments resulted in the least number of individuals classed as indeterminate. Conversely, the control salinity of 36 ppt returned a higher proportion of males and comparatively more gender indeterminate broodstock (Figure 3.2). The prevalence of indeterminate oysters is notably reduced in the later weeks of 9 to 12 within the 20 and 25 ppt treatments (Figure 3.2).

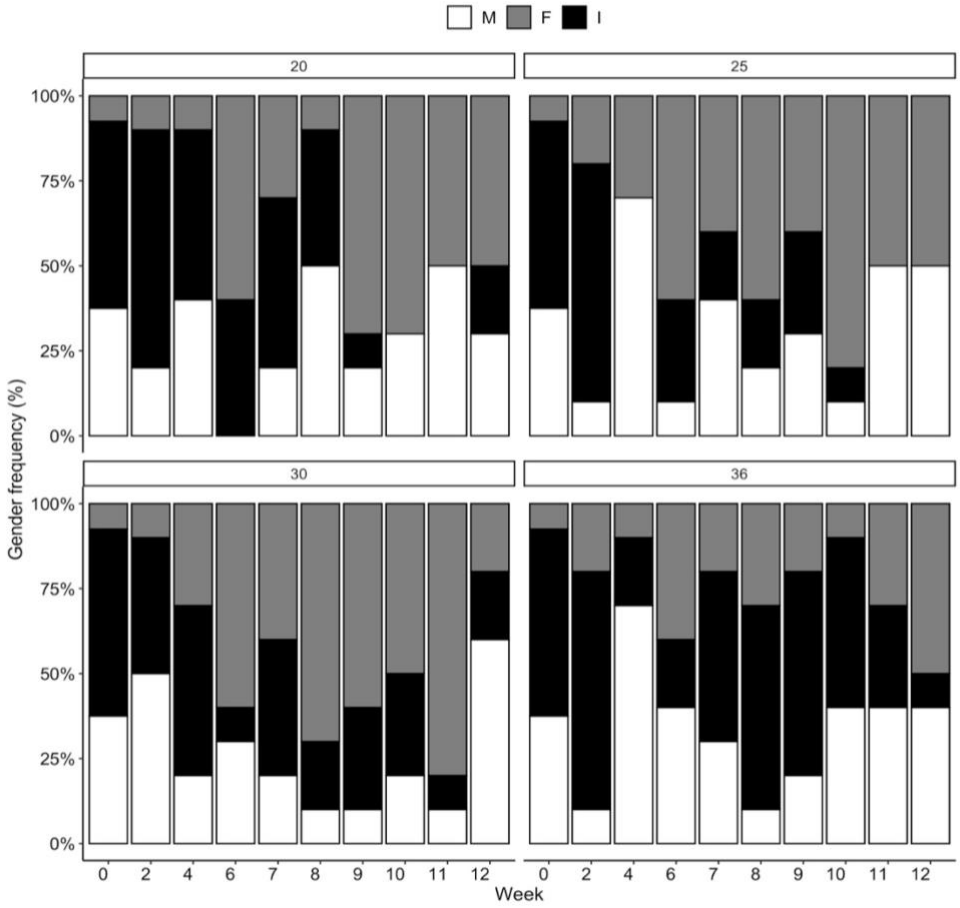


Figure 3.2. Gender frequency of *Saccostrea echinata* (M= Male; F= Female; I= Indeterminate) for 20, 25, 30 and 36 ppt salinity treatments, at each sampling week.

Mean CI of broodstock oysters returned a maximum of 77.61 and a minimum of 62.14, and no significant differences were recorded between treatments (F = 0.609; *df 1/357*; P = 0.436) (Figure 3.3). All four treatments displayed a similar pattern of mean CI variance throughout the 12 week period. The treatment of 25 ppt displayed a significant increase in CI (F = 2.183; *df* 8/81; P = 0.037) and a Tukey HSD test highlighted this to occur between weeks 4 and 10 (P = 0.016). The treatments of 20, 30 and 36 ppt, did not experience a significant change in CI between weeks (Figure 3.3).

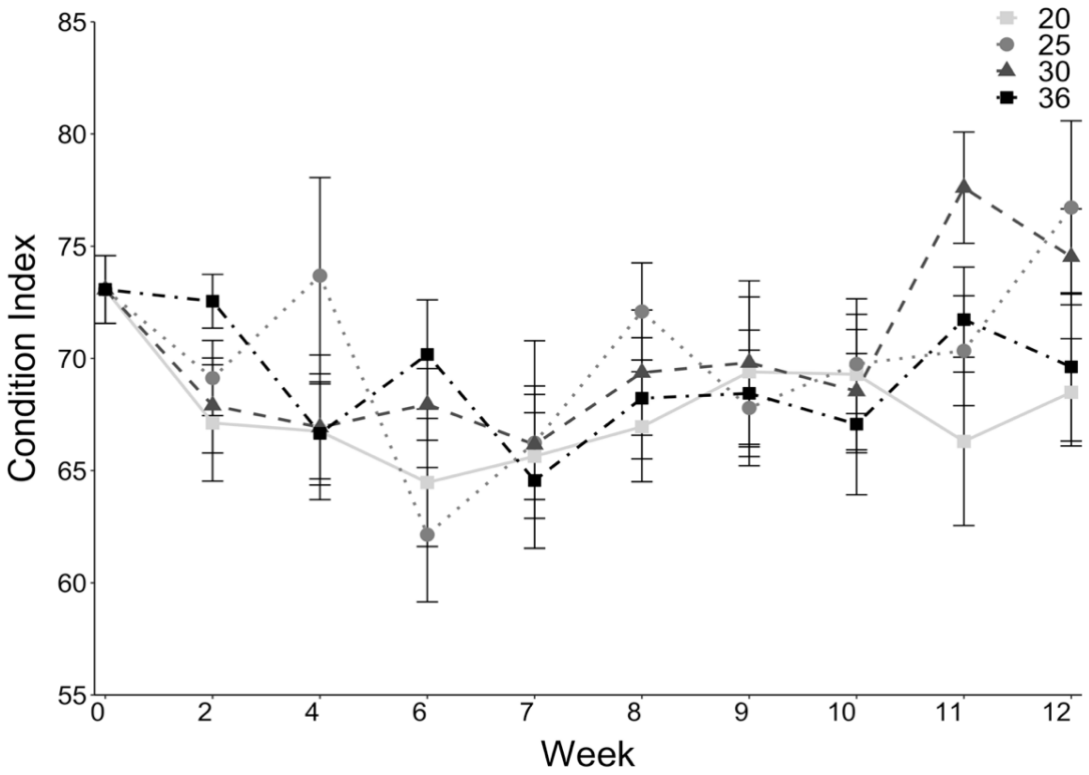


Figure 3.3. Changes in mean CI (± SE) of *Saccostrea echinata* from 20, 25, 30 and 36 ppt treatments, over 12 weeks.

The mean gonad index (GI) fluctuated throughout the 12 week period for all four treatments. The treatments of 20 and 25 ppt displayed a similar pattern of development, with both remaining relatively stable following week 4 and with the highest GIs recorded at the end of the 12 week trial (Figure 3.4). The 25 ppt treatment produced the maximum mean GI recorded in the trial, of 2.10 ± 0.28 during week 4, followed by a value of 2.0 ± 0.67 in week 7. The GI recorded within treatment 20 ppt remained relatively stable for a period of time, before experiencing some minor fluctuations during the final four weeks. The 30 and 36 ppt treatments both displayed periods of notable GI decline from week 6 to 9 and 6 to 10, respectively. Whilst the broodstock within the 30 ppt treatment experienced two rapid declines in GI following weeks 8 and 11, the oysters within the 36 ppt treatment displayed a gradual rise in GI at both the beginning and conclusion of the conditioning period, with a gradual decline throughout the middle weeks (Figure 3.4). A two-way ANOVA including treatment and week as fixed effects revealed that week did not significantly influence mean GI ($p = 0.13$), however, the effect of treatment was significant ($p = 0.00687$). Following this, a post-hoc tukey HDS test revealed that the 25 ppt treatment was significantly different from the control of 36 ppt ($p = 0.007$). A Pearson's Correlation coefficient of $R = - 0.397$ demonstrated a moderate negative correlative relationship existed between GI and salinity.

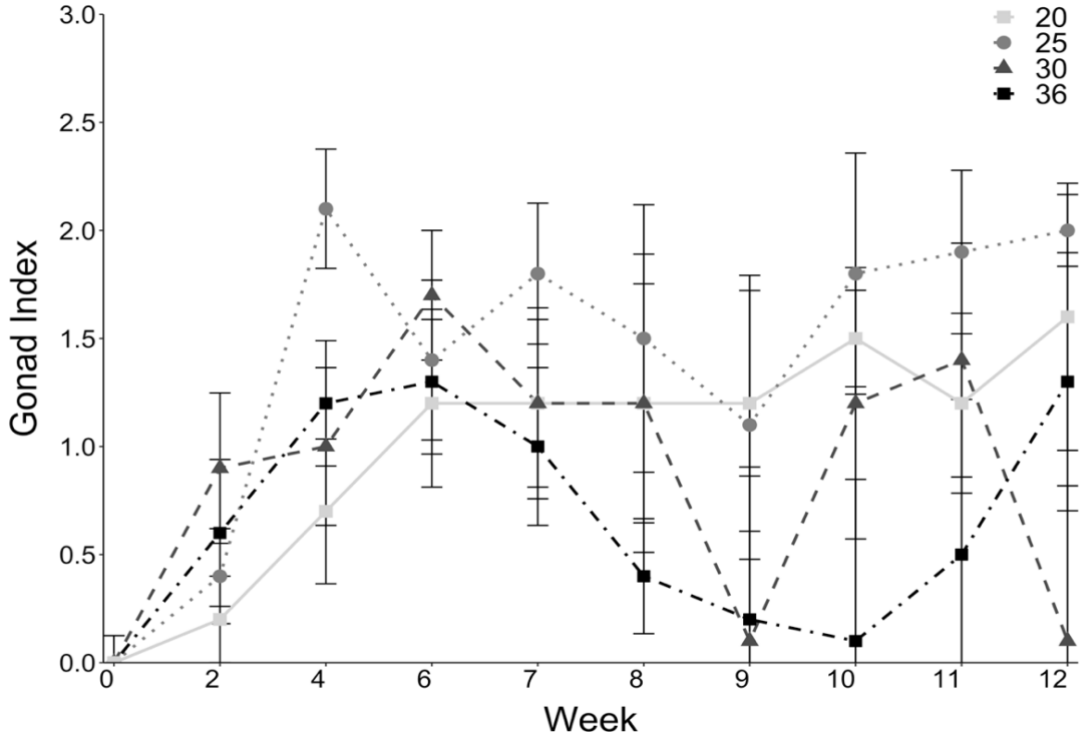


Figure 3.4. Changes in mean Gonad Index (GI) (± SE) of *Saccostrea echinata* from 20, 25, 30 and 36 ppt treatments, over 12 weeks.

All four salinity treatments displayed a transition in gonad phase frequency across the 12 week conditioning period. With respect to ripe broodstock (G3-4), the 20 and 25 ppt treatments both first displayed this stage at week 8 (Figure 3.5). A significant difference in the proportion of ripe (G3-4) individuals between treatment groups, was detected using a general linear model with binomial response ($X^2 = 25.13$; $df = 3$; Pr ($> X^2$) < 0.001. The salinity treatments of 20 ppt (P = 0.007) and 25 ppt (P = 0.001) displayed a significantly higher number of ripe individuals (G3-4), when compared to the 36 ppt treatment. Additionally, the 25 ppt treatment was significantly different overall to the 30 ppt treatment (P = 0.01).

The higher salinity treatments of 30 and 36 ppt revealed a greater frequency of the regressive (R) stage. A feature consistent across all four salinity treatments is that broodstock displayed signs of partial or complete spawning. Partially spawned broodstock (G5) were first identified in week 8 for 20, 25 and 30 ppt treatments, while they appeared later in week 9 for 36 ppt. The relative frequency of stage G5 varied between treatments. The 20 ppt treatment produced the most partially spawned (G5) individuals, while the 36 ppt treatment produced the least. The higher salinity treatments, particularly 36 ppt, displayed the greatest prevalence of residual (Gx) individuals, whilst this stage was less frequent in the 20 and 25 ppt treatments. Furthermore, the resting (R) phase persisted most notably in the higher salinities throughout the later stage of the trials (Figure 3.5). Of the 10 oysters sampled

from the farm location at the trial conclusion, nine were identified as female and one as male. A mean GI of 2.4 ± 0.56 was obtained, with 10% in stage G1 – G2, 50% G3-G4 (ripe) and 30% in the G5 (partially spawned) stage.

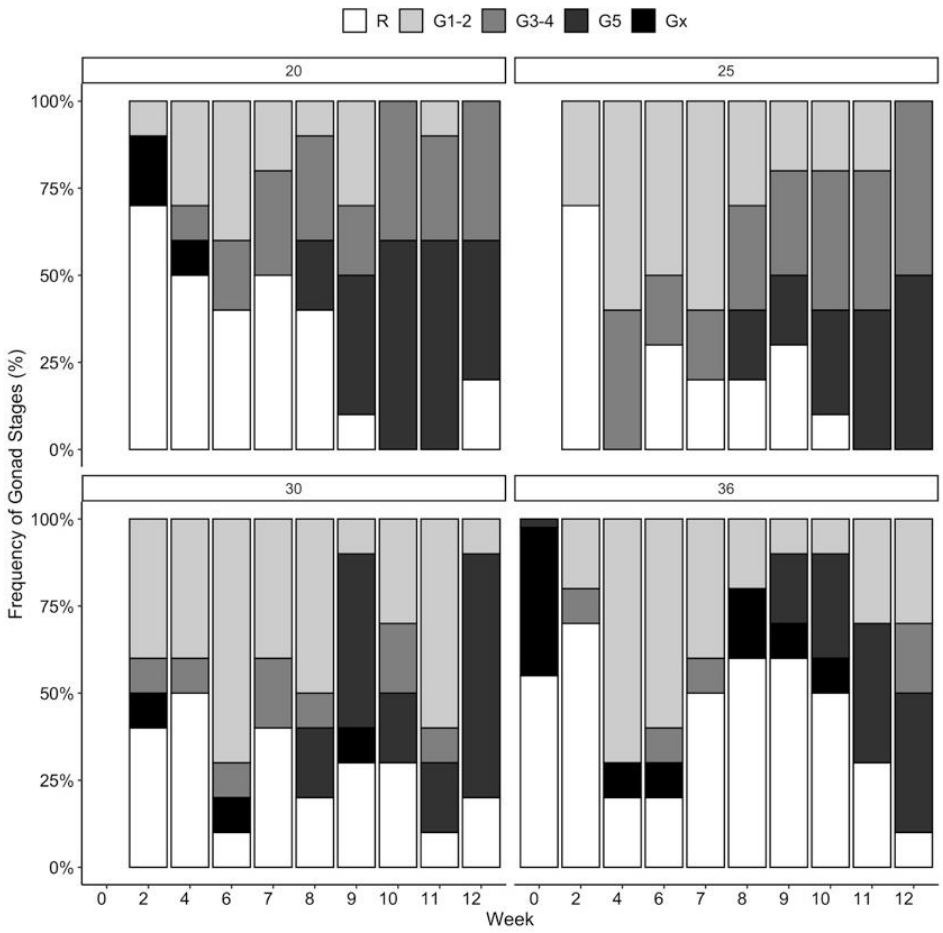


Figure 3.5. Frequency of *Saccostrea echinata* gonad stages (R, resting; G1-G2, early development; G3-G4, late development to ripe; G5, discharged; and Gx, residual) for 20, 25, 30 and 36 ppt salinity treatments, over 12 weeks.

3.3.4 Spawning trial and fecundity assessment

Of the 40 broodstock oysters that underwent spawning induction, 11 oysters (two females and nine males) were successfully induced to spawn. Five males spawned on the first day of spawning induction and three males and two females spawned on the second day. Broodstock from the lower salinity treatments, 20 ppt and 25 ppt, contributed to nine of the 11 spawning individuals (Tables 3.5. Of the two females that successfully spawned, the female from the 30 ppt treatment produced over 12 times the number of eggs than the female from the 20 ppt treatment, and produced slightly larger average D stage larvae. The hatch rate between the two females was similar.

Table 3.5. Relative number of males and female *Saccostrea echinata,* spawned across four treatments following salinity shock.

Salinity treatment (ppt)	Number of males spawned	Number of females spawned
20	5	1
25	3	0
30	0	1
36	1	0

3.4 DISCUSSION

This is the first conditioning trial conducted to assess the effect of salinity on gonad maturation and reproductive condition in Blacklip rock oyster broodstock. This broodstock conditioning trial determined that holding broodstock at lower salinities is an effective technique to condition Blacklip rock oysters. The lower salinity treatments of 25 ppt promoted a greater proportion of ripe individuals, when compared to the higher salinity treatments of 30 and 36 ppt. This result is in accordance with data collected from wild *C. gasar* in the Amazon region, which displayed the greatest prevalence of mature individuals in the months following a salinity drop from approximately 35 ppt to 20 ppt (Paixão et al., 2013).

The CI values from the current study displayed minimal variation, with all four treatments presenting a comparable pattern throughout the 12 week duration. Broodstock produced CI values of approximately 62 to 77 which is in accordance with wild sampled *S. echinata* broodstock from Melville Island, Goulburn Island and Mooroongga Island, in the Northern Territory, Australia; which recorded CI values of 60 to 85 (Nowland et al., 2019). An additional CI data set obtained from the temperate species, *S. glomerata,* provided values of between 45 during November to December and 90 in June to July, from wild oyster populations in New South Wales, Australia (Dove & O'Connor, 2012). This demonstrates that the broodstock within the trial were reaching adequate condition under hatchery conditions and were successfully consuming feed.

Whilst the GI values were an improvement from Chapter 2, which investigated the effects of temperature, they were low in comparison to subsequent wild sourced samples of *S. echinata*. Over a period of 18 months, Nowland et al., (2019) recorded GI values of *S. echinata* ranging from 0 to 4 from wild populations in the Northern Territory, Australia, including a rapid increase from 0.4 to 3.8 during the months of September to October. Similarly, Lindsay (1994) also recorded GI ranging from 0 to 4 from a local population of *S. echinata* on Orpheus Island, in north Queensland, peaking at 4 between the months of November to January, as the gonad tissue became fully developed in the lead up to spawning. The moderate negative correlation established between salinity and GI is a key

outcome of this study, and confirms that salinity is a critical exogenous factor stimulating reproductive development in this species. The presence of regressive stage individuals was noted to persist throughout the 12 week period in the higher salinity treatments, while in the lower treatments of 20 and 25 ppt they were seen to be phased out. This is an additional argument that strongly advocates for the 25 ppt treatment to be optimal for *S. echinata.* The majority of commonly cultured oyster species undergo reproductive development at salinities greater than 25 ppt (Helm et al., 2004). The temperate species of *S. glomerata* is typically conditioned at 33 to 35 ppt (O'Connor et al., 2008), *C. gigas* at 34.5 ppt (Fabioux et al., 2005) and *Crassostrea angulata* at 35 ppt (Anjos et al., 2017); all of which are significantly higher than the salinity recommended from this trial.

Oysters within all four treatments displayed signs that partial and full spawning took place during the trial. Within the three treatments with lowest salinities, spawned individuals were first identified after eight weeks of conditioning. No obvious human errors, such as overfeeding or disturbance, could be linked to the unplanned spawnings (O'Connor et al., 2008). In their natural setting, mature Blacklip oysters undergo a semicontinuous spawning pattern throughout the monsoon season (Nowland et al., 2019). It is therefore likely that mature broodstock held at low salinity for a prolonged period spawned. At the completion of the current study spawning was induced, and a comparatively higher number of oysters spawned from the lower salinities of 20 and 25 ppt, compared to the salinities of 30 and 36 ppt. This outcome was expected as these treatments contained the highest percentage of ripe individuals at the time of spawning.

Successful hatchery production of bivalves is unmistakably linked to feeding regimes, dietary composition and quantity, and is often highly species specific (Helm et al., 2004; Utting & Millican, 1997). Previous studies and trials with *S. echinata* have utilised a live microalgal diet developed for *S. glomerata* by O'Connor et al. (2008). Whilst this diet composition has been successfully used with temperate species, there may still be a key nutritional component specific to tropical species yet to be identified. In contrast to many previous bivalve conditioning trials, the diet used for this study comprised predominately of formulated microalgal paste. Due to resource restrictions and available infrastructure, the production of live microalgae was limited and subsequently comprised approximately 25% daily cell intake during the later stages of the trial (Appendix 2). Pastes have often been regarded as a secondary nutrition choice behind live microalgae (Robert et al., 2001), however, a key outcome of this trial was that a diet of 75 - 100% formulated paste was found to be a viable method for achieving reproductive conditioning of *S. echinata* broodstock. Based on the potential cost advantages of using formulated algal pastes, a future conditioning trial that compares the effects of live feeds and pastes on broodstock fecundity and larval viability is necessary to determine the overall benefits of paste substitution.

In summary, the salinity conditioning methods employed in this study proved to be successful in promoting reproductive maturation and enabled spawning of *S. echinata*. The use of two replicate systems per salinity treatment is not believed to have impacted the findings or compromised significant differences. The negative correlation present between salinity and GI and the greater number ripe (G4 stage) individuals in the treatments of 20 and 25 ppt, confirm that low salinities are more successful at conditioning broodstock. Whilst unintentional spawning during the conditioning trial was not a desired outcome, it demonstrated the ability to condition broodstock, fed mostly on algal paste, to a point at which they were able to successfully spawn. This study indicates that lower salinities contribute to successful broodstock conditioning of Blacklip rock oysters, and highlights the salinity of 25 ppt to be most effective. From an industry perspective, these findings represent a positive step towards the commercialisation of this species.

4 CHAPTER 4. THE INFLUENCE OF TEMPERATURE ON THE FILTRATION RATE OF BLACKLIP ROCK OYSTERS (*SACCOSTREA ECHINATA*) AND AN ASSESSMENT OF THEIR BIOREMEDIATORY ABILITY IN PRAWN AQUACULTURE WASTE WATER

4.1 Introduction

4.1.1 Ecological challenges facing aquaculture

The global aquaculture industry is one of the fastest growing primary production sectors, with an average annual production increase of 5.3% in the period of 2001 – 2018 (FAO, 2020). There is an increasing desire to expand and intensify land based production facilities culturing species such as prawns, that in turn require a large volume of water for growout phases (Webb et al., 2012). In many countries, intensive aquaculture facilities take in water from the natural environment and then discharge culture water back into these environments, via settlement ponds (Eng et al., 1989). Significant planning and water flow testing is required to select suitable production areas, with adequate tidal currents required to effectively disperse effluent (Nasir et al., 2015). If unsuccessful, the nutrient-rich waste water (e.g. high in nitrogen and phosphorus) can degrade marine environments through the processes of eutrophication, algal blooms and smothering benthic environments (Ziemann et al., 1992). As the environmental sustainability of the global aquaculture industry draws increased attention, such negative ecological impacts are impacting its social license (Chua Thia Eng et al., 1989).

The Great Barrier Reef Marine Park (GBRMP) is adjacent to the Queensland (Australia) coastline and includes numerous key catchment areas. Despite the relatively small amount of nutrients discharged from the aquaculture sector in comparison to other industries, such as sugarcane farming (Jegatheesan et al., 2007), the rapid growth of the aquaculture industry demands research on the reuse and treatment of farm waste water entering the GBRMP. All land based farms in Australia are required to follow discharge standards, that strictly limit the release of culture effluent back into the environment (Brennan, 2002). These discharge restrictions can ultimately limit the stocking density, overall capacity and future expansion for land-based aquaculture facilities (Brennan, 2002). However, due to the small size of suspended organic and inorganic matter within effluent water, mechanical filtration processes are often difficult and expensive (Hopkins et al., 1995).

4.1.2 Bivalves as biofilters

The ecological concern surrounding effluent discharge, in addition to costly water treatment practices, have prompted interest into biological filtration methods. One possibility includes using marine bivalves to remove particulate matter from suspension and decrease nutrient levels (Shpigel & Blaylock, 1991; Shpigel et al., 1993). Bioremediation refers to the process of removing contaminants

through a biological means (Peña-Messina et al., 2009). Bivalves are a preferred bioremediatory species due to their tolerance to a wide range of environmental conditions and resilience to poor water quality (Martinez-Cordova & Martinez-Porchas, 2006). While the organic content of wastewater from aquaculture environments (e.g. faecal matter and undigested food) can provide a rich supply of food for bivalves, such as oysters, they can also assist in the removal of fine sediments from the water column. This is because, during their natural feeding process fine inorganic particles are ingested along with food before they are coagulated together into larger, heavier particles and excreted as pseudofaeces (Tenore & Dunstan, 1973). Previous studies investigating the positive effects of oyster filtration, have demonstrated the ability of the Sydney rock oyster, *Saccostrea glomerata,* to significantly reduce the concentration of total nitrogen (N), total phosphorus (P), bacteria, phytoplankton and other particulate matter within prawn culture effluent (Jones and Preston 1999). In addition to the ecological benefits and reduced strain on mechanical filtration methods, the recovery of nutrients from uneaten pellet feeds and prawn waste could provide economic benefit by production of a second cash crop.

4.1.3 The influence of temperature on filtration rate

The effects of environmental factors such as temperature, salinity and food availability on temperate oyster (Bayne et al., 1999; Calvo et al., 2001) and tropical oyster (Enríquez-Ocaña et al., 2012; Guzmán-Agüero et al., 2013) feeding patterns have been extensively reviewed in the literature. As an emerging tropical aquaculture species, the feeding patterns of adult *Saccostrea echinata* remain unstudied. Temperature-reliant filtration rate is a key factor that governs the aerobic capacity of oysters (Eymann et al., 2020). Filtration is a fundamental process to both oxygen uptake and nutrient retention, and therefore influences the organisms metabolic capacity, energy budget and subsequent growth capacity (Kittner & Riisgård, 2005). Quantifying the amount of suspended solids or nutrients removed from the water column via filtration enables calculation of the overall ecosystem service of both oyster farms and bioremediation programs. The quantification of filtration rate has prompted some controversy and debate, resulting from the variation in methodology used and this makes it difficult to make direct comparisons between species (Peña-Messina et al., 2009). Several studies investigating oyster feeding behaviour, have described a pattern of steady filtration rate increase as water temperatures were increased, provided that the animals were within their identified thermal tolerance window (Enríquez-Ocaña et al., 2012; Eymann et al., 2020; Guzmán-Agüero et al., 2013). This trend is typically followed by an inflexion point indicating maximal filtration rate, as water warms beyond an optimal temperature. As *S. echinata* is a species with high thermal tolerance, it is suggested that they may be capable of reaching greater filtration rates compared to those of temperate species.

4.1.4 Assessing bioremediatory potential

Multiple studies have investigated the bioremediatory potential of bivalves, using a variety of methods and applications. Filtration rate is a key component of evaluating bioremediatory potential and is typically quantified by the collection of biodeposits (Hoellein et al., 2015; Tenore & Dunstan, 1973) or measuring the reduction in algae cell density over time (Bayne et al., 1999; Guzmán-Agüero et al., 2013). Those studies investigating the net uptake and removal of nutrients and pollutants by bivalve farms, undertake tissue analyses to determine nutrient and heavy metal concentrations and to estimate their removal through harvest. Trials assessing the overall ecosystem service of bivalves to the environment, tend to analyse the deposition rate and the subsequent impact of this to the substrate (Beseres Pollack et al., 2013). When assessing the applied use of bivalves in a commercial aquaculture setting, the most common methodology involves culture effluent being pumped through a series of aquaria containing shellfish. Water samples are collected before and after bivalve filtration and key water quality parameters are measured (Jones et al., 2001; Jones et al., 2002).

4.1.5 The Blacklip rock oyster

The Blacklip rock oyster, *S. echinata*, is an emerging species of tropical oyster displaying several characteristics that suggest it would be well-suited to bioremediatory applications. These include a large size and fast growth rate, furthermore, the species also displays resilience to fluctuations in temperature and salinity (Lindsay, 1994; Nowland, 2019; Chapters 2 and 3). This species displays a wide geographic distribution throughout the tropics (Lindsay, 1994). Within Australia, it has been reported from waters as far south as Bowen on the east coast of Queensland, and extending north into Cape York and the Northern Territory and as far west as Cone Bay in Western Australia (Nowland, 2019). This naturally occurring range coincides with many successful prawn farming regions, and suggests that this species would be compatible with the seasonal variation of these locations.

4.1.6 Study aims

The aims of the present study were as follows: (i) to evaluate the effect of temperature on *S. echinata* filtration rate; (ii) to describe and quantify the uptake in total nitrogen, total phosphorus, total suspended solids and chlorophyll A, using prawn pond effluent and two levels of oyster stocking density. These results will quantify the filtration rate of Blacklip rock oysters with respect to temperature change and will enable evaluation of their potential for bioremediation of prawn aquaculture effluent.

4.2 Materials and methods

Part A. Assessing filtration rate

4.2.1 Experimental design

Market size (>70 mm) oysters (n=70) were purchased from Bowen Fresh Oysters north Queensland, Australia. During acclimation, the oysters were divided among four separate recirculating holding systems, each comprising of one 290 L sump and six 35 L aquaria. The tanks were heated using element heaters (Scintex, Australia) and the oysters were acclimated from the ambient farm temperature of 28°C until their treatment temperature was reached, at a rate of 2°C per day (Table 4.1).

Table 4.1. Temperature acclimation schedule for experimental animals, *Saccostrea echinata*.

	20°C treatment	24°C treatment	28°C treatment	32°C treatment
Day 1	Collection	Collection	Collection	Collection
Day 2	28°C	28°C	28°C	28°C
Day 3	26°C	26°C	28°C	30°C
Day 4	24°C	24°C	28°C	32°C
Day 5	22°C	24°C	28°C	32°C
Day 6	20°C	24°C	28°C	32°C
Day 7	20°C	24°C	28°C	32°C

4.2.2 System design

Using a static design, 18 aquaria were each filled with 15 L of 0.2 µm filtered seawater at 36 ppt. The specific trial temperature was maintained by placing six aquaria within three respective 290 L holding tanks containing an element heater (Scintex, Australia) and a 240 volt submersible pump (Aqua-nova, Poland) to ensure water temperature was evenly distributed. Room temperature was maintained by setting the air conditioning to 2°C below the desired treatment water temperature the day prior, to cool treatment water and ensure temperatures below ambient could be successfully maintained. Each aquarium contained a single oyster and known quantity of algae; 375,000 cells/mL of *Chaetoceros muelleri* (Gray & Langdon, 2018). Aeration stones were added to each aquarium to keep algae cells in suspension. Each of the three sumps contained one control aquarium, without oysters, which also received the same quantity of algal cells.

4.2.3 Sampling and biological data

Oysters were allowed to filter for a period of five hours. Three replicate 1.4 mL samples of water were taken from each aquarium at the commencement of the trial and at the end of each hour (one,

two, three, four and five). Samples were fixed using 0.1 mL of 10% seawater buffered formalin to preserve algal cells, and allow the concentration of cells to be later determined using a microscope and hemocytometer. The temperature of each individual tank was recorded hourly using a handheld thermometer (Soffritto, UK).

At the completion of the trial period, all bivalves were measured for shell dorso-ventral measurement (DVM), antero-posterior measurement (APM) and depth. Whole oyster weight was recorded, followed by the removal of tissue and wet weight recorded to a precision of 0.01 g. Using a pre-weighed and dried paper liner, oyster tissues were dried using a drying oven at 70°C for 24 h to achieve a constant dry weight. The dried tissues were weighed using scales accurate to 0.01 g.

4.2.4 Data analysis

All data were tested for heterogeneity of variance and data for oyster dry weights required log transformation to meet the normality assumption of ANOVA. To determine the influence of temperature on oyster filtration rate over time, a fixed effect model was used with hour and treatment as fixed effects and sump and aquaria as random effects. Repeated measure analysis (analysis of deviance) was conducted using a type II Wald chi-square test, to determine significance. The filtration rate was obtained by using the indirect method of measuring algae cell concentration decrease over time. The filtration rate formula (Coughlan 1969) (Eq. 4.1) describes the hourly volume of water filtered by an oyster, per gram of dry tissue weight.

$$FR = (V/nt)\ln(C0/Ct)/w \qquad \text{Eq 4.1}$$

V = volume of water per aquarium (mL)
n = number of animals per aquarium
t = time (hour)
ln = log base e
C0 and Ct = algal concentrations at time t-1 and at time t (hour) respectively
w = dry oyster tissue weight (g)

Part B: Bioremediation of prawn culture water

4.2.5 Experimental design

An additional 100 market size (>70 mm) oysters were sourced from Bowen Fresh Oysters. Initially, 10 were chosen at random to be dissected and analysed for total tissue nitrogen. The remaining 90 oysters were acclimated to 28°C at a rate of 0.5°C per day, over a period of two days. Using a static system, a total of nine rectangular aquaria were filled to 30 L from a sump containing prawn culture effluent, obtained from Pacific Reef prawn farm, Ayr, Queensland, Australia, 12 hours before the experiment commenced. The water temperature was recorded at 31°C and the salinity was 35 ppt at the time of collection.

Three treatments were compared; a high density, containing 20 oysters (0.66 oysters / L), a medium density containing 10 oysters (0.33 oysters / L) and a control containing no oysters. Each treatment contained three replicate aquaria. Water temperature was controlled via air conditioning and was set to 28°C in order to reflect the yearly average farm water temperature. Oysters were suspended on layered mesh in the water column of the tanks, approximately 15 cm above the bottom, to allow water circulation.

4.2.6 Sampling and biological data

Taking care not to disturb the feeding oysters, initial samples were collected, including; 500 mL for analysis of total suspended solids (TTS), 500 mL for analysis of chlorophyll A and 60 mL for analysis of both total nitrogen (TN) and total phosphorus (TP). Three replicate water samples per parameter were collected from the sump prior to pumping into the aquaria. Once all aquaria were filled, oysters were allowed ten minutes to resume their natural feeding pattern, before the three hour filtration trial period commenced. Each aquaria's filtration start time was staggered by 10 minutes to allow time for accurate sample collection. Samples for TSS, chlorophyll A, TN and TP were collected at the completion of each hour (one, two and three). At the completion of the three hour period, all oysters were measured for shell length (DVM), width (APM) and depth to the nearest mm and whole oyster weight to 0.1 g.

4.2.7 Total suspended solids (TSS)

The total suspended solids analysis was conducted in a laboratory at the Centre for Tropical Fisheries and Aquaculture (CSTFA) in Townsville, Australia. To determine the total mass of suspended solids, each sample was passed through a pre-dried and weighed Whatman glass microfiber filter, using a buccal flask and funnel assembly. These filters were dried for a period of 24 hours at 104°C to ensure they achieved a constant weight and reweighed using a scale accurate to 0.0001g. The overall mass of TSS was determined by comparing the initial and final weights.

4.2.8 Total nitrogen (TN) and total phosphorus (TP)

Total nitrogen and total phosphorus samples were sent to the Centre for Tropical Water & Aquatic Ecosystem Research (TropWATER) laboratory in Townsville, Australia for analysis. Using a stock calibration standard containing both nitrate and orthophosphates, five standards, over the desired calibration range, were prepared. Where necessary, samples were diluted with deionized water so that expected nitrogen and phosphorus levels fell within the range of the calibration standards. Quality control standards containing organic nitrogen and phosphorus were analysed on each run. These standards provided a reference check on the calibration and tested the efficiency. Using a pipette, 6.0 mL of sample was transferred to the culture tubes. Secondly, 1.25 mL of oxidizing reagent was added to each tube using a repeating pipettor, before tubes were covered with loose-fitting caps. To prepare autoanalyzer wash water, oxidation agent was added to deionized water in an Erlenmeyer flask, in the same proportion that was added to the samples, before covering in foil. The samples and wash water were autoclaved at 120°C for 55 minutes before being allowed to cool to room temperature. Each tube received 0.05 mL of 3M NaOH before proceeding to nitrate + nitrite analyses. The same proportion of 3M NaOH was added to digested wash water. The automated cadmium reduction method was used for the nitrate-nitrite determination after digestion. The automated ascorbic acid reduction method was used for the determination of phosphate after digestion (APHA standard methods, as adapted by TropWATER). Nitrogen and phosphorus standard curves were created by plotting the instrument response of standards against standard concentrations. These concentrations were compared to the sample response with the standard curve.

4.2.9 Chlorophyll-A

Chlorophyll A samples were also analysed by the TropWATER laboratory. To determine the concentration of chlorophyll A, samples were first filtered through glass fiber filters. Each sample was placed in a tissue grinder, with 2-3 mL of 90% aqueous acetone solution and macerated at 500 rpm for 1 minute. Each sample was then transferred to a screw-cap centrifuge tube and the total volume adjusted to 10 mL with 90% aqueous acetone. Samples were allowed to steep for 2 hours at 4°C in the dark. Samples were then clarified by filtering through a solvent-resistant disposable filter. Following this, 3 mL of clarified extract were transferred to a 1 cm cuvette and the optical density (OD) was measured at 750 and 664 nm using a spectrophotometer. The extract was then acidified in the cuvette with 0.1 ml 0.1M HCl, before being agitated and read at OD 750 and 665 nm, 90 seconds after acidification. From these values, the concentration of chlorophyll *a* per cubic meter were calculated.

4.2.10 Total tissue nitrogen

Oyster tissue samples were analysed by the Environmental Analysis Laboratory, Southern Cross University, Lismore, NSW, Australia. Oyster whole weights were recorded to 0.01g, before being

sacrificed and the tissue removed from the shells. Wet tissue weight was also recorded to 0.01g. Tissue samples were frozen at -25°C prior to transport. Samples were defrosted and dried at 40°C for 48 hours prior to crushing and analysis. Total nitrogen analysis followed the methods described by Rayment & Lyons (2012).

4.2.11 Data analysis

All data were tested for heterogeneity of variance, whole oyster weight was log transformed to meet assumptions of normality. To analyse the effect of oyster density on water quality parameters (TN, TP, TSS and chlorophyll-A), a fixed effect model was used with hour and treatment as fixed effects and aquaria as a random effect. To determine the effect of oyster density on water quality parameters TSS, TN, TP and chlorophyll-A, a repeated measure analysis (analysis of deviance) was conducted using a type II Wald chi-square test, to determine significance. All analyses were completed and plots created using R (R Core Team 2019).

4.3 Results

Part A. Assessing filtration rate

4.3.1 Biometric data and filtration rate

No significant differences in mean oyster whole weights, dry tissue weight, DVM or APM were recorded between the four temperature treatments. All four treatments reached the desired temperature (±1 °C) and experienced minimal variation (≤ 0.5 °C) throughout the five hour trial period (Table 4.2).

Table 4.2. Mean dry tissue weight of oysters, *Saccostrea echinata*, and the maximum and minimum temperature recorded for each treatment.

Treatment (°C)	Mean dry tissue weight (g) ± SE	Maximum (°C)	Minimum (°C)	Variation (°C)
20	1.08 ± 0.117	20.8	20.6	0.2
24	1.02 ± 0.06	24.3	23.9	0.4
28	1.13 ± 0.07	27.8	27.3	0.5
32	1.06 ± 0.08	32.0	31.5	0.5

Oyster filtration rate differed significantly between temperature treatments ($X^2 = 86.21$, df = 3, $p < 2.2 \times 10^{-16}$), in addition to time ($X^2 = 213.81$, df = 2, $p < 2.2 \times 10^{-16}$), and the interaction of treatment and time was also significant ($X^2 = 44.98$, df = 6, $p = 4.715 \times 10^{-8}$). Oysters within the 32 °C treatment assumed the fastest filtration rate within the first hour reaching 3.54 L/hr/g and steadily increased until hour three, before plateauing at hour four, at a maximum filtration rate of 14.67 L/hr/g. The oysters within the 24 °C and 28 °C treatments both displayed a similar pattern of filtration rate increasing with time, completing the five hour trial period without plateauing, and at rates of 15.20 and 12.68 L/hr/g, respectively. The oysters in the 20 °C treatment demonstrated a more gradual increase in filtration rate and remained lower than the three warmer treatments, before reaching a maximum rate of 4.89 L/hr/g at hour four (Figure 4.1).

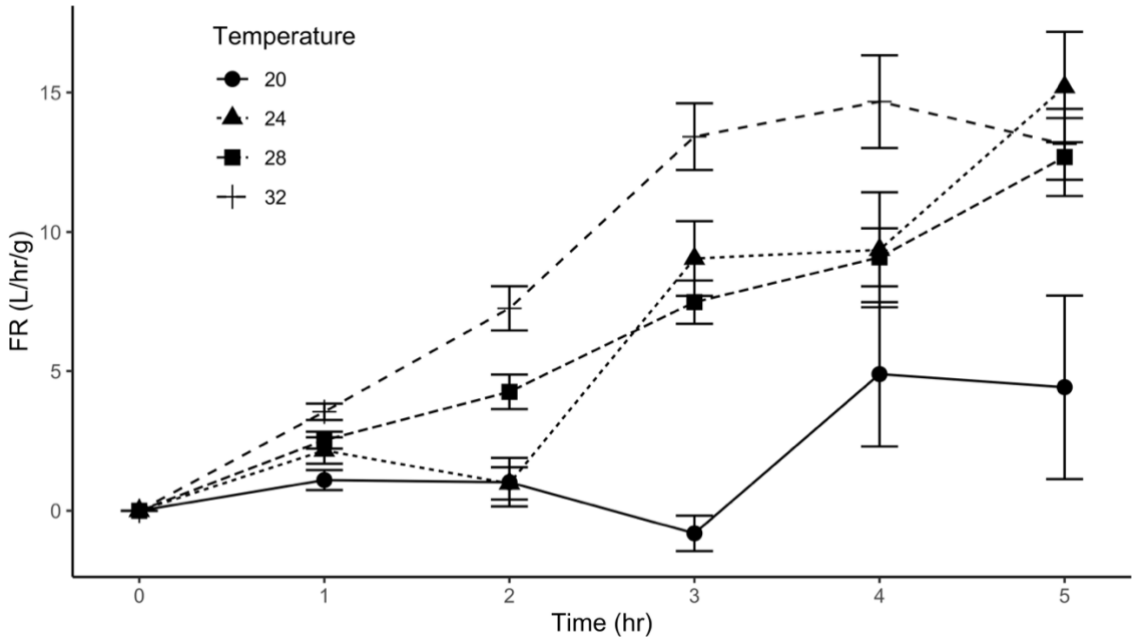


Figure 4.1. Filtration rate (L/hr/g) ± SE of *Saccostrea echinata*, across four temperatures (20, 24, 28 and 32 °C), over a five hour trial period.

Part B. Evaluating bioremediation potential

4.3.2 Biometric data

No significant difference in oyster mean whole weights, DVM or APM was detected between tanks or treatments (Table 4.3).

Table 4.3. Mean whole weight, DVM and APM of oysters, *Saccostrea echinata*. Tanks 1, 2 and 3 were control tanks and contained no oysters.

Tank	Density	Mean whole weight (g) ± SE	Mean DVM (mm) ± SE	Mean APM (mm) ± SE
4	Medium	82.77 ± 4.49	72.0 ± 1.5	58.8 ± 2.2
5	Medium	74.78 ± 3.21	67.6 ± 1.5	54.2 ± 1.7
6	Medium	81.66 ± 2.13	71.3 ± 0.9	56.0 ± 1.8
7	High	75.01 ± 2.67	70.6 ± 1.0	53.5 ± 1.6
8	High	82.62 ± 3.60	71.7 ± 1.7	55.3 ± 1.3
9	High	80.32 ± 2.74	72.3 ± 1.2	53.5 ± 1.4

4.3.3 Bioremediation

Filtration by oysters removed a significant quantity of suspended organic matter from the water column, and the amount removed tended to be larger with higher stocking densities. The high density of stocked oysters resulted in TN being reduced by 21%, TP was reduced by 27%, chlorophyll-A was reduced by 39% and TSS was reduced by 99%. The control treatment containing no oysters, highlighted that comparatively more particulate matter remained suspended in the water column for the three hour duration. Within the control treatment, TN was reduced by 9%, TP was reduced 13%, chlorophyll-A was reduced by 14% and TSS was reduced 71%.

The change in total phosphorus did not differ significantly between treatment ($X^2 = 4.31$, df = 2, p = 0.115), however it did differ significantly with time ($X^2 = 71.18$, df = 2, p = 3.496×10^{-16}) and with the interaction of treatment and time ($X^2 = 13.52$, df = 4, p = 0.009). Total phosphorus decreased gradually across all three treatments within the first hour. In the final two hours, both the medium and high density treatments displayed accelerated rates of phosphorus removal from the water column (Figure 4.3).

Total suspended solids were shown to differ significantly between treatment ($X^2 = 44.69$, df = 2, p = 1.974×10^{-10}), in addition to time ($X^2 = 11.92$, df = 2, p = 0.003) and the interaction of treatment and time ($X^2 = 16.86$, df = 4, p = 0.002). For the final two hours, the control treatment displayed little to no change. Both the medium and high density treatments displayed notable reductions in suspended solids throughout hours two to three, with the high density treatment concluding the trial period with the lowest total suspended solids (Figure 4.4).

Total chlorophyll-A was shown to differ significantly between treatment ($X^2 = 76.16$, df = 2, $p < 2.2 \times 10^{-16}$), in addition to time ($X^2 = 109.63$, df = 2, $p < 2.2 \times 10^{-16}$), and the interaction of treatment and time ($X^2 = 30.51$, df = 4, p = 3.849×10^{-6}) (Figure 4.5). The control treatment remained relatively constant for the first two hours before declining in the final hour. In the first hour, the high density treatment exhibited the most rapid reduction in total chlorophyll A. For the final two hours, the medium and high treatments both displayed comparable trends in reducing total chlorophyll A.

The total nitrogen dataset exhibited a similar trend to the previous three parameters. In the first hour, both the medium and high density treatment displayed a slight increase in TN, possibly due to oyster excretion. At the conclusion of the trial period, the medium and high density treatments both exhibited greater declines in TN than the control. Little can be drawn from this however, as the dataset remained bimodal and could not be successfully transformed using log or square root methods. In this scenario, no non-parametric alternatives were available.

There are several common trends among these four water quality parameters. All treatments experienced a reduction across all four parameters, following the three hour period. The high density oyster treatment produced the fastest rate of reduction of all water quality parameters. In accordance, at the conclusion of the trial the lowest concentrations of water quality parameters occurred in the high density treatment.

Total nitrogen percentage by dry weight of the ten oysters sampled, revealed a range from 6.66 - 9.50% and a mean value of 8.27% (Table 4.4). With a mean dry tissue weight of 1.08 g, this equates to a mass of 0.09 g of nitrogen removed per oyster. Upon scaling, this equates to approximately 1.2 kg of nitrogen removed per harvest tonne.

Table 4.4. Percentage by dry weight, of total nitrogen stored within *Saccostrea echinata* tissue.

Sample number	Whole oyster weight (g)	% N of dry tissue
1	65.22	9.50
2	61.02	9.07
3	82.03	6.95
4	87.64	8.10
5	74.15	9.24
6	79.29	6.66
7	70.47	8.91
8	74.92	7.57
9	76.98	8.64
10	82.53	8.06

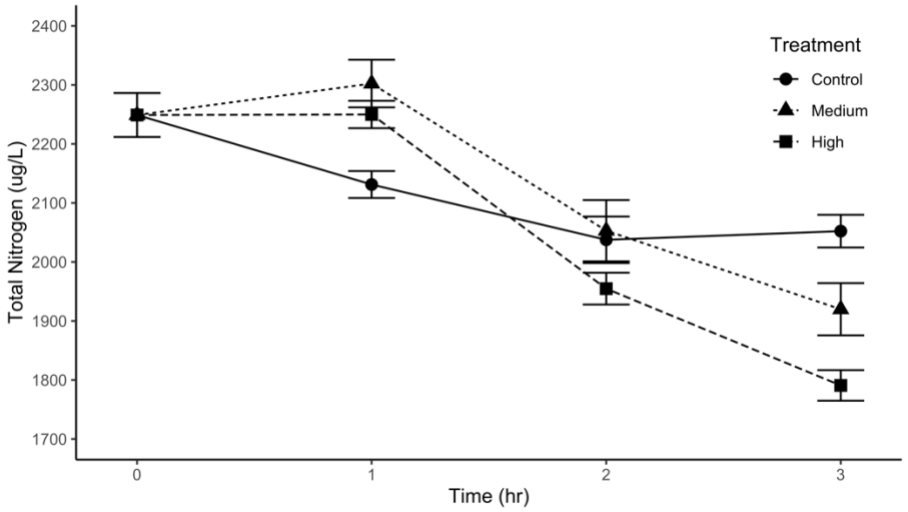


Figure 4.3. Changes in total nitrogen (μg/L) (± SE) of the control, medium and high treatments over the three hour trial period.

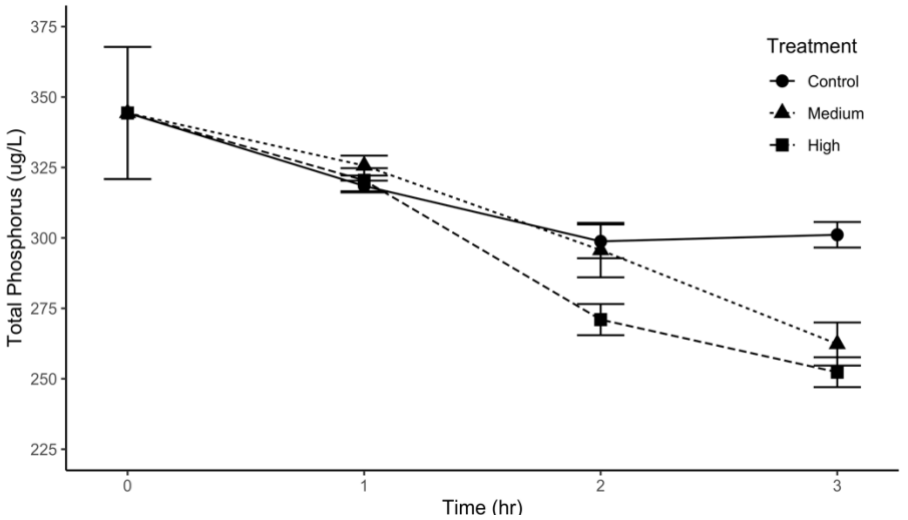


Figure 4.2. Changes in total phosphorus (μg/L) (± SE) of the control, medium and high treatments over the three hour trial period.

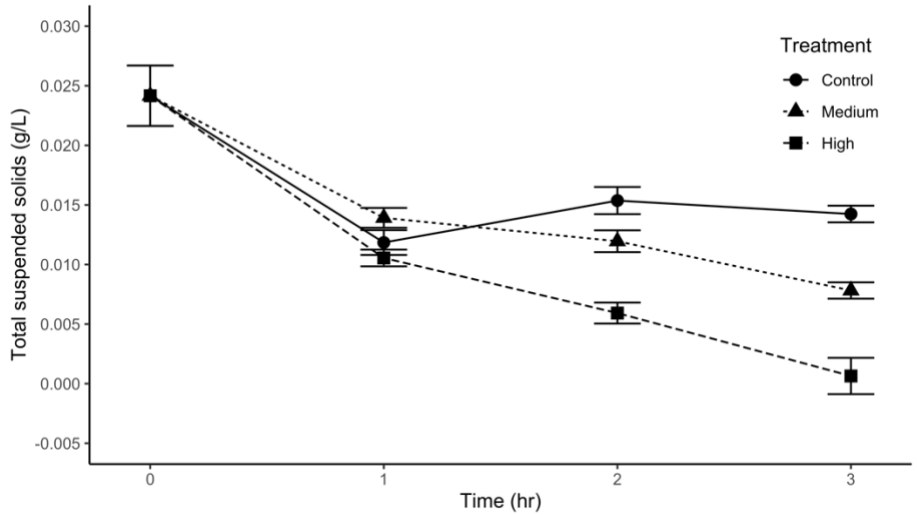


Figure 4.5. Changes in total suspended solids (g/L) (± SE) of the control, medium and high treatments over the three hour trial period.

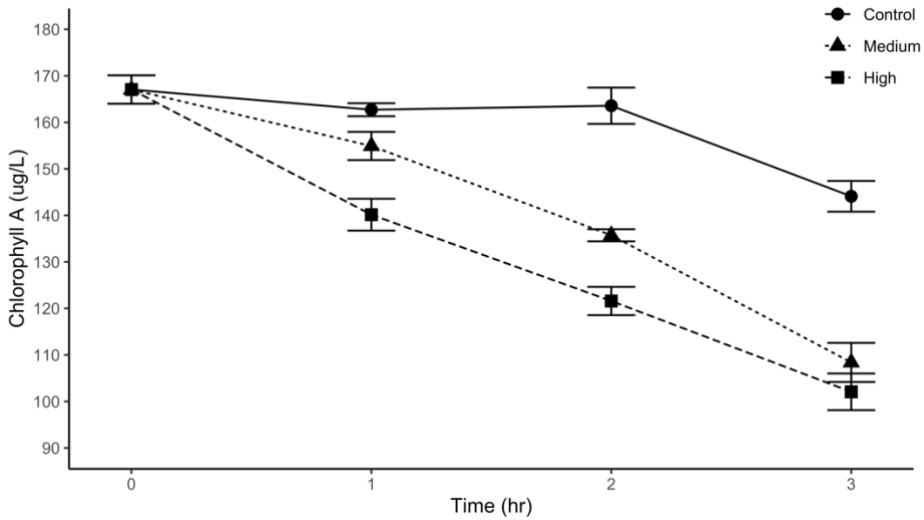


Figure 4.4. Changes in total chlorophyll A (μg/L) (± SE) of the control, medium and high treatments over the three hour trial period.

4.4 Discussion

4.4.1 Filtration rate

The findings highlight that the filtration rate of *S. echinata* is closely linked to temperature, corroborating the outcomes of studies on the European flat oyster, *Ostrea edulis,* (Eymann et al., 2020) and the Mangrove oyster, *Crassostrea corteziensis* (Enríquez-Ocaña et al., 2012; Guzmán-Agüero et al., 2013). The filtration rates of the 24 and 28°C treatments displayed comparable patterns over time and neither reached a point of inflection. While the 32 °C treatment returned the highest filtration rate for much of the five hour period, it eventually reached an inflection point and plateaued. The inflection point may indicate that the food source available within the 32°C treatment had diminished and therefore causing the filtration rate to peak. Food availability is an important factor influencing filtration rate and should be taken into account when estimating oyster performance in a specific location (Guzmán-Agüero et al., 2013). The oysters within the 20°C treatment appeared to display an inhibited filtration rate, most likely due to reduced metabolic rate (Specht & Fuchs, 2018).

When compared to trials that followed a similar methodology, the filtration rate of the Blacklip oyster was between three to five times that of other frequently cultured species (Table 4.5). The influence of temperature on the filtration rate of bivalves has been attributed to several different physiological mechanisms (Enríquez-Ocaña et al., 2012). One explanation suggests that as water temperature increases, the water viscosity decreases which assists with improved filtration capacity (Specht & Fuchs, 2018). Elevated temperatures induce higher metabolic rates but also result in less dissolved oxygen in water, resulting in bivalves pumping harder in order to compensate (Specht & Fuchs, 2018). There is also likely an interaction effect between temperature and food availability, which both display large seasonal variation. Food availability typically peaks during periods of high water temperature in northern Australia (Munksgaard et al., 2017). These larger filtration rates in comparison to those of other species suggest that Blacklip oysters may be well suited to biofiltration roles.

The results of the present study suggest that the Blacklip oyster has a tolerance to high water temperatures. In accordance with the findings reported by Cáceres-Puig et al. (2007) on the tropical oyster species *C. corteziensis,* the Blacklip oyster similarly displays a thermal tolerance above 32°C. Future studies investigating the maximal filtration rate of Blacklip oysters would benefit from including temperatures higher than 32°C, to find the temperature at which filtration rate is negatively affected. This represents an important study topic as it may assist in future site selection and identify locations that are not suitable.

Table 4.5. Comparison of four oyster species filtration rates (FR) and the temperature recorded.

Species	Water temperature	FR (L/hr/g)	Reference
Sydney rock oyster (*Saccostrea glomerata)*	20 °C	3.10	Bayne et al., 1999
European flat oyster (*Ostrea edulis)*	22 °C	7.70	Eymann et al., 2020
Cortez oyster (*Crassostrea corteziensis*)	29 °C	5.34	Guzmán-Agüero et al., 2013
Blacklip rock oyster (*Saccostrea echinata)*	24 °C	15.20	Present study

4.4.2 Bioremediation

The key water quality parameters contained within prawn farm effluent, undergo significant variation as the number of ponds and their stocking density vary with seasonal demand (Brennan, 2002) (Table 4.6). Typically, Australian farms have the highest stocking densities from October to February, as prawns are in peak demand during the summer months. This bioremediation trial was conducted during the month of May, however the parameters of TN, TP, TSS and chlorophyll-A were higher than the same month in previous years, due to the prolonged growing season. The level of chlorophyll-A in the effluent was indicative of high phytoplankton productivity within the culture ponds.

Table 4.6. Yearly water quality parameter range for a north Queensland prawn farm in 2019 (data provided confidentially).

Water quality parameter	Mean	Minimum	Maximum
TSS (g/L)	0.032	0.003	0.084
Chlorophyll-A (ug/L)	3.05	0.1	8.5
TN (ug/L)	1136	2000	290
TP (ug/L)	139	20	270

The net effect of oyster filtration on the effluent nutrient load, reveals the exchange between nutrient uptake and faecal excretion (Jones & Preston 1999). The reduction of total phosphorus by 27% within the high density treatment, was likely due to the removal of phosphorus bound to organic and inorganic particulates. The reduction of total nitrogen by 21% was less effective by comparison, and could be attributed to nitrogenous waste in oyster faeces, lessening the overall net reduction within the system.

The absence of significant settlement of suspended solids following the first hour as demonstrated by the control treatment, suggests that much of the inorganic matter is primarily comprised of small particles. This is in agreement with the findings of Ziemann et al., (1992) which highlighted that a high proportion of fine inorganic particles is characteristic of unlined earthen prawn ponds. During the process of filtration, oysters categorize particles based on size, weight and nutritional value. Organic particles and microalgae are typically ingested and retained as a source of food, while inorganic particles are ingested, flocculated together and then expelled as pseudofaeces through the inhalant opening (Dumbauld et al., 2009; Tenore & Dunstan 1973). Therefore, oysters can assist in the removal and settlement of small inorganic particles, as after flocculation they are heavier and fall out of suspension faster (Jones & Preston 1999). This mechanism of reducing fine suspended solids, appears to have been present in this study as evidenced by the success of the high density treatment.

4.4.3 Efficiency as biofilters

In a previous study investigating the ability of Sydney rock oysters, *S. glomerata*, Jones & Preston (1999) highlighted that the parameters of TN, TP, TSS and Chlorophyll-A from prawn effluent were all successfully reduced utilising a stocking density of 0.7 oysters / L, over a two hour period in a static system (Table 4.7). The percentage reduction of TN and TP appears to be comparable between the two trials, however the Sydney rock oyster trial returned a far greater reduction in chlorophyll-A, while the Blacklip study returned a superior reduction in TSS (Table 4.7). It is key to note however, that the effluent used in the Blacklip study contained over three times the level of chlorophyll A, while the effluent used in the Sydney rock oyster study contained over five times the level of TSS. High sediment loads have been shown to reduce or even inhibit oyster filtration and in some case can lead to mortalities due to smothering (Hopkins et al., 1995). Further studies are required to assess the consequences of high sediment loads on oyster filtration rates and survival, in addition to settlement techniques that may reduce suspended silt prior to oyster filtration (Jones & Preston 1999).

The factor of flow rate has been highlighted by several studies to be an important consideration for oyster bioremediation. In a comparative study investigating the effects of flow rate and system design, results indicated that the use of a flow-through system can successfully improve *S. glomerata* filtration efficiency. Jones et al, (2002) described that under a flow-through system, Sydney rock oysters were approximately six times more effective at reducing suspended solids and 1.3 times more effective at reducing chlorophyll A, compared to the static water system used in the 1999 trial (Bayne et al., 1999). Higher flow rates have been shown to enhance oyster filtration rates (Tenore & Dunstan, 1973), however, lower flow rates are more conducive for settling particulate matter (Unger & Brinker, 2013). This suggests that north Queensland prawn farms implementing oyster filtration would benefit most from incorporating a settlement period, followed by biofiltration under flow-through conditions

Table 4.7. Comparison table of Sydney rock oyster, *Saccostrea glomerata*, and Blacklip oyster, *Saccostrea echinata*, bioremediation studies, detailing the system type, filtration time, stocking density and percentage reduction of water quality parameters.

Species	System	Time	Stocking density (oysters / L)	Raw effluent levels				% reduction				Reference
				TN (mg/)	TP (mg/L)	TSS (g/L)	Chl-A (ug/L)	TN	TP	TSS	Chl-A	
Sydney rock oyster (*Saccostrea glomerata)*	Static	2 hr	0.70	1400	150	0.13	44.1	20%	33%	51%	92%	(Jones & Preston 1999)
Blacklip rock oyster (*Saccostrea echinata)*	Static	3 hr	0.66	2250	340	0.025	165	21%	27%	99%	39%	Present study
Sydney rock oyster (*Saccostrea glomerata)*	Flow through	2 hr	0.72					34%	44%	71%	61%	(Jones et al., 2002)
Sydney rock oyster (*Saccostrea glomerata)*	Recirculation	2 hr	0.1					NA	NA	84%	96%	(Jones et al., 2002)

4.4.4 Net removal via harvest

Whilst a proportion of the suspended material filtered by oysters is excreted as either faeces or pseudofaeces, the retained particles are incorporated into oyster tissue. In order to eliminate these nutrients from the system completely, removal of the sediment and bacterial denitrification is required, as well as harvesting the oysters. In addition to the positive effects of oyster culture on factors such as turbidity, the net removal of nitrogen through harvesting can further assist to quantify the ecosystem services provided by oyster farms within waterways (Dewey et al., 2011; Petersen et al., 2016). With a mean harvest oyster weight of 75.4 g, this equates to a mass of 0.09 g of nitrogen stored within each oyster. When scaled up to a yearly Blacklip oyster harvest of one tonne (13,260 oysters), a total weight of 1.20 kg of nitrogen is subsequently removed from the growout water way. These findings are comparable to a similar study investigating the ability of *Crassostrea virginica* to take up and remove nitrogen from coastal estuaries. The mean percentage of nitrogen within oyster dry tissue was highlighted to be 8.6%, in comparison to 8.27% as found in *S. echinata* specimens (Carmichael et al., 2012).

4.4.5 Nutrient trading

Nutrient credit trading is a concept that has been proposed, and in some countries already implemented, as a strategy to achieve water quality improvement targets and provide supplementary income to those involved. Such programs use a market-based approach and provide economic incentives to complete nutrient load and pollution reduction targets (Bricker et al., 2018). The process of nutrient trading enables point-source dischargers who reduce their nutrient discharge below target levels, to then on-sell their surplus reductions or 'credits' to other dischargers who are unable to do so, or face more expensive treatment options (Wijsman et al., 2018). A nutrient credit is a transferable

unit that represents a volume of pollutant that has been prevented from entering the environment, and is defined by the difference between the discharge allowance and the measured discharge from that source (Wijsman et al., 2018). This process may lead to new income opportunities for farmers, businesses or investors, who are able to quantify their net positive effect on water way health (Petersen et al., 2016).

The Chesapeake Bay program (USA) is a model example of such an initiative, which is evaluating the success of assigning nutrient credits to cultured oysters and restored shellfish reefs (Calvo et al., 2001; Ferreira & Bricker, 2016). During 2018, the use of harvested oyster tissue was approved as a nutrient reduction best management practice. The town of Mashpee, Massachusetts, USA, is currently utilising oysters for nutrient reduction and is working towards the harvest of 500,000 oysters in an effort to remove 50% of the 5000 kg N per year goal (Bricker et al., 2018).

In Australia, an application of nutrient trading known as Reef Credits, was developed in partnership with the Queensland Governments' Reef 2050 Plan. Primarily involving stakeholders from the agricultural sector, Reef Credits aims to incentivize land users to refine practices and prioritise improvements to water quality. At present, the two primary goals of the Reef Credits scheme are targeting a reduction in sediment run-off through gully rehabilitation and a reduction in nutrient run-off through managed fertilizer application. The outcomes of this bioremediation study closely align with goals laid out by the Reef Credits scheme. This study has helped quantify the ability of oysters to take up and reduce TN, TP, TSS and chlorophyll A. This is a progression step towards evaluating the value of this species for the purpose of bioremediation. In future, this knowledge will hopefully provide an opportunity for primary production sectors in northern Australia, to work collaboratively towards water quality improvements and diversify income sources.

4.4.6 Future considerations

There is considerable scope to further investigate the bioremediatory ability of the Blacklip rock oyster, within a laboratory setting. Based on previous findings, the inclusion of a sedimentation phase prior to biofiltration, will likely improve efficiency and survivability of oysters (Jones et al., 2001). An additional key point for future studies, is investigating the optimal flow rate for this species. While this study used a basic static system, when integrated within a flow through system design, the maximum capacity of Blacklip oysters to reduce nutrients and suspended particulates is expected to be further enhanced.

An additional element that requires investigation is integrated multi-trophic aquaculture, combining macroalgae grown in conjunction with oysters to assimilate dissolved nutrients and balance oyster excretion. Previous studies have highlighted that after sedimentation and oyster filtration have

reduced suspended particulates, macroalgae has the ability to rapidly reduced dissolved nutrient loads from prawn culture effluent (Jones et al., 2001; 2002). As a form of integrated multi-trophic aquaculture, both oysters and macroalgae are highly marketable products with uses for human consumption and within other animal feeds (Jones et al., 2001; Patel et al., 2021). As well as offering a potentially cheaper alternative to mechanical filtration and water treatment, polyculture provides an opportunity to diversify crops and create supplementary income. Subsequent *in-situ* farm based trials are the next step, to build on the findings from laboratory based studies, such as the current study, and identify barriers to biofilter integration.

4.4.7 Conclusions

The outcomes of this study detail the ability of Blacklip oysters to successfully reduce concentrations of TN, TP, TSS and chlorophyll-A from prawn pond effluent. This laboratory based trial provides important baseline data regarding filtration rates, sedimentation and nutrient reduction and uptake within a static system. The Blacklip oyster demonstrated substantially higher filtration rates than several other oyster species, while operating within its optimal temperature range. Further laboratory based trials investigating the success of a pre-settlement phase, varied flow-through rates and macroalgae are suggested to refine optimal conditions, before farm-based trials begin. Based on its high filtration rate, tolerance to elevated temperatures and salinity fluctuations and filtration ability, the Blacklip oyster is a promising candidate for integrated bioremediation in a tropical aquaculture setting.

5 5.1CHAPTER 5 GENERAL DISCUSSION

5.1 Introduction

This study assessed the key factors influencing reproductive maturation in tropical Blacklip rock oyster, *Saccostrea echinata,* broodstock. The overarching project objective was to inform and develop a series of broodstock conditioning protocols to optimise hatchery techniques which will enhance spat supply to northern Australia. Of complementary interest, was to assess the capacity to incorporate *S. echinata* to pre-existing aquaculture facilities as a form of biofiltration. The studies used to target these project objectives included: (chapter 2) assess the influence of temperature manipulation on broodstock reproductive condition; (chapter 3) determine the effects of salinity manipulation on broodstock reproductive condition; (chapter 4a) quantify the filtration rate of *S. echinata* and how it is impacted by temperature; (chapter 4b) evaluate the bioremediatory capacity of *S. echinata* with prawn farm effluent water. The research presented in this thesis has contributed towards hatchery protocol developments and recent spawning success at the Darwin Aquaculture Centre in the Northern Territory. The major outcomes of this research project, and their potential applications are summarised in Table 5.2 and are described below.

Table 5.1. Chapters 2 and 3 points of comparison.

Trial	Time of year	Temperature	Salinity	Maximum GI	CI range
Chapter 2 Temperature	3rd July – 25th September	24.4 – 27.6 °C (Control) 31 ±1 °C (Heated)	36 ppt	0.5	51.4 – 65.4 (Control) 43.3 – 57.8 (Heated)
Chapter 3 Salinity	20th July – 19th October	29 ±1 °C	20 – 36 ppt	2.1	62.1 – 77.7

5.2 Broodstock conditioning

A lack of a broodstock conditioning protocol remains the greatest impediment to the commercialisation of the tropical Blacklip rock oyster, *Saccostrea echinata* (Nowland, 2019). Consequently, inconsistent and inadequate spat supply continues to hinder the potential for growout and future expansion. A further understanding of the species physical requirements is key to overcoming this bottleneck and enable conditioning outside of their natural spawning seasons (Helm et al., 2004). It is therefore high priority for the industry, to develop a standard protocol for manipulating the physical holding environment of *S. echinata* broodstock.

5.2.1 Temperature

Due to the well-established method of conditioning the Sydney rock oyster*, Saccostrea glomerata,* via temperature manipulation, this was the logical starting point to evaluate exogenous factors of

influence (O'Connor et al., 2008). The major finding from this first pilot study was that temperature alone, was not successful at conditioning broodstock to a point at which spawning could occur (Table 5.1, Figure 5.1). This contrasted with links to a seasonal pattern of spawning and a strong positive correlation between temperature and reproductive condition in wild populations (Nowland et al., 2019).

Seasonal trends of water temperature tend to display smaller variation in the tropics than compared to temperate locations (Fabioux et al., 2005). This pattern is highlighted when comparing water temperature data from successful oyster culture sites in Australia, including Sydney which fluctuated between 17.2 – 25.4 °C and Hobart which fluctuated between 11.9 – 18.1°C. In contrast, a study conducted in the Amazon basin into the Mangrove oyster, described an annual fluctuation of between 28.4 – 30.1 °C and found no evidence to suggest that the reproductive patterns of oysters were linked to fluctuations in water temperature (Paixão et al., 2013). The chosen treatment temperature of 31°C was selected based on temperature data that coincides with periods of reproductive development in local wild populations, and is therefore unlikely to be responsible for the overall poor condition of broodstock observed in Chapter 2. While a slight increase in gonad index was observed in the later stage of the heated treatment, the absence of ripe broodstock indicates that temperate is not a key exogenous factor to broodstock conditioning of *S. echinata*.

5.2.2 Salinity

Following on from the temperature pilot study, the physical factor of salinity was selected to be the next parameter of interest. Due to salinity fluctuation in the tropics, and semi-continuous pattern of spawning throughout the monsoon season, this was the logical next exogenous parameter to investigate (Nowland et al., 2019). The key finding from Chapter 3 was that salinity manipulation proved to be an effective technique to condition broodstock Blacklip oysters (Table 5.1, Figure 5.1). A negative correlation between salinity and GI was identified (R = - 0.397), where lower salinity appeared to promote higher GI throughout the trial period. The treatment of 25 ppt produced a significantly greater proportion of ripe individuals than the control of 36 ppt and was therefore identified as the optimal salinity for *S. echinata* broodstock conditioning.

Partial spawning was an unintentional occurrence during this study, and was responsible for the comparatively lower GI than in wild populations described by Nowland et al., (2019). As *S. echinata* exhibit a semi-continuous spawning during the monsoon season, it is therefore expected that broodstock will inevitably spawn once reproductive maturation is attained. Whilst not an optimal outcome, the presence of partial spawning highlights that broodstock were achieving maturation earlier than predicted, and that subsequent studies would likely benefit from a shorter conditioning period of between six to eight weeks. A key point of difference from Chapter 2, was the presence of

ripe broodstock, and this enabled spawning induction to occur during Chapter 3. Of the 40 oysters used in the spawning trial, 11 broodstock were successfully induced to spawn, with nine of the 11 having undergone low salinity treatments. This outcome was expected as the low salinity treatments contained comparatively more ripe oysters at the time of spawning.

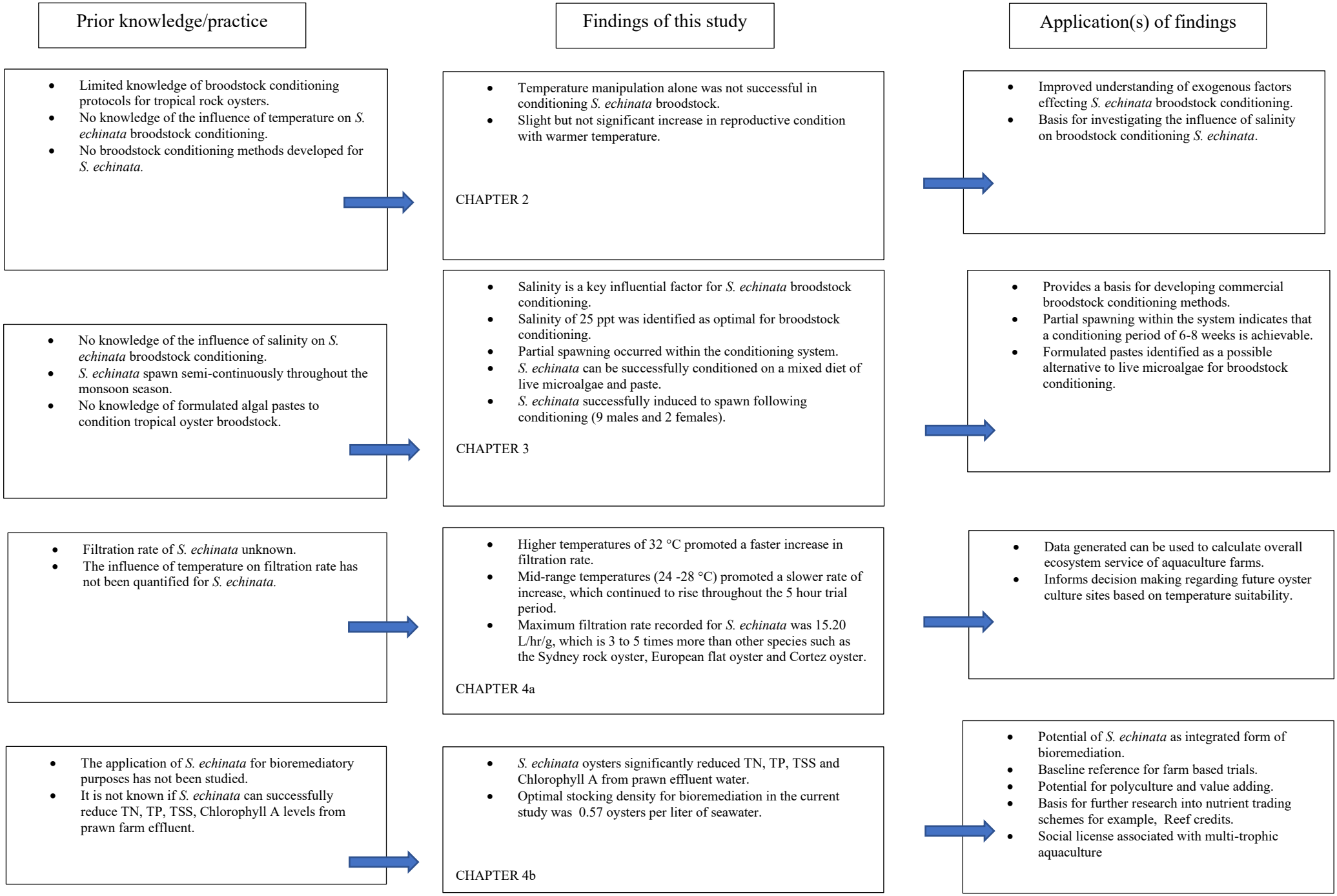
The positive results from conditioning broodstock Blacklip oysters using salinity manipulation, are already being implemented in further studies and assisting the development of hatchery protocols. A trial within the CRCNA tropical rock oyster research and development project, currently underway in the Northern Territory has been successful at conditioning broodstock at 25 ppt in a period of six to eight weeks, and has experienced positive spawning outcomes (S. Nowland, per comm 2021).

5.2.3 Diet alternatives

In addition to the knowledge gap regarding key exogenous factors to *S. echinata* conditioning, there is currently minimal species-specific knowledge of optimal broodstock diet. Successful hatchery production of bivalves is highly dependent on feeding regimes, while the dietary composition and quantity is often highly species specific (Helm et al., 2004; Utting & Millican, 1997). The present dietary composition for Blacklip rock oysters (employed in chapters 2 and 3) is based on the Sydney rock oyster diet and there is likely scope for improving the specificity, to best reflect the nutritional requirements of Blacklip rock oyster broodstock. The diet used in Chapter 3 included formulated algal pastes, to supplement live microalgae. Formulated pastes have often been regarded as a secondary nutritional choice, in favour of a mixed live microalgal diet (Ponis et al., 2003). However, a key outcome of Chapter 3 was that formulated algal pastes proved to be a viable alternative diet, as broodstock were successfully conditioned on a high percentage paste diet.

The process of microalgae culture is often typified by its technical methods and high costs, often constituting between 15 – 85% of overall hatchery costs depending upon the species, scale of production and culture procedures (Ponis et al., 2003). Consequently, there is a demand for substitutes to live cultured microalgae, that are nutritionally complete, of adequate size to allow ingestion, have a shelf-life spanning rearing cycles and provide a cost effective alternative to on site algae production (Nell, 1993). The use of formulated algal pastes represents a considerable reduction in production cost, labour and infrastructure to a hatchery facility. In addition, they are a stable option that eliminates the risk of live feed shortages in the event of an algal culture crash. Their supplementary use would also permit algae culture facilities to undergo a complete dry out period to allow for sterilisation and cleaning, without compromising feed availability.

Figure 5.1. Major outcomes of this research project and their potential.



5.3 Filtration rate

The first component of Chapter 4, Part A bioremediation study, was to quantify and investigate the effects of temperature on the filtration rate of *S. echinata*. The influence of physical water parameters upon bivalve filtration rate has been extensively documented in the literature (Kittner & Riisgård, 2005; Peña-Messina et al., 2009), however, they remain unstudied for *S. echinata.* Temperature reliant filtration rate is a key factor that determines the oysters aerobic capacity, and subsequently governs their biofiltration potential and feeding ability (Eymann et al., 2020). The key finding of this chapter component was that the filtration rate of *S. echinata* has a tolerance to high water temperatures, and has an optimal performance window between 24 – 32 °C.

The maximum filtration rate recorded in this study, was between three to five time that of several other described species, and suggests that Blacklip oysters may be well suited to biofiltration roles. This data is a key component required to calculate the overall ecosystem service, provided by oyster farms situated within tidal estuaries. The optimal performance window of between 24 – 32 °C will likely provide a baseline for subsequent filtration rate studies and inform grow-out site selection into the future. For example, many coastal locations are forecast to experience temperature increases over the coming years (Lough & Hobday, 2011). Previously established oyster grow-out sites located on the northern fringe of temperate waters, are at risk of experiencing water temperatures beyond optimal for their culture species. These farms may look to incorporate Blacklip oysters, as water temperatures continue to warm and sites become unsuitable for temperate species.

5.4 Bioremediatory potential

Results from Chapter 4 Part B confirmed that *S. echinata* displays potential as a bioremediatory species, both as a stand-alone product and integrated within pre-existing farms as a form of multi-trophic aquaculture. The Blacklip oyster successfully reduced levels of total nitrogen, total phosphorus, total suspended solids and chlorophyll-A, all of which are key discharge parameters of interest to prawn culture facilities. Their integration into a commercial aquaculture setting as a form of biofiltration, represents a potential alternative or inclusion to a facilities waste-water treatment protocol, which is typically an expensive process and requires considerable infrastructure (Shpigel & Blaylock, 1991).

In comparison to a similar study using prawn effluent and the Sydney rock oyster, both species returned comparable reductions in total nitrogen and total phosphorus (Jones and Preston 1999). Additional studies have highlighted that the bioremediatory ability of oysters can be improved by incorporating a period of settlement, followed by oyster filtration under flow-through conditions (Jones et al., 2001; Jones et al., 2002). The paired culture of macroalgae in conjunction with oysters

has demonstrated promise, by rapidly reducing dissolved nutrient loads and balancing nitrogen waste from oyster excretion (Jones et al., 2002). Further to providing a potentially cheaper form of waste water treatment, polyculture offers the capacity to diversify crops and supplement income through the culture of additional products.

The quantification of net nitrogen removal through the harvest of *S. echinata*, forms a baseline measurement to further develop the concept of nutrient trading for aquaculture farmers in northern Australia. Nutrient trading is an innovative model that has been proposed and, in some countries, already implemented, as an approach to meet water quality improvement targets and provide supplemental income to businesses involved (Bricker et al., 2018). The concept of nutrient trading is achieved when point source dischargers are able to reduce their nutrient levels below the required target. This surplus reduction is then able to be on-sold in the form of 'credits' to other dischargers who are not able to meet target levels or face more expensive water treatment costs (Wijsman et al., 2018). This trading scheme aims to provide an additional income source to farmers, businesses or investors, who are in a position to quantify their net positive effect on water way health (Petersen et al., 2016). In accordance with the Reef 2050 Plan (Queensland Government, 2018), a parallel concept known as Reef Credits was developed. It was conceived in response to the emerging consensus that a market-mechanism was urgently needed to incentivize water quality improvements across the Great Barrier Reef catchment area (Reef Credit Guide version 1.0, 2019). The key focus of reducing sediment and fertilizer in agricultural run-off, closely aligns with the outcome of this study. Oysters demonstrated an ability to remediate suspended solids and nutrients in the form of N and P, and therefore display potential for integration into the Reef Credits scheme. The further development of this program seeks to build positive and sustainable relationships between Queensland's various primary industries.

5.5 Future studies

5.5.1 Conditioning

While salinity has been identified as a key exogenous factor influencing broodstock maturation in *S. echinata*, there is likely to be an interaction between salinity and additional exogenous and endogenous factors. Future studies should investigate the synergistic effects of salinity and temperature on broodstock conditioning. Furthermore, conditioning methods involving salinity manipulation will benefit from continual evaluation and may include refinement of conditioning duration, or include a gradual salinity decline throughout the maturation phase.

5.5.2 Diet

While the exogenous factors influencing oyster reproduction are an important consideration, the role

of nutrition in conditioning broodstock represents a crucial area for future research (Anjos et al., 2017). Effective hatchery production of bivalves is clearly linked to broodstock feeding rates and dietary composition (Helm et al., 2004; Utting & Millican, 1997). As such, future research objectives should aim to determine broodstock feeding requirements specific to *S. echinata*, in an effort to optimise gonad development. Microalgae species differ in their nutritional content and therefore can have varying effects on gametogenesis and subsequent larval growth and viability (Ponis et al., 2003). Future studies should investigate dietary composition of tropical oysters in the wild and the potential of incorporating native algae feed into conditioning diets. Additional studies could also seek to enhance gonad development and larval success by analysing digestion rates and the nutritional demand of spawning. The suitability of formulated algal pastes is also an area of interest for future conditioning studies, to determine if suitable maturation, spawning and survival rates can be achieved on a 100% paste diet. Such studies and their outcomes would inform optimal broodstock conditioning procedures to support the development of *S. echinata* as a commercial aquaculture species. Formulated pastes represent a potential alternative, particularly when considering the intensive process of microalgae culture and the remote locations in northern Australia being considered for future culture sites.

5.5.3 Filtration rate

A subsequent study into the filtration rate of *S. echinata*, would benefit from conducting the trial over a timeframe greater than five hours to find the maximal filtration rate achieved, particularly between the temperatures of 24 – 28 °C. The inclusion of temperatures greater than 32 °C would be beneficial, to find the point at which high temperatures negatively affect filtration performance. Due to the improved efficiency of oyster filtration in flowing water (Jones et al., 2002), a future study investigating the benefits of a flow-through and recirculating system following a settlement period, would be highly advantageous in identifying an appropriate setting for integration of oysters into waste water treatment.

5.5.4 Bioremediation

New findings gained from Chapter 4 on the bioremediatory ability of *S. echinata,* enables further research to better understand challenges associated with integrating oysters into existing prawn farms. Further studies should investigate the optimal settings and long term effects of Blacklip oysters used for bioremediation. Blacklip oysters represent one of the most promising candidates for integration into northern Australian and Indo-Pacific aquaculture, based on their tolerance to wide temperature and salinity fluctuations. Future research into Blacklip oyster bioremediation, should include *in-situ* trials on farms, to investigate the long-term effects to oyster health and polyculture of oysters with macroalgae to enhance nutrient uptake and assimilation.

5.6 Other issues

The commercial hatchery production and growout of *S. echinata* remains a novel prospect to tropical aquaculture and as such, there are numerous aspects that require research and development attention including:

- Selective breeding programs
- Optimising larval settlement rates
- Investigating various grow out methods and applied technology
- Screening of suitable sites for growout
- Market research and economic suitability comparisons to other available bivalve species
- Quality assurance, food and safety standards for oysters
- Community ownership and land tenure agreements

5.7 Overall conclusions

This study forms part of a broader aquaculture research program, with the goal to support and promote the development of tropical oyster culture in remote Indigenous communities in northern Australia. This project was undertaken as part of the CRCNA Northern Australian Tropical Rock Oyster research and development program, and more specifically Sub-Project 2 – Securing commercial spat (juvenile) supply. The research chapters focussed primarily on accomplishing the goals of reliable spat production and year round spawning, and have achieved significant progression towards broodstock management in a hatchery setting. The outcomes from this thesis provide the baseline information required to enable broodstock conditioning of the Blacklip rock oyster. Successfully initiating reproductive maturation outside of natural spawning seasons is a key stepping stone to securing commercial quantities of spat. Early findings indicated that the Blacklip Rock Oyster has different physical requirements to the highly studied and commercialised Sydney Rock Oyster and Pacific Oyster. As such, the established hatchery manuals developed for Australian temperate species, are not effective for producing adequate quantitites of *S. echinata* spat. The technique of temperature manipulation was identified to be ineffective at conditioning broodstock. In subsequent trials, the factor of salinity was highlighted to be highly influential on the reproductive maturation process. A moderate negative correlation was established between salinity and GI, with an optimal salinity of 25 ppt identified. Of further note was the inclusion of formulated algal diets which represents an area of research interest for further optimisation for future trials. Like many oyster species, the filtration rate of *S. echinata* displayed a high degree of temperature dependence, with accelerated rates achieved with higher temperatures. The subsequent bioremediation study emphasised multi-trophic aquaculture as a viable grow out procedure and described the potential for nutrient trading schemes to occur within the prospective new industry. The bioremediatory ability of

S. echinata displayed promise, with reductions to the parameters of TN, TP, TSS and chlorophyll-A. This species displays suitability for integration into prawn farm water treatment and application to nutrient trading schemes such as Reef Credits.

Appendix 1. Chapter 3 gender frequency of *Saccostrea echinata* over ten sampling events. (M = Male, F = Female, I = Indeterminate).

sample	gender	20	25	30	36
1	M	NA	NA	NA	34
1	F	NA	NA	NA	4
1	I	NA	NA	NA	2
2	M	3	4	5	1
2	F	7	6	5	6
2	I	0	0	0	3
3	M	4	7	8	6
3	F	5	3	2	4
3	I	1	0	0	0
4	M	1	2	3	4
4	F	9	8	7	5
4	I	0	0	0	1
5	M	3	5	3	6
5	F	7	5	5	3
5	I	0	0	2	1
6	M	8	4	4	7
6	F	2	6	6	3
6	I	0	0	0	0
7	M	2	3	3	7
7	F	8	5	6	2
7	I	0	2	1	1
8	M	3	1	2	8
8	F	7	9	8	2
8	I	0	0	0	0
9	M	5	5	1	7
9	F	5	5	9	3
9	I	0	0	0	0
10	M	5	5	8	4
10	F	5	5	2	6
10	I	0	0	0	0

Appendix 2. Feed schedule detailing live algae and paste contributions of Chapter 3. (Tiso = *Tisochrysis lutea*; Muel = *Chaetoceros muelleri*; Pav = *Diacronema lutheri*).

Week	Cells/oyster/day	Number of days on 100% paste	Number of days on 75% paste, 25% live algae	Algae spp. used
1	2 billion	7	0	-
2	2 billion	7	0	-
3	2 billion	7	0	-
4	2 billion	7	0	-
5	2.5 billion	7	0	-
6	2.5 billion	5	2	*Pav*
7	3 billion	4	3	*Tiso*
8	3 billion	4	3	*Tiso, Pav*
9	3.5 billion	3	4	*Tiso, Pav, Muel*
10	3.5 billion	0	7	*Tiso, Pav, Muel*
11	3.5 billion	0	7	*Tiso, Pav, Muel*
12	3.5 billion	0	7	*Tiso, Pav, Muel*

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