# Reproduction and larval ecology of the toheroa, *Paphies ventricosa*, from Oreti Beach, Southland, New Zealand

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# Abstract

*Paphies ventricosa* is a large surf clam endemic to New Zealand with a patchy distribution and whose populations have substantially declined during the past century owing to overfishing and habitat degradation. Poor recruitment is now evident, and therefore, understanding the larval recruitment of *P. ventricosa* is key to developing and implementing conservation strategies for the species. In order to identify factors driving larval recruitment in toheroa, *Paphies ventricosa*, from Oreti Beach, Southland, New Zealand, the southernmost known extent of the species, various studies were carried out from 2011 to 2014 in the field and the laboratory

In 2011, the reproductive cycle of *P. ventricosa* was examined over one year in a population at Oreti Beach. In 2012, the spatial variation in reproduction among four sites along Oreti Beach, including the site from 2011, was quantified from body indices and the histological examination of gonads. Based on changes in oocyte size, gametogenic stage and condition index, we observed a species with a primary spawning in spring and a second spawning event in late summer/autumn, with no resting phase but minimal reproductive activity over winter. Seasonal reproduction corresponded with warmer sea surface temperature and a peak in chlorophyll-*a* concentrations in the region. Small-scale (< 15 km) variation in the timing of spawning was also evident along Oreti Beach, with a degree of asynchrony that could affect fertilisation success in the population. These patterns may be an important consideration when identifying areas that may be considered for conserving source populations.

Using scanning electron microscopy and light microscopy, fertilisation, and embryonic and larval development were observed at three culturing temperatures

(12, 16 and 20 ºC). The progress of development follows that previously described for the family Mesodesmatidae, with *P. ventricosa* having a small egg (63–70 mm), with an 83–102 mm trochophore stage observed at 15 h, and a 100 mm D-veliger larva observed at 22 h at 12 and 16 ºC, and 37 h at 20 ºC. At 20 ºC, the pediveliger larval stage was reached by 31 d. While the morphology of the embryonic and larval stages of *P. ventricosa* is typical for bivalves, we show that in this species the shell field invagination occurs in the gastrula stage and that the expansion of the dorsal shell field occurs during gastrulation, with the early trochophore having a well-developed shell field that has a clearly defined axial line between the two shell lobes. The growth of *P. ventricosa* larvae cultured at 12, 16 or 20 ºC over 39, 33 and 31 d respectively, was faster at warmer temperatures. Using the temperature quotient $Q_{10}$ at day 27 to quantify the response to temperature, values of $Q_{10}$ = 1.82 for the range 12–16 ºC and $Q_{10}$ = 2.33 for the range 16–20 ºC were calculated. Larval shape was not temperature dependent, suggesting that the smaller larvae found at colder temperatures reflect a slowing of larval development, rather than physiological damage by temperature resulting in abnormal larval development.

Temperature is one of the most important environmental factors controlling development in marine invertebrates, and thus likely plays a critical in recruitment dynamics. The temperature thermal tolerance of fertilisation and early larval development in *Paphies ventricosa* was examined to understand the role of temperature in early larval recruitment success. Fertilisation was examined across a thermal gradient of 10.5 to 30 ºC in an aluminium heat block. Fertilisation was considered successful by microscopic observance of the breakdown of the germinal vesicle, and the appearance of the fertilisation envelope and polar body. The thermal tolerance of development was examined across a thermal gradient of 8.0 to 25.5 ºC in

an aluminium heat block at 2, 15, 22, and 37 h post-fertilisation. Fixed samples were examined using light microscopy and classified into the developmental stages of unfertilised, fertilised, embryonic (2-64 cell embryos), blastula, trochophore, veliger, and abnormal. There was a significant effect of temperature on the fertilisation success which ranged from 4.6% to 46.7%. Fertilisation was > 30% successful between 16.0 and 21.0 ºC, and was successful beyond the natural temperature range of the species. *P. ventricosa* larva were tolerant to temperatures beyond the naturally occurring temperatures during spawning/development periods, but were most successful around 15 ºC.

While temperature is important in the recruitment of marine invertebrate larvae, feed availability is also crucial, and often thought to be more important in overall larval development. The combined effects of temperature (12, 16, and 20 ºC) and feed concentration (1:1 mixed algal diet of *Tetraselmis chui* and *Isochrysis galbana*; 1,000, 10,000, and 20,000 cells $ml^{-1}$) were examined in *P. ventricosa* larvae over 17 days. There was found to be significant combine effect of sampling day, temperature, and feed concentration on larval shell length. By 17-d post-fertilisation, the combined effect of feed concentration on larvae in each temperature became more apparent. Unlike the results of Chapter 3 when larvae were fed a single species (*T. chui*) diet at 10,000 cells $ml^{-1}$, larvae reared at the colder temperatures had the largest shell lengths by 17-d post-fertilisation. At 17-d fertilisation, larvae at 12 ºC grew best when fed 20,000 cells $ml^{-1}$, and 16 and 20 ºC grew best at 10,000 and 1,000 cells $ml^{-1}$, respectively.

Overall, the results of this research fill in many gaps in our knowledge about the life history of *Paphies ventricosa.* In particular, the reproduction of the local Oreti Beach population and the early larval ecology, with implications for both the

northern and southern populations, have been explored in depth. This is the first study of its kind for many of its components, including the detailed microscopic (both scanning electron and light microscopy) examination and description of the early larval stages of toheroa, and the identification of the fertilisation and thermal development windows in the species. In addition, it is the first study in toheroa to examine the combined effects of temperature and feed concentration. The present study has greater implications in regards to other bivalves of similar distributions and habitats, and provides insight into the conservation and management of the species.

# Chapter 1

# General Introduction

## Background and taxonomy

*Paphies ventricosa* (Gray, 1843) is a bivalve (Veneroida: Mesodesmatidae), endemic to New Zealand. It is a large surf clam, occurring as fragmented populations on dissipating beaches. The species is commonly known by the Maori name toheroa, meaning long tongue (Hoby, 1933). This thesis examines the recruitment dynamics of *Paphies ventricosa* at Oreti Beach, Southland, New Zealand, by examining the factors affecting reproduction, fertilisation, and early larval ecology.

Toheroa were first described by Gray in 1843 as a true gill bearing mollusc (Eulamellibranchiata) of the genus *Amphidesma* (Lamarck, 1818), calling it *Amphidesma ventricosum* (Hoby, 1933; Smith, 2003). Dawson (1959) suggested that *Amphidesma* was an incorrect classification, belonging to Family Mesodesmatidae (Gray, 1840) rather than the current, at the time, Family Semelidae (Stoliczka, 1870), and that *Donacilla* (de Blainville, 1819) replace *Amphidesma*. Beu (1971) thought that *Donacilla* should not include large New Zealand Mesodesmatidae, and placed them in genus *Paphies* (Lesson, 1831). Toheroa are one of four clams belonging to the genus *Paphies*, all of which are large, edible, saltwater clams endemic to New Zealand; the others genus members being the pipi (*P. australis*), northern tuatua (*P. subtriangulata*), and southern tuatua (*P. donacina*). Of the four *Paphies* species, toheroa are the largest, with the shell reaching lengths of up to 150 mm. The current taxonomic hierarchy of toheroa is as follows:

Kingdom: Animalia

Phylum: Mollusca

Class: Bivalvia

Order: Veneroida

Family: Mesodesmatidae

Genus: *Paphies*

Species: *P. ventricosa* (Gray, 1843)

## Distribution

*P. ventricosa* is a surf clam, in that it is a clam that lives in the intertidal swash zone. It has a broad longitudinal but highly fragmented distribution, ranging between 34º 31' S and 46º 20' S (Figure 1.1A). Only three significant populations are still present – Northland, Wellington, and Southland – and are separated by up to 700 km and up to ~ 6 ºC (Figure 1.1B). Historic populations were also located along both Northland coasts, and the southern shore of the Bay of Plenty; a possible historic population was located at Moeraki on the South Island. The species is currently restricted to high-energy dissipating beaches that are on western-facing fully-exposed coasts made of fine sand (250 µm average grain size) (Rapson, 1952; Cassie, 1955). Adult size classes (≥ 100 mm) are concentrated at mid-tide levels, sub-adults (40 – 99 mm) are generally found higher on the beach, while juveniles (< 40 mm) have a wider and more even vertical distribution across the entire range, but are most abundant higher up the beach (Beentjes & Gilbert, 2006; Beentjes *et al.*, 2006). Along the beach, adults are often found aggregated into distinct beds (Redfearn, 1974).

The distribution and habitat characteristics of *P. ventricosa* are similar to those of more commonly known genera, such as the well-studied genus *Donax*, also of order Veneroida. Similar size class vertical distributions have been reported for *D. faba* (Alagarswami, 1966), *D. trunculus* (Ansell & Lagardère, 1980); and *D. serra* (McLachlan, 1996). *Donax* surf clams are also found on exposed, sandy beaches in tropical to temperate latitudes (Ansell, 1983; Donn *et* al., 1986). In particular, *D. serra* have been observed to populate long, exposed, dissipating sandy beaches (Shoeman & Richardson, 2002); to form distinct dense beds along dissipating beaches (McLachlan, 1996; Laudien *et al.*, 2003); and to prefer a grain size of 200 to 300 µm (McLachlan, 1996). Unlike *P. ventricosa*, and the genus *Paphies*, *Donax* clams have a global distribution.

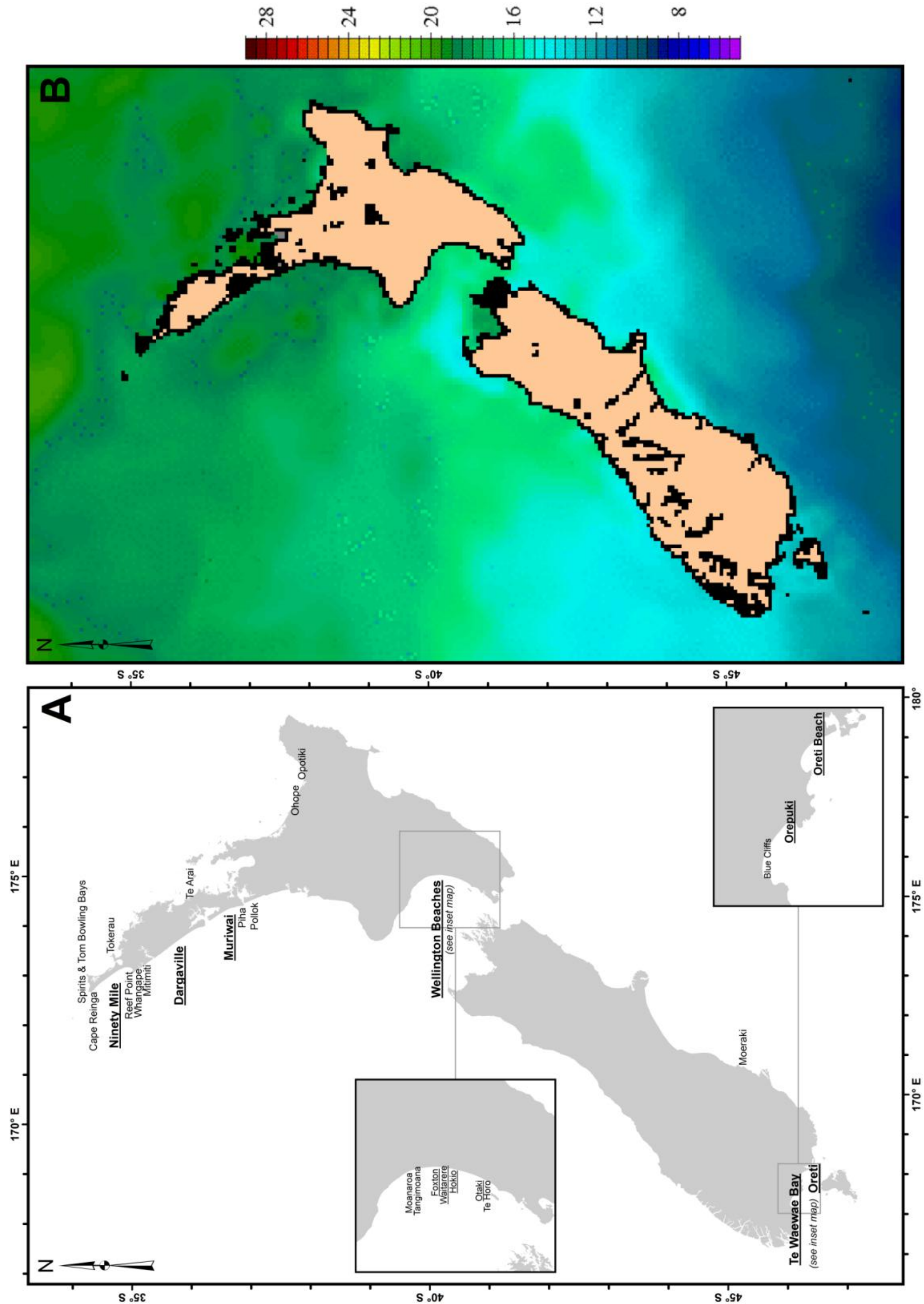



**Figure 1.1. (A) Historic distribution of *Paphies ventricosa* within New Zealand, with current major populations both underlined and in bold (after Redfearn, 1974; Williams *et al.*, 2013). (B) Sea surface temperature of New Zealand waters, on December 15, 2012 (1/8º Global HCOM dataset;** www.ocean.nrlssc.navy.mil**).**

## Biology and ecology

*Paphies ventricosa* is one of New Zealand's largest bivalve species. The shell is solid and shaped like a wedge with the umbos closer to the posterior end than the anterior end (Figure 1.2). Adult toheroa are usually found up to 200 mm beneath the substratum (Redfearn, 1974), or at depth roughly equivalent to its shell length (Hoby, 1933; Kondo & Stace, 1995; Various, 2011 pers. comm.). The toheroa is known for its large, muscular, triangular in shape foot, or tongue, which allows for rapid burrowing into the sand. They have been known to dig faster than the person trying to dig them up, often when the digger is inexperienced (Various, 2011 pers. comm.). The ability to bury themselves so quickly is a defence against predation (Haddon *et al.*, 1987). Like many bivalves, *P. ventricosa* is an infaunal filter feeder. They have two independent extendable siphons that can either sit flush with the substratum (Figure 1.3), or extend slightly above. The larger inhalant siphon is topped with a network of tentacles, or papillae, that act as a primary filter against larger particles through which water and food particles are drawn into the mantle cavity for processing (Figure 1.3) (Hoby, 1933 Redfearn, 1974). The smaller exhalent siphon ejects deoxygenated water, faeces, and pseudofaeces comprised of non-digestible and excess food particles bound in mucous (Figure 1.3). The siphons and foot are usually drawn into the mantle cavity when removed from the substratum, though will often be ejected in an exploratory manner to test the substrate. *In situ*, the siphons will also be withdrawn from the substrate surface when disturbed. Toheroa have a fused ventral mantle edge and the inability to completely close their valves, with a constant gape at the posterior end where the siphons emerge from the shell. Reproductively, toheroa are gonochoristic broadcast spawners, showing distinct

stages of gonad development (Redfearn, 1974; Mandeno, 1999; Smith, 2003; Gadomski & Lamare, 2015).

The wedge shell shape, like that found in toheroa and other surf clams, has been noted to have been adapted to provide easy penetration of the sediment and fast reburial in the surf clam genus *Donax* (Ansell, 1983). Fast reburial and recovery when buried too deeply may also be an adaptation to the high energy environments in which *Donax* spp., toheroa and other surf clams are found (Ansell, 1983). These environments are prone to frequent sediment disturbance, with a high potential for animal displacement. A major factor in the success of *Donax* spp., toheroa, and likely other surf clams, is the ability to coordinate their movements to maintain or reattain ideal burial depth and position (Ansell, 1983; Kondo & Stace, 1995).

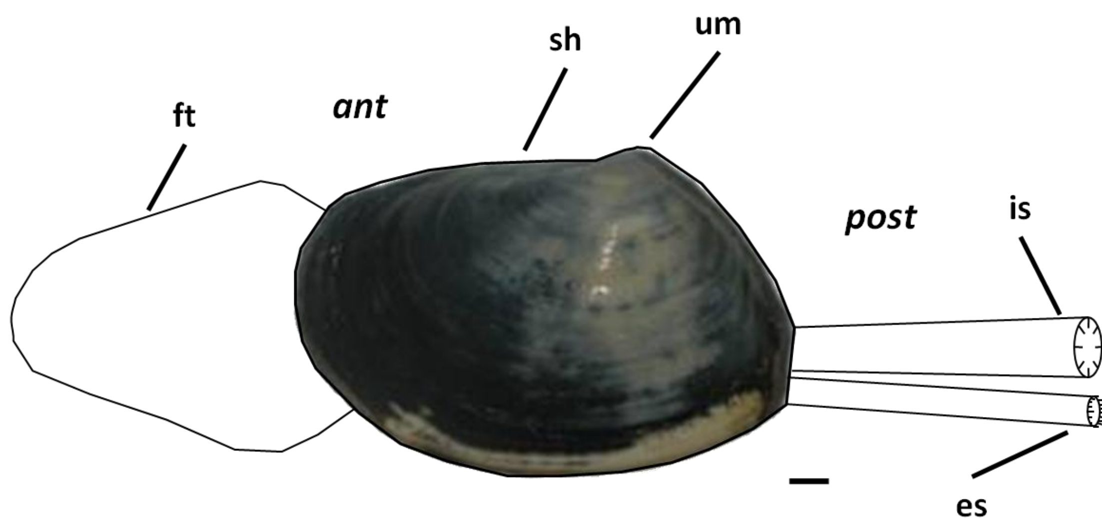



**Figure 1.2. Line drawing of a toheroa with the foot (or tongue; ft), umbo (um), inhalant siphon (is), and exhalent siphon (es) extended over a photo of a live adult *Paphies ventricosa* showing the shell (sh) and posterior (*post*) and anterior (*ant*) ends. Scale bar = 1 cm.**

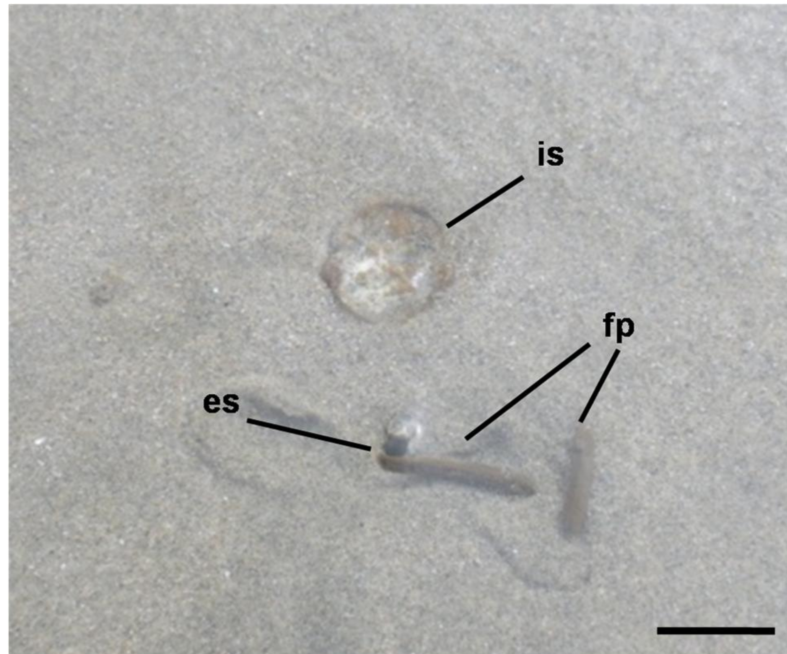



**Figure 1.3. Buried *Paphies ventricosa* showing inhalant siphon (is), exhalent siphon (es), and faeces/pseudofaeces (fp) on Oreti Beach, Southland, New Zealand.**

## Exploitation and population decline

*Paphies ventricosa* populations have decreased substantially over the past century, although the reasons for the decline are poorly understood (Williams *et al.*, 2013). Substantial overfishing in the first half of the 20th century (Rapson, 1952), habitat degradation (Beentjes *et al.*, 2006) and disturbance from beach traffic (Brunton, 1978; Beentjes *et al.*, 2006) are thought to be contributors.

Commercial harvest is thought to be a significant contributing factor to population decrease in the northern populations. Commercial harvest of toheroa began in the late 1800s, but numbers remained low until commercial canning began in the early 1900s (Williams *et al.*, 2013). The first cannery opened in 1904 on Dargaville Beach (Figure 1.1A), followed by a second in 1911, with one closing in 1923 having

become uneconomic (Williams et al, 2013). In 1923, a new cannery opened on Ninety Mile Beach (Figure 1.1A), and though only open for three months of the year, processed almost 10,000 toheroa per day (576,000 per annum) (Williams *et al.*, 2013). The Ninety Mile Beach factory closed in 1945 having become uneconomic, but briefly reopened between 1962 and 1964 (Williams *et al.*, 2013). Canning peaked in the 1940s, followed by a consistent decline until 1957; production briefly resumed in 1960 to a small peak in 1963, before declining again through the rest of the decade, until all commercial harvest ceased in 1969 (Williams *et al.*, 2013). Commercial production averaged around 20 tonnes per annum, with a record of 77 tonnes in 1940 (McLachlan *et al.*, 1996). Toheroa were also briefly canned at Muriwai Beach, Wellington beaches, and at Te Waewae Bay (Figure 1.1A) at various times for short durations, though nowhere near the scale as at Dargaville and Ninety Mile Beach (Rapson, 1952; Redfearn, 1974, 1987). The majority of toheroa commercially harvested were larger than 80 mm in shell length (McKinnon & Olsen, 1994). Initially, commercial harvest was managed under licenses issued by the Minister of the Marine Department, and only permitted in licensed areas, with maximum allowable catch determined by size and condition of a population within a licensed area (Williams *et al.*, 2013). The license system was replaced by commercial quotas and restricted harvesting seasons in 1962 (Williams *et al.*, 2013), both of which were based on annual surveys.

Recreational harvest restrictions were first implemented in 1932, and were limited to: (a) a daily limit of 80; (b) a size limit of 76 mm; (c) a 2 month closed season (October and November); and (d) banning the use of metal implements in digging (Williams *et al.*, 2013). The first closure of a northern beach was in 1940, due to increased exploitation (Williams *et al.*, 2013). In 1955, recreational restrictions were

updated to (a) an individual daily limit of 20 toheroa and (b) a closed season of 10 months (September to June) (Redfearn, 1974). The use of any implement, other than hands, was prohibited in 1962 (Williams *et al.*, 2013). All Northland beaches were closed for the year in 1972, and restrictions were further amended to (a) a daily individual limit of 10 toheroa, or daily vehicle limit of 30 toheroa, and (b) a season limited to 2 weeks in September (Greenway, 1972). Recreational harvesting was prohibited from Ninety Mile Beach in 1971, followed by Muriwai, Wellington beaches, and Dargaville Beach in 1976, 1978, and 1980, respectively (Williams *et al.*, 2013). Southland beaches maintained sporadic one day seasons per year since 1972, with the last seasons held in 1980 and 1993 at Te Waewae Bay and Oreti Beach, respectively (Beentjes, 2010; Williams *et al.*, 2013). The legal size limit increased to the current size of 100 mm in 1979 (Williams *et al.*, 2013). Currently, all toheroa harvest is under customary permiture, with Kaitiaki from local iwi managing harvest by issuing permits with the oversight of the Ministry for Primary Industries (MPI) (Williams *et al.*, 2013). There is little information on the extent of any illegal harvest.

### Population surveys

One result of the commercial harvest/canning, was estimates of abundance from cannery records and subsequent surveys of abundance, which began in the later years of commercial harvest, as a means to set limits. Northland beaches have abundance data going back to the 1930s, with intensive surveying from the early 1960s to the late 1980s, before resuming in 2000 (Williams et al, 2013). The surveys show a general decreasing trend in population estimates, with greater fluctuations in the Ninety Mile Beach and Dargaville populations after the cessation of commercial

harvest. For example, Greenway (1972) estimated the northern population had declined from an estimated 10,000,000 individuals in 1964 to an estimated 1,000,000 in 1971. Wellington beaches have survey data primarily from the late 1960s and early 1970s, with a few estimates going back to 1940 (Williams *et al.*, 2013). Though these surveys are fewer in number than the Northland surveys, they do show a large population decrease around 1970 (Williams *et al.*, 2013). Despite the cessation of commercial harvesting and recreational open seasons, population numbers have not recovered on the North Island.

Surveys on the southern population at Bluecliffs Beach between 1966 and 2005 show a declining population, with numbers decreasing from 2,000,000 to 80,000 adults in the early 1990s (Beentjes *et al.*, 2006). A 2009 survey estimated the adult population at Bluecliffs Beach to be only 34,000 adults, ascribing the continuing decline to further habitat loss caused by beach erosion (Beentjes, 2010). The most recent survey, concluded in 2014, estimated the adult population to be 8,000 (Berkenbusch *et al.*, 2015). The Oreti Beach population showed natural fluctuations around an overall general declining trend in abundance until the mid-1990s, when the population appeared to have stabilised (Beentjes, 2010). The population of adults at Oreti Beach has remained around 1,000,000 between 2009 and 2014, while the population of juveniles has increased from around 600,000 in 2009 to over 2,000,000 in 2014 (Berkenbusch *et al.*, 2015).

## Research history

The toheroa has been important historically as a commercial, recreational, and customary fishery (Stace, 1991) for generations of New Zealanders. This, combined

with the continued population decline despite a near total ban on harvest over the last 20 years, has created a push for greater understanding in regards to the biology and ecology of the species.

Previous studies, other than government funded surveys, have focused on population dynamics (Rapson, 1954; Cassie, 1955), distribution (Cassie, 1951; Waugh & Greenway, 1969; Akroyd *et al.*, 2002), burying behaviour (Haddon *et al.*, 1987; Kondo & Stace, 1995), predation (Rapson, 1954; Brunton, 1978; Haddon *et al.*, 1987), and taxonomy (Dawson, 1959; Beu, 1971). Hoby (1933) provided the first detailed examination of internal structures using extensive histology. Redfearn (1974) summarised the biology of the species, and is a basis for most of the more recent studies. Smith (2003) examined the reproduction and recruitment in toheroa from Ripiro Beach, North Island (Figure 1.1A), and was the first to examine traditional ecological knowledge regarding spawning. Mandeno (1999) was the first to examine the reproduction and spawning induction of toheroa from Oreti Beach, South Island (Oreti Beach; Figure 1.1A).

## Research aims

Given the relatively small, generally decreasing population sizes, and the geographically fragmented distribution of the three main populations, recruitment has been identified as an important factor in the future of the species (Beentjes *et al.*, 2006). Recruitment is influenced by many factors, including fertilisation success, temperature, food availability, spatial distribution, and hydrodynamics. The location of the study, Oreti Beach, Southland, was chosen for its proximity to the University of Otago, the stability of the population, and interest in the research by local Kaitiaki.

The aim of this body of research is to provide a detailed examination of factors likely to effect the early recruitment of *Paphies ventricosa* at Oreti Beach, Southland.

***In Chapter 2:***

Larval recruitment begins with fertilisation, which in turn begins with reproduction. Knowing the physical conditions and time when reproduction occurs is the first step in understanding the recruitment within a population. The reproductive stage of mature *Paphies ventricosa* at Oreti Beach is examined in terms of both temporal and spatial variation, in order to identify spawning events and how these events relate to environmental factors. Histological examination of the gonad is used to describe gonad developmental stage, sex ratio, oocyte density, and average oocyte density in toheroa. In addition, the condition index of collected adults is also compared spatially and temporally in relation to environmental factors.

***In Chapter 3:***

Once the reproductive physical conditions and timing have been identified, the *in situ* conditions in which embryonic and larval development occur can be deduced. One of the critical physical factors to consider is temperature. The effect of temperature on the rate of larval development at environmentally relevant temperatures to the Oreti Beach population (12º, 16º, and 20º C) is examined to estimate how long toheroa larvae remain in the water column. Developmental rates are compared to results from Redfearn (1982) at 25º C. All experimental temperature treatments were fed the same single species cultured algae diet. A detailed description of fertilisation and embryonic and larval development is made using light and scanning electron microscopy, the first examination of its kind for *Paphies ventricosa*. Additionally, a new method for spawning induction in toheroa is described.

***In Chapter 4:***

One of the first things a broadcast egg is exposed to is *in situ* temperatures. Temperature can fluctuate *in situ*, and larval sensitivity can affect the success of larval recruitment to the veliger stage. However, before larval development can occur, fertilisation and successful embryological development must first occur; other than predation, temperature is one of the biggest factors limiting this success. The success of fertilisation and early larval development of *Paphies ventricosa* across a thermal gradient, the first study of its kind, is described. Additionally, a new method of lab conditioning for toheroa broodstock is described. This chapter is in prep to be submitted for publication.

***In Chapter 5:***

Even more important than temperature in limiting larval development is food availability. Free-swimming, feeding larvae experience mixed diets of fluctuating concentrations and fluctuating temperatures *in situ,* which can affect development and recruitment success to varying degrees, and is critical in understanding recruitment in a given species. The combined effect of temperature and feed concentration (cultured, 1:1 mixed algae diet) on the larval development of southern *Paphies ventricosa* is examined. Understanding the combined effect these factors have on larval development is critical in understanding the early recruitment processes of toheroa.

# Chapter 2

# Reproduction in toheroa (*Paphies ventricosa*) from Oreti Beach, Southland

This chapter is adapted from:

Gadomski K. & Lamare, M. (2015). Spatial variation in reproduction in southern populations of the New Zealand bivalve *Paphies ventricosa* (Veneroida: Mesodesmatidae). *Invertebrate Reproduction and Development*, **59**(2): 81-95.

## Introduction

Reproduction has been examined in a range of temperate bivalve orders such as Veneroida (Newell & Bayne, 1980; Harvey & Vincent, 1989; Gaspar *et al.*, 1999), Mytiloida (Wilson & Hodgkin, 1967), Osteroida (Ruiz *et al.*, 1992), and Myoida (Zaidman *et al.*, 2012). This includes a number of bivalve species inhabiting New Zealand soft sand environments, such as *Ruditapes largillierti* (Gribben *et al.*, 2001), *Panopea zelandica* (Gribben *et al.*, 2004) and *Zenatia acinaces* (Gribben, 2005). For the New Zealand species, reproduction follows relatively well-defined annual cycles with spawning occurring within the spring and summer periods.

Of interest in this study is the family Mesodesmatidae (Bivalvia: Veneroida), in which the reproduction has been described in a small number of species including the Australian *Donacilla cuneata* (Roberts, 1984), Asian *Coecella chinensis* (Kim *et al.*, 2013), the South American *Mesodesma donacium* (Peredo et al, 1987; Riascos et al, 2009; Uribe *et al.*, 2012) and *Mesodesma mactroides* (Herrmann *et al.*, 2009) and the tropical species *Atactodea striata* (Baron, 1992). Reproduction in New Zealand

mesodesmatids, all in the endemic genus *Paphies*, has been described for *P. ventricosa* (Redfearn, 1974), *P. australis* (Hooker & Creese, 1995), *P. donacina* (Marsden, 1999), and *P. subtriangulata* (Grant & Creese, 1995).

Most of what is known about *P. ventricosa* reproduction comes from the North Island. Hoby (1933) found that egg diameter increased from June (59 – 65 μm) to October (75 – 80 μm) and that sperm activity increased from "fairly active" at the end of July to "extremely active" in October. Additionally, Hoby (1933) found that at the beginning of October, few animals had spawned, but by the end of the month, most animals had spawned. Redfearn (1974) identified four stages of gametogenic development in *P. ventricosa*, classified as early active (EA), late active (LA), mature, and partially spawned/spent. The study found that two to three spawning events occurred during the summer season, with an initial maturation period in September followed by a second maturation period in December/January when mean sea surface temperature (SST) was the highest. Smith (2003) classified five gametogenic developmental stages (EA, LA, ripe, partially spawned/spent, and rapid redevelopment) and also observed two ripening/spawning events, one in August/September, and a second from December to March. No published accounts exist for reproduction in southern New Zealand, although an unpublished account by Mandeno (1999) examined reproduction of the South Island population from a single site. Mandeno (1999) classified southern *P. ventricosa* into the same four gametogenic stages as Redfearn (1974) and identified two major ripening events, one in September, and a second in February.

The current study aims to increase the understanding of the reproduction of *P. ventricosa*, particularly in terms of temporal and spatial patterns at Oreti Beach, Southland, New Zealand. Here, a detailed description of the reproduction of *P.*

*ventricosa* is made using a condition index (CI) and detailed histological study of the gonad over a period of two years at four different sites (one site in 2011, and four sites in 2012) at Oreti Beach. Reproductive activity over the period was compared with the physical environmental conditions from the study sites.

This study is the first to detail the reproductive cycle in southern populations of *P. ventricosa* and provides insight into the spatial in variations in reproduction, both across latitude and at smaller scales (i.e. < 5 km). This information is required for the effective management of threatened bivalve species such as *P. ventricosa*, where knowledge of spatial and temporal patterns of reproduction can be used in conservation strategies to maximise future recruitment by identifying larval source populations (Gaines *et al.*, 2010).

## Materials and Methods

### Study site and animal collection

Reproduction in *P. ventricosa* was examined at four sites along Oreti Beach, Southland, New Zealand. Oreti Beach is a southwest facing, 26 km long, dissipating surf beach on the shores of Foveaux Strait (Figure 2.1). Each end of the beach is bordered by two river/estuary systems – New River Estuary to the south, and Jacobs River Estuary to the north.

To examine reproduction over one year, 20 mature animals were collected from a single site (Site B) every month between January 2011 and December 2011 (Figure 2.1), excluding April. Sampling was unable to occur in the month of April 2011, due to a combination of vehicle failure in the field; a University holiday closure period;

inclement weather; and lack of workable tides during daylight hours. In 2012, the spatial and seasonal patterns of reproduction in *P. ventricosa* along Oreti Beach were examined at four sample sites along the length of the beach (Sites A – D) for autumn (March), winter (June), spring (September) and summer (December) (Figure 2.1). For this study, 20 mature animals were collected per site per season. All animals collected in 2011 and 2012 were greater than 95 mm in shell length, exceeding the minimum size of sexual maturity (76 mm shell length) reported by Rapson (1952). All collections were made under the appropriate customary permit, and thus all extractions were made by hand. Following extraction, animals were packed in local sand and seawater for transportation to Portobello Marine Laboratory (PML), Dunedin for dissection within 2 days. Collections in both years were made at low tide, ± 2 h.

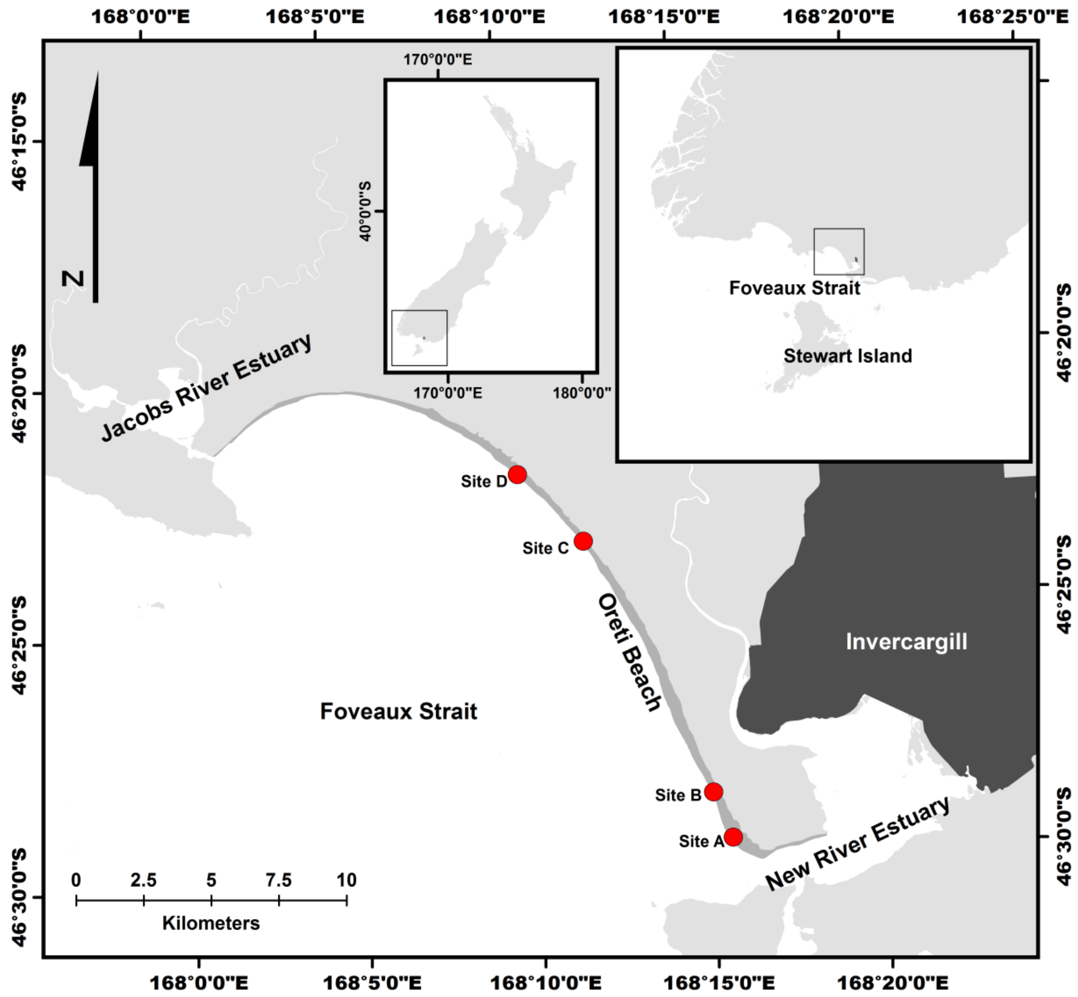



**Figure 2.1. Location of the study sites at Oreti Beach, Southland for 2011 (Site B (46.4790º S, 168.2522º E)) and 2012 (Sites A (46.4943º S, 168.2604º E), B (46.4790º S, 168.2522º E), C (46.3935º S, 168.1970º E), and D (46.3701º S, 168.1673º E).**

## Individual condition index

For each animal, live individuals were blotted dry and the total wet mass ($M_t$) was determined to the nearest 0.1 g. Next, animals were dissected by removing the soft tissues from the shell in one piece, and removing residual sand from the mantle cavity. The soft tissues were patted dry and the combined weight of the foot and visceral mass was determined ($M_v$), also to the nearest 0.1 g. Using these weights and methods, as described by Herrmann *et al.* (2009; after de Villiers, 1975), a CI for each individual was calculated as:

$$\mathrm{CI}\ (\%) = 100\left(\frac{Mv}{Mt - Mv}\right)$$

## Histological preparation

Since the gonad tissue of *P. ventricosa* is diffuse within the body, an intact 15 mm cross-sectional potion of foot, gonad, and gut was excised adjacent to the anterior adductor muscle (AAM) and immediately fixed in 10% neutral buffered formalin (30 g sodium tetraborate $l^{-1}$) in seawater. Following fixation, a section of gonad tissue (< 15 mm side length, surrounded by foot tissue to stabilise the gonad tissue) was removed and placed into tissue cassettes for tissue processing. Any portion of the crystalline style was removed from the sections, and care was taken to avoid gut tissue (as it tends to be full of sand) in the preparations.

Excised sections were dehydrated via an alcohol series and re-saturated using xylene. Sections were embedded in Paraplast® wax (Sigma-Aldrich), sectioned at 7-10 µm thickness and mounted on chrome alum gelatine coated slides. Sections were first stained with Meyer's haematoxylin, counterstained with eosin and mounted using DPX Mountant (Sigma-Aldrich).

### Determination of sex and reproductive stage

Mounted gonad sections were photographed using a BX51 compound microscope fitted with an Olympus Colorview III$^{TM}$ camera, controlled by the software AnalySIS LS (Olympus Corporation). Animals were sexed and assigned to one of four reproductive stages (Figure 2.2, Table 2.1), namely early active (EA), late active (LA), ripe (RI), and spawned/partially spent (SP). Definitions of the stages were adapted from earlier bivalve studies (Ropes, 1968; Redfearn, 1974; Grant & Creese 1995; Hooker & Creese, 1995; Mandeno, 1999) to apply to toheroa.

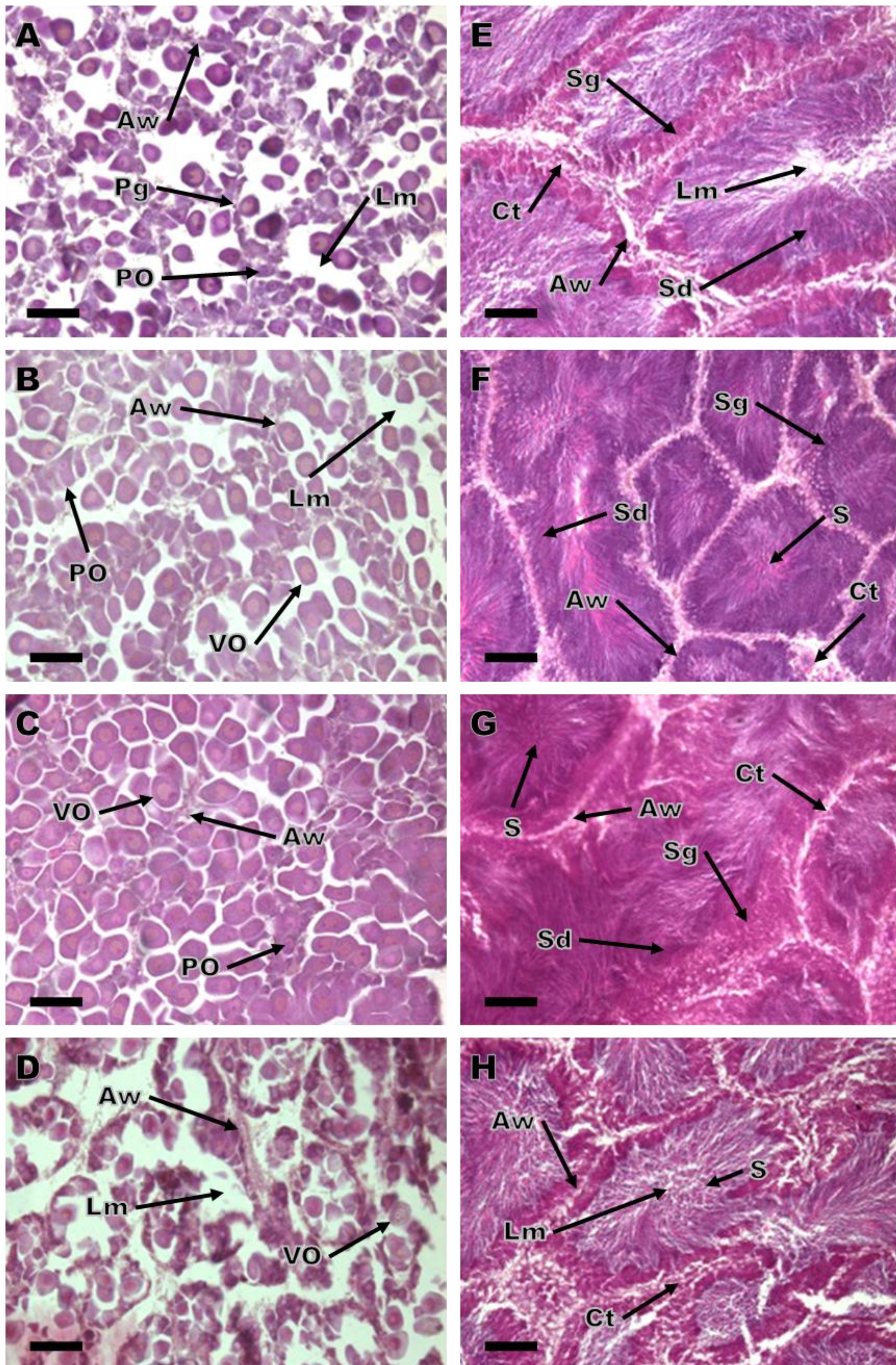



**Figure 2.2. Histological sections of female and male *Paphies ventricosa* gonads. Female stages: EA (A); LA (B); ripe (C); and partially spawned/spent (D). Aw – Alveoli wall; Pg = protogonia; PO = pre-vitellogenic oocyte; VO = vitellogenic oocyte; Lm = lumen. Male stages: EA (E); LA (F); ripe (G); and partially spawned/spent (H). Aw = Alveoli wall; Sp = Spermatogonia; Sd = Spermatid; S = Spermatozoa; Ct = Connective tissue; Lm = Lumen. See Table 2.1 for detailed descriptions of female and male reproductive stage. Scale bars = 100 µm.**

**Table 2.1. Description of four reproductive stages assigned to female and male *Paphies ventricosa* gonad sections. Abbreviations refer to Figure 2.2.**

| Stage | Females | Males |
|---|---|---|
| Early active (EA) | The alveoli walls (Aw) are thick and contain numerous protogonia (Pg). Many pre-vitellogenic oocytes (PO) are present, attached to the alveoli walls by broad cytoplasmic bases. Free vitellogenic oocytes (VO) may remain in the lumen (Lm) from the last ripe cycle. | The alveoli walls (Aw) are thin and have a layer of spermatagonia (Sg). Spermatids (Sd) fill the lumen (Lm). A few spermatozoa (S) still remain in many lumen centres. |
| Late active (LA) | The alveoli walls (Aw) are less dense. Vitellogenic oocytes (VO) are larger and quite numerous. Remaining basal attachments are much thinner in pre-vitellogenic oocytes (PO). | The alveoli walls (Aw) are lines with spermatagonia (Sg). Roughly one-third of the lumen (Lm) is filled with spermatids (Sd). Lumen centres are loosely packed with radiating bands of spermatozoa (S). Little lumen is visible. |
| Ripe (RI) | The alveoli walls (Aw) are thin and may contain protogonia (Pg). Vitellogenic oocytes (VO) are at their largest and fill the lumen of most follicles. Pre-vitellogenic oocytes (PO) are few and are broadly attached to the alveoli walls (Aw). | The lumen (Lm) is mostly filled with densely packed spermatozoa (S). A thin layer of spermatids (Sd) fill the rest of the lumen space. |
| Partially spawned/spent (SP) | The alveoli walls (Aw) are thin and contracted, with some partially ruptured. Some pre-vitellogenic oocytes (PO) remain attached to the alveoli walls (Aw). Few vitellogenic oocytes (VO) may remain in the lumen. | The alveoli walls (Aw) may be slightly contracted; have few spermatagonia (Sg); and have a marbled appearance. Spermatids (Sd) form a thin layer around the outer edges of the lumen (Lm). Remaining spermatozoa (S) are distributed throughout the lumen in loosely packed groups. |

## Oocyte diameter and density

For each female, the size of up to 50 oocytes sectioned through the nucleolus was measured as the average of two perpendicular measurements of the longest ($d_1$) and shortest ($d_2$) axes (Figure 2.3). From this, the oocyte size frequency and average oocyte size was calculated for each female at each month. In addition, for each month, all oocyte size measurements were pooled to obtain the size-frequency distribution. All measurements were made using the software ImageJ (NIH, USA). For each section, the average free oocyte density (per $mm^2$ of gonad tissue) was estimated for each slide.

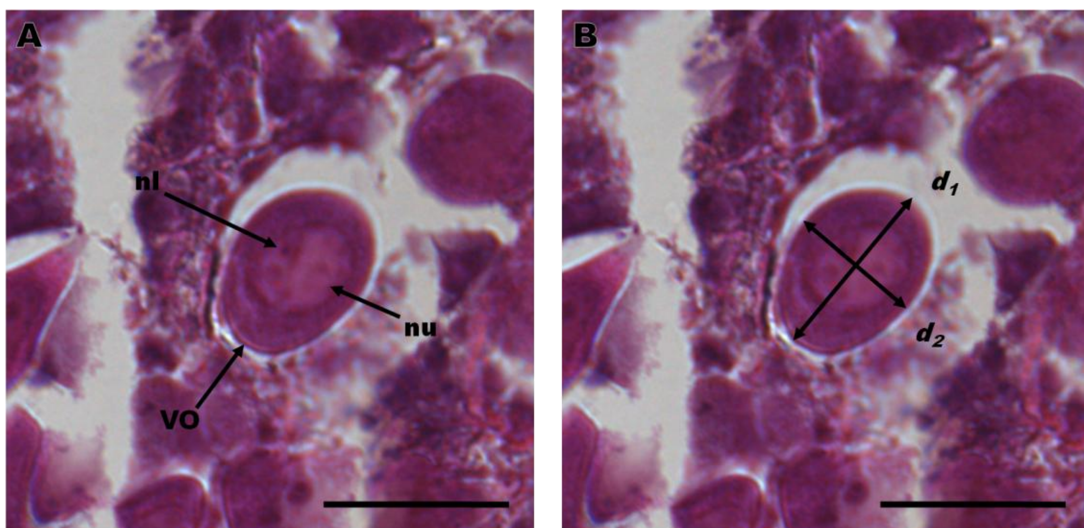



**Figure 2.3. (A) Cross-section of a vitellogenic oocyte (VO), showing the sectioned nucleus (nu) and nucleolus (nl). (B) Cross-section of an oocyte showing the diameter measurements ($d_1$, $d_2$). Scale bar = 50 µm.**

## Environmental measurements

Sea surface temperature (SST) and salinity at each site was collected using a HORIBA U-50 multi-parameter water quality meter (HORIBA, Ltd.) at each sampling time in 2011 and 2012. Regional SST was derived from the monthly composite Aqua MODIS SST (11 μ daytime) 9 km dataset (www.oceancolor.gsfc.nasa.gov). Regional averages were calculated from the same 6 by 6 pixel area (2916 $km^2$) for each month, stretching from Colac Bay to Bluff, Southland and fully encompassing the study area (Figure 2.4), by determining water pixel values using the provided scale. Site-specific chlorophyll-*a* (chl-*a*) data, calculated following the method of Strickland and Parsons (1972), averaged from two 2 l water samples collected in the surf, were compared to regional satellite derived chl-*a* concentrations from the monthly composite Aqua MODIS chl-*a* concentration 9 km dataset (www.oceancolor.gsfc.nasa.gov) in both 2011 and 2012. Regional averages were calculated as previously mentioned for SST. Day length was obtained for the 15th day of each collection month.

## Statistical analyses

The sex ratio of *P. ventricosa* at the four sampling sites (months pooled) and for all samples pooled was tested using the $X^2$-test. Statistical differences ($\partial$ = 5%) in CI (arcsine square-root transformed) between sexes and among months were tested using a two-way ANOVA, whilst the average oocyte/ova size (ln(*x*) transformed) and abundance (ln(*x*) transformed) among months in 2011 were tested using a one-way ANOVA, with significant pairwise differences between months identified using Tukey's *post hoc* test. Due to heterogeneity of variances, a Welch's test was used to assess statistical differences in the CI and oocyte density among sites and months in 2011. Differences in the CI and oocyte density among sites and months in 2012

were tested using two-way ANOVA and where a significant interaction between the two factors occurred, differences among months within each site were testing for normality visually via normal quantile-quantile plots and homoscedasticity was confirmed by Levene's test. All statistical analyses were carried out using the JMP 10.0 statistical package (SAS Institute).

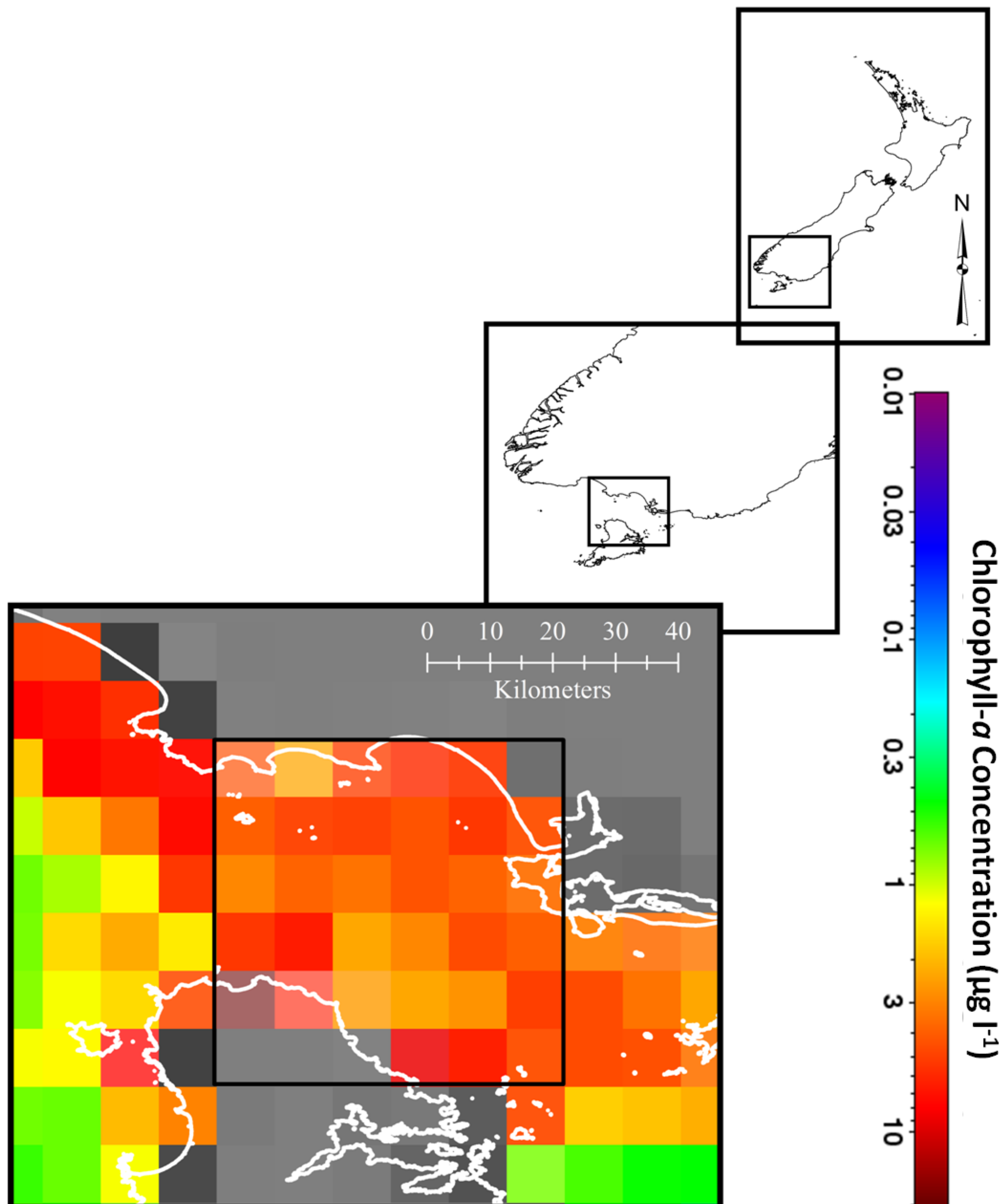



**Figure 2.4. Map showing the area of interest (internal black box) used for the analysis of the Aqua MODIS chl-*a* dataset, with the coastline of New Zealand overlaid (in white) to show the approximate location of the study area. The same extent was used in the analysis of the Aqua MODIS SST dataset..**

# Results

## Sex ratio

The ratio of males and females sampled over 2011 at Site B (103 males: 93 females) was not significantly different ($X^2$ = 0.5102, $p$ = 0.4751) from 1:1 (Table 2.2). Similarly, there was no significant difference in the male:female ratio for the four sample sites in 2012 (Table 2.2), or when all four sites were pooled despite a bias towards males (304 males: 264 females).

**Table 2.2. Number and ratio of male (M) and female (F) *Paphies ventricosa* from Site B collected during 2011, and from four sites along Oreti Beach, Southland at four times in 2012. $X^2$-values of the comparison between the observed sex ratio and an expected 1:1 (M:F) sex ratio are indicated for each population for the pooled populations. *P*-values based on 1 degree of freedom.**

| | Males | Females | Ratio (M:F) | $X^2$-values | $p$-value |
|---|---|---|---|---|---|
| Site B (2011) | 103 | 93 | 1.11 | 0.510 | 0.475 |
| | | | | | |
| Site A (2012) | 44 | 32 | 1.38 | 1.895 | 0.168 |
| Site B (2012) | 41 | 29 | 1.41 | 2.057 | 0.152 |
| Site C (2012) | 44 | 35 | 1.26 | 1.025 | 0.311 |
| Site D (2012) | 38 | 39 | 0.97 | 0.013 | 0.909 |
| Sites Pooled (2012) | 304 | 264 | 1.15 | 2.817 | 0.093 |

## Condition indices

The average monthly CI during 2011 ranged from 41.6 to 73.7% for males and 43.9% to 72.6% for females (Figure 2.5). Two-way ANOVA indicated that there was no significant difference in the CI between sexes ($F_{(1, 194)} = 1.125$, $p = 0.291$) across all months (Sex × Month, $F_{(10, 194)} = 1.052$, $p = 0.402$). When males and females were pooled, there was a significant difference in the CI among months ($F_{(10, 194)} = 9.4460$, $p < 0.001$), with a Tukey's *post hoc* comparison of means indicating that the CI in January (42.9%) and December (43.1%) were significantly lower than all other months (Figure 2.5). The greatest CI occurred in October (73.2%), although the difference in the CI from May to October was generally not significant.

The CI for *P. ventricosa* collected during 2012 (Figure 2.6) varied significantly among sites ($F_{(3, 288)} = 20.989$, $p < 0.001$), although the effect of site depended on the month of collection ($F_{(9, 288)} = 7.582$, $p < 0.001$). In this respect, there was no significant difference among sites in September ($F_{(3, 79)} = 2.257$, $p = 0.063$) and December ($F_{(3, 78)} = 1.271$, $p = 0.291$), with the CI ranging between 32.2 and 45.6% during these times. The CI was greater in March and June, ranging between 47.0 and 75.1%, and was significantly lower at Site A than Site D in both March ($F_{(3, 72)} = 13.853$, $p < 0.001$) and June ($F_{(3, 72)} = 16.22$, $p < 0.001$).

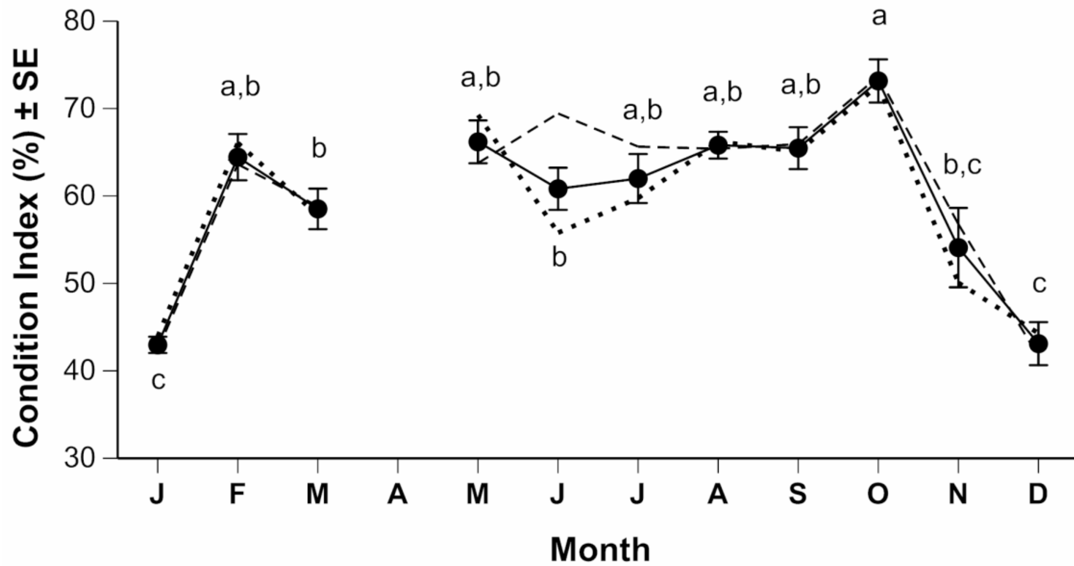



**Figure 2.5. Changes is male (- - - -) and female (····) CI, and the index for sexes pooled (●) for *Paphies ventricosa* from Oreti Beach, Southland, between January 2011 and December 2011. Standard errors are given for the CI of pooled data, but error bars for the individual sexes are omitted for clarity. Significant differences in the pooled CI among months are indicated by lower case lettering.**

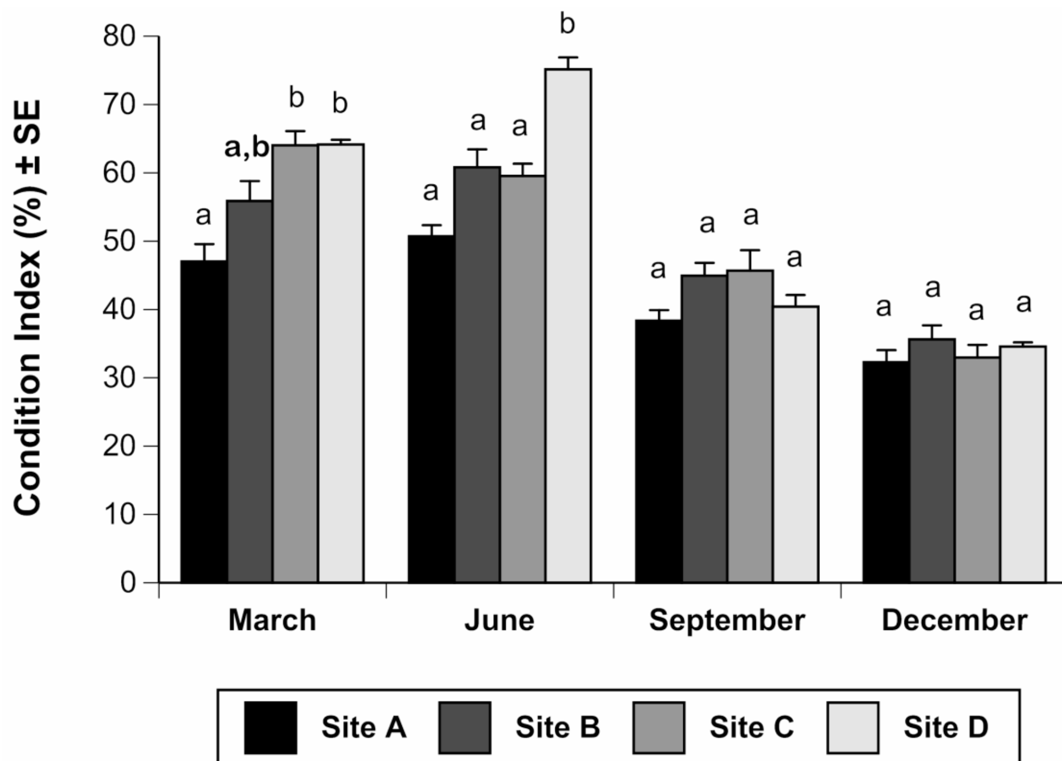



**Figure 2.6. Average CI (± SE) for *Paphies ventricosa* at four sites along Oreti Beach, Southland, during four months in 2012. Significant differences in the pooled CI among sites within each month are indicated by lower case lettering.**

### Gametogenic cycle

For females, histological examination of oogenic stages over a one-year period in 2011 (Figure 2.7A) indicated that ripe females occurred in all months (ranging from 9 to 70% of the sample), but were most common in September and October (64 and 70%, respectively). There was a smaller proportion of ripe females in February (40%), which was followed by a decrease in the proportion ripe to 14% by June. The proceeding EA and LA gametogenic stages were most abundant in the months between ripe maxima, making up to 86% (EA and LA pooled) in June and 73% in December. Spawned females were present in May (20%), but were most common from September to December, making up 50% of the females in November. As shown in Figure 2.7A, there appears to be two spawning events, indicated by the appearance of spawned females in May, and again from September to December.

For males, histological examination of spermatogenic stages over a one-year period (Figure 2.7B) indicated a clearer pattern in the abundance of ripe individuals, with peaks in February and March (90 and 93%, respectively) and troughs in October and November (100% and 83%, respectively). EA and LA stages were predominant in the remaining months, whilst spawned males were present in May and August, but most common in December when they made up 44% of males. As shown in Figure 2.7B, like the females, there appears to be two main spawning events, indicated by the appearance of spawned males in May and again in August, November and December.

Spatial variation in the gametogenic stages was examined at four sites along Oreti Beach for four months in 2012 (Figure 2.8). There was a substantial amount of variation among sites for both sexes, particularly in March and December, the time likely to be near or following spawning, suggest a degree of spawning variability

along Oreti Beach. For both sexes, a greater degree of gametogenic synchrony was evident among sites in June and September, although there was a tendency for individuals to be more advanced gametogenically with increasing distance north along the beach (i.e. from Site A to Site D). This is reflected in a typically greater proportion of ripe and late active stages, and fewer spawned individuals at Site D in most months.

## Oocyte/ova size frequencies

Oocyte and ova size-frequency distributions over the 2011 sampling period (Figure 2.9) were often bimodal and generally left-skewed. During the months of January to March, the distributions were relatively stable in shape although there was a progression of the distribution into larger size classes, showing the emergence of the mature cohort, in preparation of spawning. A clear change in size-frequency occurred in May with a broader distribution (≈ 12 – 55 µm), and a reduction in the abundance of larger oocytes and ova. From June to August, there was a distinct bimodal distribution, with a slight broadening of the distribution, to a clear emergence of a maturing cohort by September. In the following three months, the distributions decreased in size and breadth, with no oocytes larger than 50 µm by November, and shifted from left-skewed to right-skewed in December.

During the 2011 period, the average oocyte size varied significantly (Welch's test, $F_{(10,\ 1479.5)} = 66.26$, $p < 0.0001$) among months (Figure 2.10A), with two distinctive peaks in average size – the first in January/March (37.8 – 38.8 µm) and a second in September (36.3 µm). The smallest average oocyte sizes occurred during the winter months when oocyte average size was as small as 29.3 µm, and in December when oocyte size was at a minimum (25.8 µm). Tukey's *post hoc* tests indicated

significant differences among the months of maximum diameter with those of minimum sizes (Figure 2.10A).

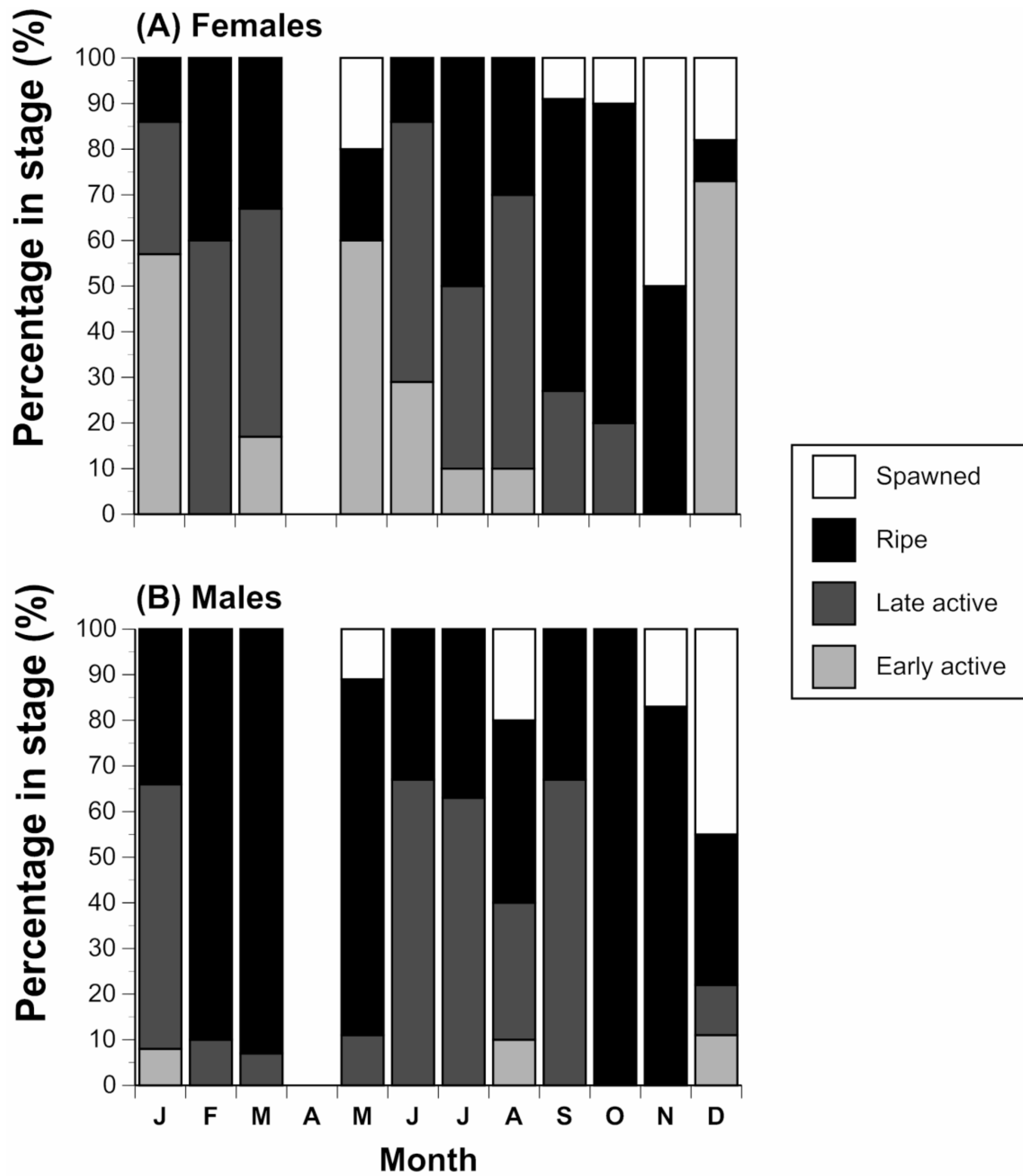



**Figure 2.1. Monthly changes in the percentage of (A) female and (B) male *Paphies ventricosa* in each of four gametogenic stages from Oreti Beach, Southland between January and December 2011.**

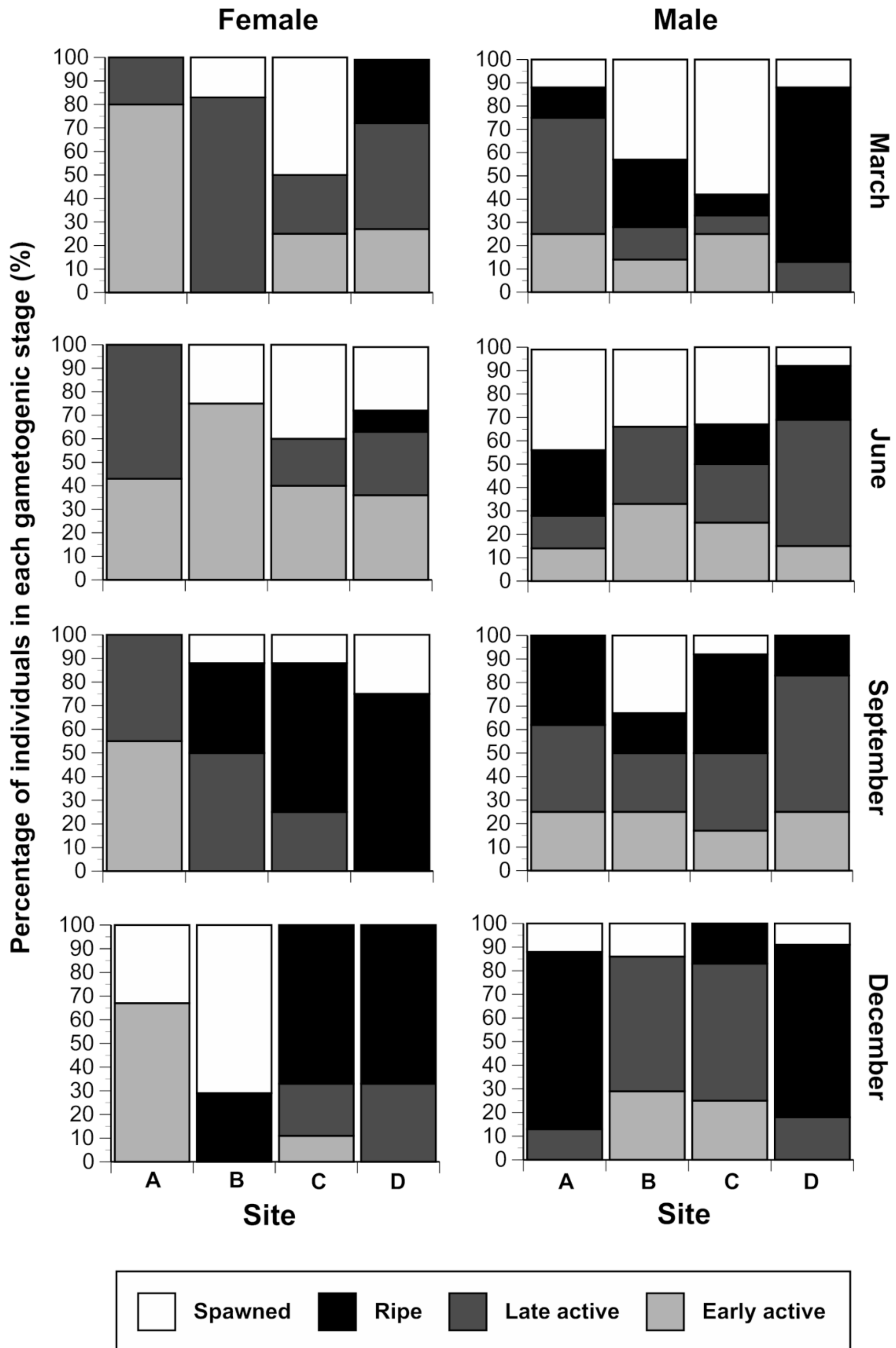



**Figure 2.2. The percentage of female (left column) and male (right column) *Paphies ventricosa* in each gametogenic stage from four sites along Oreti Beach, Southland, at four months in 2012.**

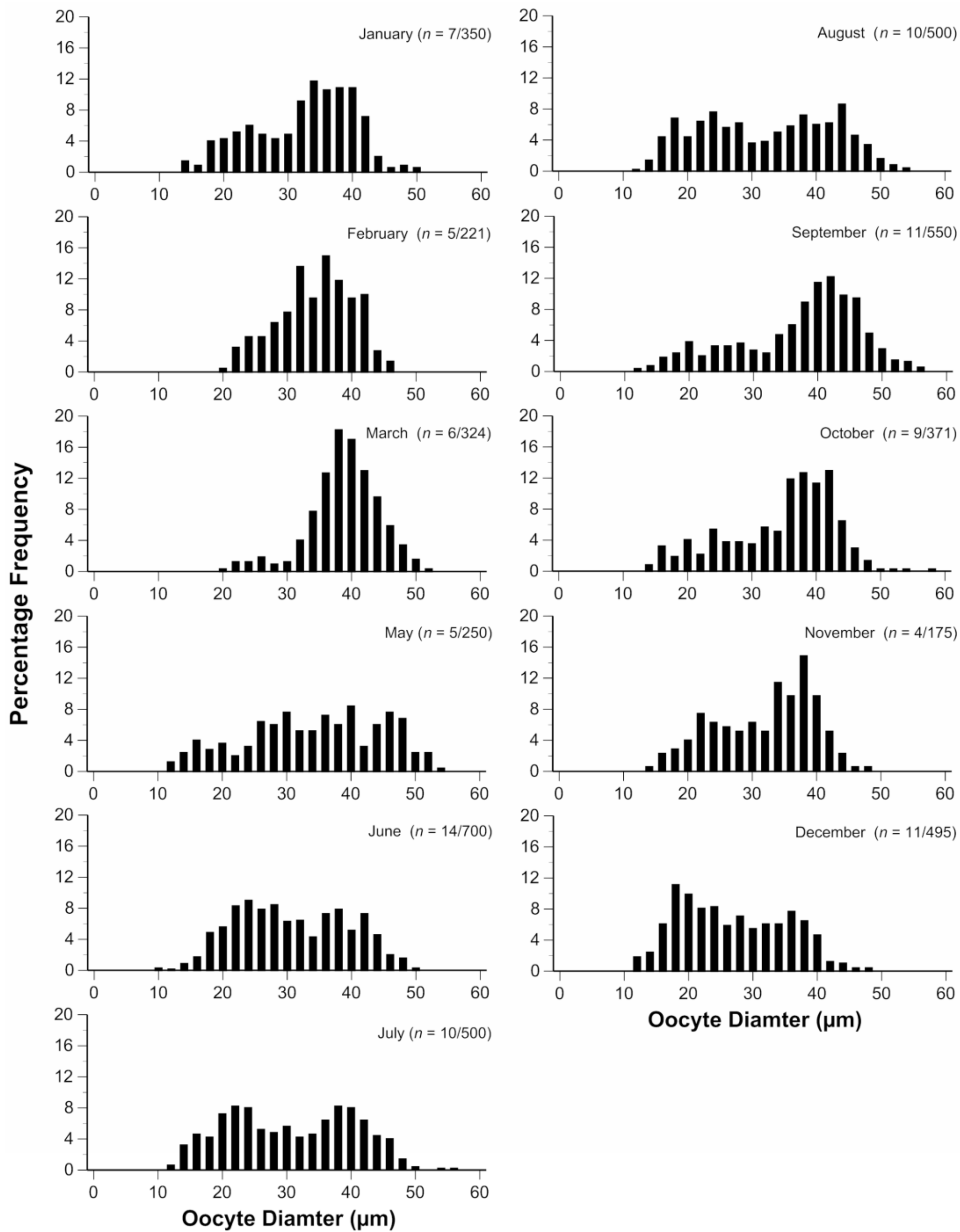
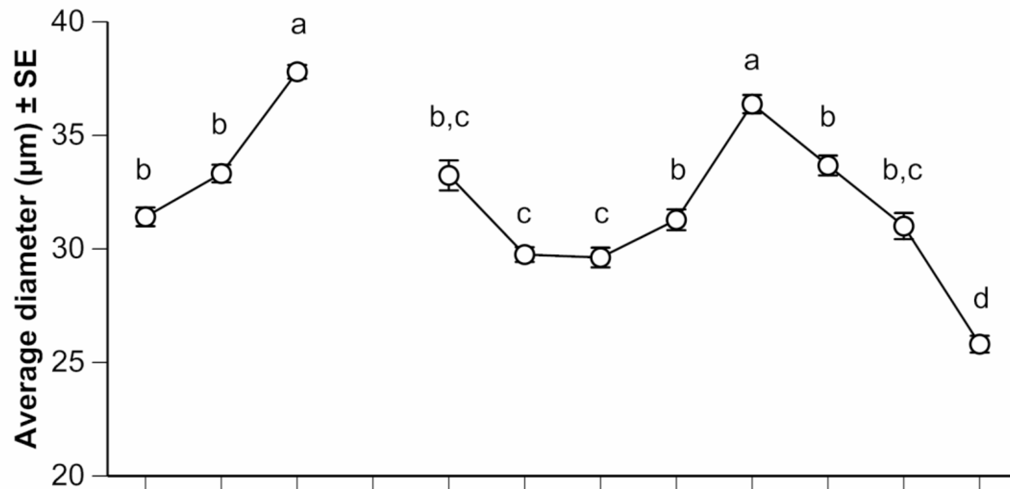



**Figure 2.3. Monthly oocyte size-frequency distributions for *Paphies ventricosa* from Oreti Beach, Southland, between January 2011 and December 2011. *n* = number of females sampled/number of oocytes measured on each sampling date.**



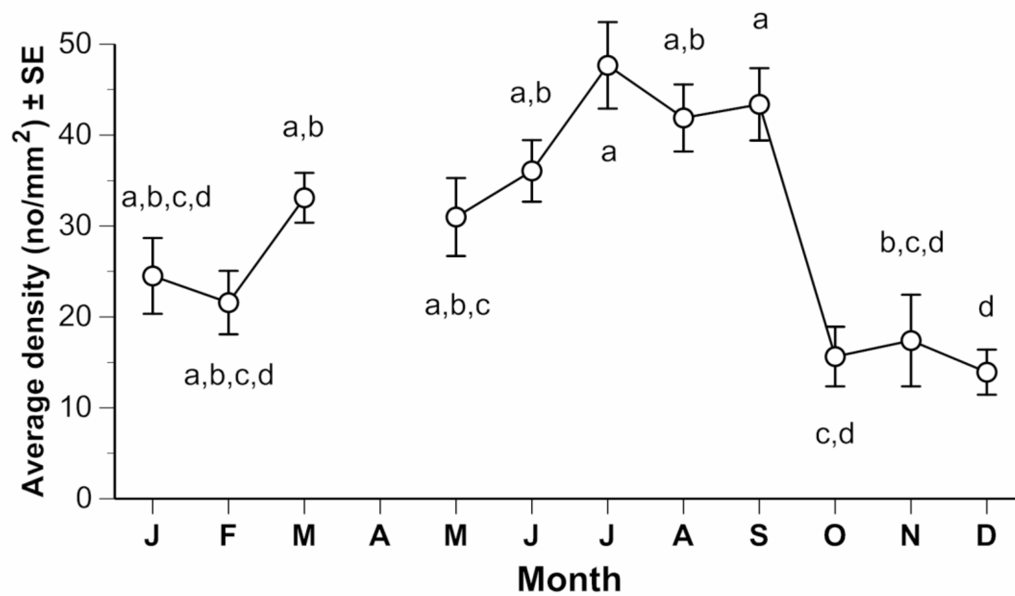




**Figure 2.4. (A) Average oocyte/ova size (± SE) and (B) average oocyte density (± SE) at monthly intervals for *Paphies ventricosa* from Oreti Beach, Southland, between January 2011 and December 2011. The sample size for each month is given in Figure 2.9. Significant differences in the oocyte measurements among months are indicated by lower case lettering.**

## Oocyte density

Oocyte density over 2011 ranged from 13.9 oocytes $mm^{-2}$ in December to 47.7 oocytes $mm^{-2}$ in July (Figure 2.10B). Densities were significantly different among months ($F_{(10, 92)} = 8.605$, $p < 0.001$), with Tukey's *post hoc* comparisons indicating that densities were significantly higher in July, August, and September than in the following three months. Oocyte densities at the four sites measured in 2012 (Figure 2.11), varied significantly among sites (ln($x$) transformed; $F_{(3, 135)} = 7.294$, $p = 0.002$), although the effect of site depended on the month of collection (Site × Month; $F_{(9, 135)} = 5.239$, $p < 0.001$). Significant differences among sites were evident at all the months: March ($F_{(3, 33)} = 5.422$, $p < 0.001$); June ($F_{(3, 32)} = 3.166$, $p = 0.013$); September ($F_{(3, 32)} = 5.542$, $p = 0.004$); and December ($F_{(3, 35)} = 5.895$, $p = 0.003$). With the exception of March, bivalves at Site B generally had significantly lower oocyte densities (Figure 2.11), whilst those at Sites A and C had significantly higher oocyte densities in March and December, respectively.

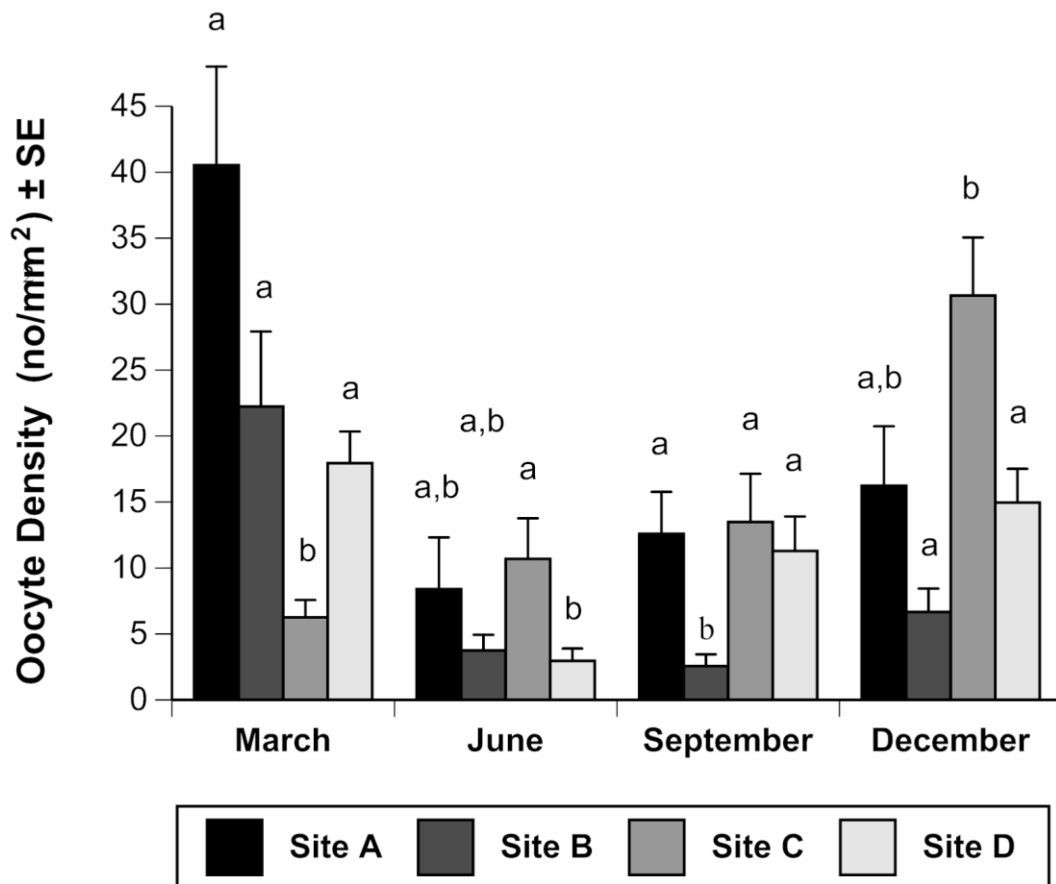



**Figure 2.5. Average oocyte density (± SE) for *Paphies ventricosa* at four sites along Oreti Beach, Southland, during four months in 2012. Significant differences in the oocyte density among sites within each month are indicated by lower case lettering.**

## Physical environment

Average SSTs in the Oreti Beach region of the Foveaux Strait (MODIS satellite measurements averaged over a 54 km × 54 km area; Figure 2.4) for 2011 ranged from 15.4 ºC in February to a minimum winter temperature of 9.6 ºC in July (Figure 2.12A). *In situ* measurements of temperature in the surf zone immediately next to the *P. ventricosa* beds were similar in the spring months, but ≈ 2.3 ºC cooler and 2.1 ºC warmer than remote measurements for the winter and spring months, respectively (Figure 2.12A). Chl-*a* concentrations for the same region of the Foveaux Strait ranged from 1.14 mg $m^{-3}$ in June to a maximum of 5.13 mg $m^{-3}$ in October, representing the peak spring bloom for 2011 (Figure 2.12B). Day length ranged from 15 h 46 min in December to 8 h 36 min in June (Figure 2.12C). The decrease in CI and spawning of *P. ventricosa* during 2011 was associated with periods of changing temperature, namely an ≈ 3 ºC warming from September to December, and an ≈ 4 ºC cooling from March to May, whilst the main spawning event in spring commenced at the time of the September chl-*a* concentration peak.

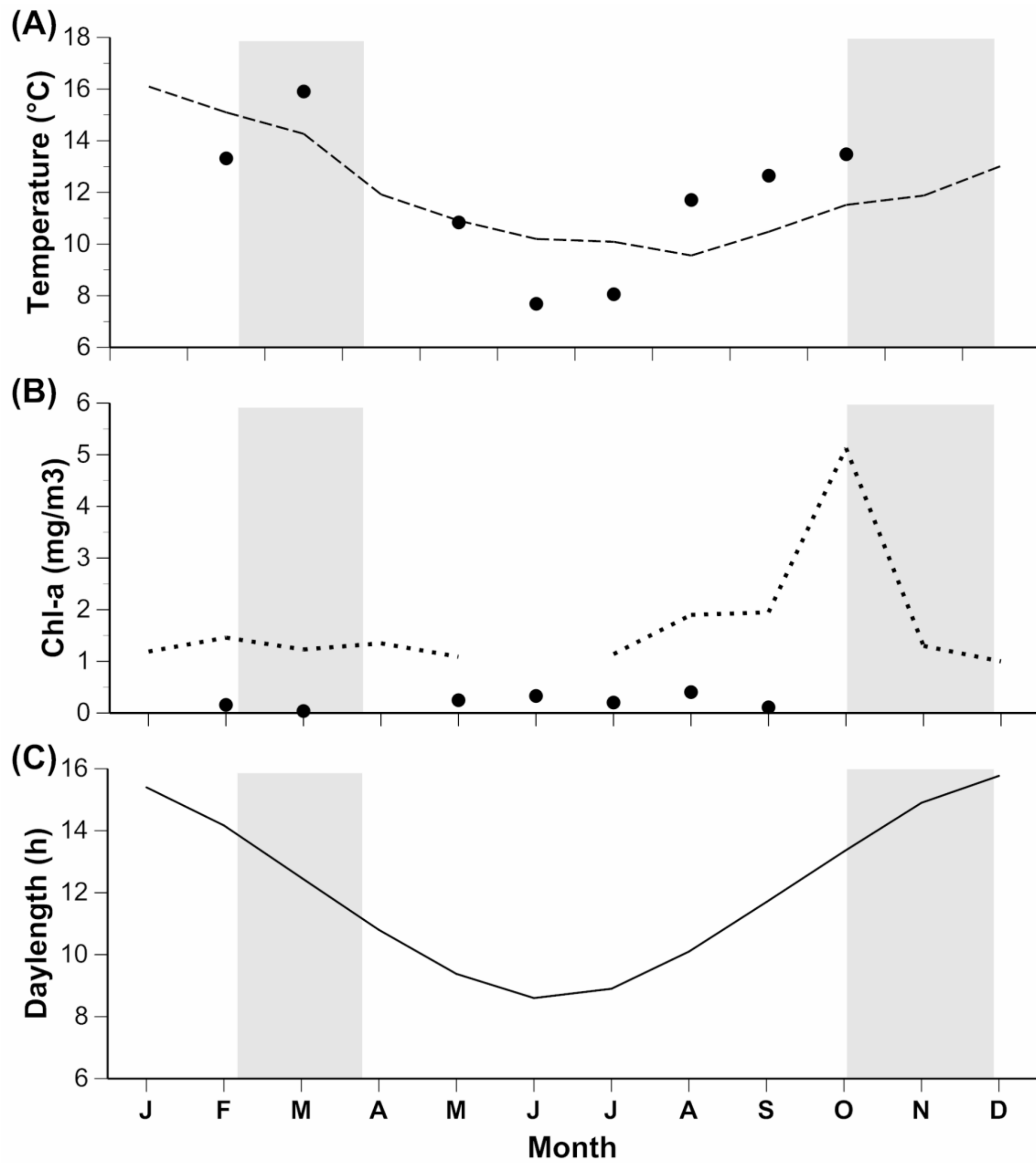



**Figure 2.6. SST (A), chlorophyll-*a* concentration (B) and day length (C) for Oreti Beach during 2011. SST (- - - -) and chlorophyll-*a* concentration (····) are graphed for both Aqua MODIS satellite estimates and *in situ* measurements (●). The estimated *P. ventricosa* spawning periods for 2011 are indicated by shaded vertical bars.**

## Discussion

### Gametogenic cycle

Annual and spatial reproductive biology of *P. ventricosa* was examined over two years along Oreti Beach, a location representing the southernmost known extent of the species range. The only published account of the reproductive cycle in *P. ventricosa* by Redfearn (1974) described an annual reproductive cycle in individuals from the species' northernmost extent, which lacked a resting phase with continuous spawning punctuated by 2 – 3 main spawning periods occurring over the warmer summer months (December to February). In this study, *P. ventricosa* at its southern location also had an annual reproductive cycle that lacked a resting phase and two main spawning periods, the first in spring and the second in late summer. Histological examination of gametogenesis indicated that whilst ripe females were found throughout the year, the proportions were greatest in September to November (up to 70%) and February (40%), with intervening months dominated by EA and LA ovaries (73%). The male cycle was more pronounced, with ripe testes prevalent (83 – 100%) in October/November and February/March. Redfearn (1974) described a similar gametogenic pattern in *P. ventricosa* ovaries, with two to three periods of predominantly mature stages over the spring to late-summer period, separated by actively growing ovaries with no resting period during the annual cycle. In contrast to the present study, Redfearn (1974) observed spawned individuals throughout the year, whilst the Oreti Beach population lacked spawned individuals in the winter and mid-summer months.

An extended spawning period that involves an intervening resting or active period is often observed in bivalve species (Sastry, 1979), including other Mesodesmatids

such as *M. Mactroides* (Herrmann, *et al.*, 2009). Within the wider *Paphies* genus, gametogenesis also follows an annual cycle, with spawning occurring over the summer periods for *P. donacina* (Marsden, 1999), *P. australis* (Hooker & Creese, 1995) and *P. subtriangulata* (Grant & Creese, 1995). As for *P. ventricosa*, the latter two species have no resting phase during gametogenesis, and for *P. subtriangulata* spawning occurred in spring and late autumn with a mid-summer active period. *P. donacina* may also undergo more than one gametogenic cycle annually. However, in contrast to other *Paphies* species, it has an inactive resting phase during winter.

Monthly changes in the oocyte size frequency distributions and associated average oocyte size and density further support the suggestion that female *P. ventricosa* undergo a major spawning in spring, followed by active oocyte development leading to a second spawning in late-summer. The unimodal oocyte distributions in *P. ventricosa* are commonly observed in bivalves (Herrmann *et al.*, 2009), with the broad oocyte range observed throughout the year reflecting gametogenic processes where active and mature females are present throughout the year. The distinct loss of larger oocytes and reduction in oocyte density in the spring and autumn months reflected two spawning episodes.

## Condition index

A CI has been applied to a range of bivalve species to describe reproductive cycles, including mesodesmatids where the gonad and visceral tissue are closely associated (Herrmann *et al.*, 2009). For *P. ventricosa*, the CI varied seasonally, with an ≈ 50% decrease in the index in spring, consistent with the major spawning event seen in the Oreti Beach population. The decrease in CI in late-summer when spawning occurred was less clear and not significant. Redfearn (1974) calculated the CI (determined by volume) in *P. ventricosa* over one year, and correlated decreases in CI with spawning

in spring and late-summer. The application of the index to other mesodesmatids has been less informative. For example, both Marsden (1999) and Herrmann *et al.* (2009) showed the CI cycled annually in *P. donacina* and *M. mactroides*, respectively, but neither study could consistently correlate changes in the CI with gametogenic changes associated with spawning events. Marsden (1999) noted that the poor correlation would result from simultaneous changes in somatic tissue, shell, and gonad tissue over a reproductive cycle.

### Spatial and temporal variation

Whilst the annual reproductive cycle of *P. ventricosa* showed a clear annual pattern, there was a degree of spatial variation among the four sites separated by 15 km. The variation was mostly owing to variation during the months associated with spawning, March and December, with individuals at the southern end of the beach (Site A) more advanced gametogenically, having the higher oocyte densities earlier, and a lower CI in March and June following spawning. This suggests that whilst annual cycles are broadly similar among sites, there may be a degree of spawning asynchrony along Oreti Beach. Spatial differences in bivalve reproduction at relatively small scales can be marked (Gosling, 2004). *Pecten maximus* populations around coastal Isle of Man, for example, showed significant variation in gonad condition, fecundity, and spawning onset at small horizontal scales of < 5 km (Hold *et al.*, 2013), whilst intertidal bivalves can vary reproductively across tidal heights within a population (Harvey & Vincent, 1989; Azouzi *et al.*, 2002).

Latitudinal differences in reproduction can also be pronounced. Examples are found in a range of bivalve species (Sastry, 1979), including Mesodesmatidae, in which Uribe *et al.* (2012) noted significant reproductive asynchrony in populations of *M. donacium* along the 4000 km long Humboldt Current upwelling ecosystem. *P.*

*ventricosa* spans 12º of latitude (1350 km), and whilst the cycles are broadly similar, the reproductive cycles of *P. ventricosa* appear more pronounced in the southern population compared with those at its northern extent (Redfearn, 1974). This is best demonstrated by the presence of spawned individuals all year round in the north, whilst spawned individuals were absent from winter and late-summer months in the southern *P. ventricosa* population. Other *Paphies* species show a similar pattern of spatial variation, with differences in spawning period in *P. subtriangulata* (Grant & Creese, 1995) and *P. australis* (Hooker & Creese, 1995) observed among locations.

Spatial differences in gametogenesis in bivalves are the result of the interaction of environmental factors, which includes primarily sea temperature and food supply, as well as photoperiod and lunar cycles (Sastry, 1979), whilst spawning episodes have been mainly associated with temperature changes, salinity, and light (Sastry, 1979; Gosling, 2004). Temperature is undoubtedly influential in mesodesmatid species inhabiting seasonal environments. Herrmann *et al.* (2009) correlated the reproductive cycle of *M. mactroides* with seasonal changes in sea temperatures which ranges from 11 to 23 ºC annually: Manzi *et al.* (1985) reported temperature influenced the gametogenic cycle in *Mercenaria mercenaria*; and Peredo *et al.* (1987) discussed sea temperature variation and annual reproduction in *M. donacium*. In this study, *P. ventricosa* spawning was associated with periods of changing sea temperature, namely an ≈ 3 ºC warming from September to December, and a 4 ºC cooling from March to May. Redfearn (1974) discussed reproductive events associated with temperature changes, noting spawning probably occurred in December/January following a period of gonad maturation over July to November when sea temperatures increased from 14 to 20º C, with a second spawning following the 22 ºC summer temperature maxima.

Temperature variation may also play a role in the latitudinal reproduction of *P. ventricosa* although it is interesting that the annual variation in temperature at both locations (8º C) is similar, despite the southern population having a shorter and more defined spawning period. Latitudinal variation in the reproductive cycles of New Zealand bivalves has been related to sea temperature. Gribben *et al.* (2004) concluded that the duration of gametogenesis and spawning period in two populations of the New Zealand geoduck, *P. zelandica,* separated by ≈ 5º of latitude was related to seasonal changes in sea temperature, with spring spawning occurring when temperature reached 15 ºC in both populations. For *Paphies* species, cold temperatures have been shown to delay gametogenesis in *P. donacina* (Marsden, 1999), and Grant and Creese (1995) suggested that the minimum temperature threshold for spawning in *P. subtriangulata* was 15 ºC. It is conceivable, therefore, that the colder temperatures in the southern population during June to September (≈ 10 ºC) suppress spawning in the species during the winter period. Such cold temperatures are not experienced by the northern populations, with temperatures remaining above 14 ºC throughout the year, during which spawning is continuous.

It is also possible that other environmental factors influence reproductive periodicity in *P. ventricosa*. Indeed, for *M. donacium*, latitudinal variation in reproductive cycles has been attributed to a range of factors in addition to temperature, including localised upwelling and changes in salinity and runoff (Uribe *et al.*, 2012). Few studies have considered the effects of day length on reproductive cycles in bivalves, as it is often difficult to separate from seasonal temperature changes (Gosling, 2004), but it is worth noting that seasonal changes in day length are more pronounced at the southern location (from 8 h 34 min to 15 h 50 min, seasonally) compared with the north (from 9 h 34 min to 14 h 36 min, seasonally). Furthermore, seasonal changes

in chl-*a* concentrations which tend to me more variable at higher latitudes, have been linked to seasonal reproduction in bivalves (Sastry, 1979; Gosling, 2004) and specifically in mesodesmatids (Peredo *et al.*, 1987; Herrmann *et al.*, 2009). Seasonal variations in chl-*a* concentration are greater in southern New Zealand (Murphy *et al.*, 2001) which ranged from 0.2 t 0.6 mg $m^{-3}$ of chl-*a* in northern offshore regions compared with 0.3 to > 1.0 mg $m^{-3}$ of chl-*a* in southern waters offshore of Oreti Beach. A chl-*a* concentration spike (> 5 mg $m^{-3}$) was observed during October, coinciding with the main spawning of *P. ventricosa*. It is possible, therefore that the more pronounced reproductive cycles in the southern population reflect greater seasonality when a range of environmental variables are considered.

For other *Paphies* species, Marsden (1999) concluded that *P. donacina* reproduction was promoted by warmer summer temperatures, with high food concentrations potentially extending gonad development. Grant and Creese (1995) suggested that warmer temperatures promoted spawning both seasonally and tidally, and Hooker and Creese (1995) noted spawning during spring coincided with short-term temperature fluctuations. The small-scale spatial variations in spawning that were observed along Oreti Beach are unlikely due to temperature variation, but may be associated with gradients in food supply. Densities of *P. ventricosa* vary significantly along Oreti Beach (Beentjes, 2010) and are greatest at the Sites A and B in the current study. *P. ventricosa* distribution is thought to be controlled by phytoplankton abundances (Redfearn, 1974), and the high densities at Sites A and B have been attributed to enhanced food supply from the adjacent New River Estuary (Beentjes, 2010; Figure 2.2). It is likely that the animals at these sites are nutritionally advantaged and therefore complete gametogenesis and spawning earlier. This suggestion would be consistent with observations in bivalves that intra-specific

variation in reproductive cycles can be related to small-scale food supply patterns (Hold *et al.*, 2013). Salinity fluctuations can also induce spawning in bivalves (Sastry, 1979), and it is equally plausible that individuals at Sites A and B experience greater fluctuations in salinity, associated with freshwater output from the estuary that could induce spawning episodes.

## Conclusions

*P. ventricosa* has experienced dramatic declines in population sizes over the past century (Greenway, 1972; Beentjes *et al.*, 2006), and the conservation and management of the species require an understanding of the reproductive biology of the species across a range of spatial scales. This study is the first to show that whilst reproductive cycles are broadly similar in the species across its latitudinal extent, southern populations have a more defined spawning period that may be associated with greater environmental seasonality. Smaller-scale variation in reproduction was also evident within the Oreti Beach population and may be an important consideration in identifying areas for conserving source populations (Gaines *et al.*, 2010).

# Chapter 3

# Embryonic and larval development of toheroa (*Paphies ventricosa*) at a range of temperatures

This chapter is adapted from:

Gadomski, K., Moller, H., Beentjes, M., & Lamare, M. (2015) Embryonic and larval development of the New Zealand bivalve *Paphies ventricosa* Gray, 1843 (Veneroida: Mesodesmatidae) at a range of temperatures. *Journal of Molluscan Studies*, **81**: 356-364.

## Introduction

This study aims to increase the understanding of embryonic and larval development in *Paphies ventricosa* and the role of temperature in the rate of development. While there are brief descriptions of various preveliger developmental stages (Rapson, 1952; Redfearn, 1974, 1982), a detailed description of early embryonic and larval development of and morphology of *P. ventricosa* is lacking. Redfearn (1982) used scanning electron microscopy (SEM) to describe larval shell development in the veliger, noting that *P. ventricosa* reaches the straight-hinged veliger stage after 24 – 48 h and completes development to metamorphosis in 22 d at 25° C. Importantly, the development processes described for *P. ventricosa* by Redfearn (1982) were at temperatures associated with the northern limit of the species (i.e. 18 – 25° C), with no description available for larvae at temperatures associated with the species' southern range (i.e. 12 – 16° C). The effects of temperature on bivalve larval development have been established in a number of species, with rates typically increasing with temperature (Cataldo *et al.*, 2005; Cragg, 2006; Sánchez-Lazo &

Martínez-Píta, 2012); it is therefore likely that development processes are slower in the colder southern regions.

Embryonic and larval development in bivalves have been well described (Sastry, 1979; Kasyanov *et al.*, 1998), with light microscopy techniques contributing to a clear understanding of patterns of fertilisation, spiral cleavage (Lambert, 2010) and the functional morphology of the blastula, gastrula, and veliger stages (e.g. in *Ostrea edulis*, Waller, 1981). This technique has also allowed the timing of these key developmental milestones to be determined for ecologically and economically important species (review by Ackerman *et al.*, 1994). The use of scanning electron microscopy (SEM) and transmission electron microscopy techniques to examine early developmental processes in bivalves is less common, but has been used to view spermatozoa (Mouëza & Frenkiel, 1995; Mouëza *et al.*, 1999), embryonic and larval morphology (Zardus & Morse, 1998; da Costa *et al.*, 2008), ciliation patterns (Eyster & Morse, 1984; Chaparro *et al.*, 1999; Mouëza *et al.*, 1999; Mouëza *et al.*, 2006), larval shell differentiation (Eyster & Morse, 1994; Mouëza *et al.*, 2006; Aranda-Burgos *et al.*, 2014), internal anatomy (Zardus & Morse, 1998), and larval shell development and hinge morphology (Redfearn, 1982; da Costa *et al.*, 2008; Arellano & Young, 2009).

Here a detailed description of the development of *P. ventricosa* is made using both light microscopy and SEM techniques. To gain an understanding of the role of temperature in embryonic and larval development across the natural thermal range (i.e. 12 – 25 ºC), we examine the effects of three environmentally relevant temperatures (12, 16, and 20 ºC) on larval growth. This is done by quantifying, at each of the three temperatures: (1) the timing of key development processes using developmental schedules; (2) larval size over the free-swimming period; and (3)

differences in larval shell morphology (to understand if temperature not only changes the pace of development, but also key developmental processes such as shell formation). The measurements from the present study are compared with those previously made for *P. ventricosa* larvae at 25 ºC by Redfearn (1982), and the role of temperature on larval development and its implications for larval supply is discussed in the species across its geographic range.

## Materials and methods

### Animal collection and conditioning

In October 2012, 20 adult *Paphies ventricosa* (100 – 110 mm total shell length [TL]) were collected from Oreti Beach (46.479º S, 168.252º E), Southland, under the appropriate customary permit. Animals were extracted by hand during low tide and transported to PML, Dunedin, in seawater-filled buckets. In the laboratory, animals were placed at densities of 10 individuals per tank into 4 circular flow-through 116 l tanks (43.5 cm × 58.5 cm, flow 3 l $min^{-1}$) filled to a 35 cm depth with Oreti Beach sand. Oxygen concentrations were maintained through continuous aeration via airstones. Animals were conditioned for spawning by daily feeding with a cultured mixed algae diet (*Tetraselmis chui, Isochrysis galbana, Pavlova lutheri, Chaetoceros muelleri,* and *Skeletonema marinoi*). At feeding, water flows were turned off, tanks were cleaned by siphoning off ≈ 5 l of water, and 5 l of mixed algae were then added to each tank to give a final algal concentration of 8 – 10 × $10^5$cells $ml^{-1}$ in each tank. The relative proportions of each algal species depended on the availability of cultures. During feeding, the tanks' aeration was maintained in order to maintain mixing and water quality. Water flows were restored once the animals had cleared

the water, or after 4 h, whichever came first. Animals were conditioned for ≈ 7 weeks.

### Spawning

For spawning, the animals were removed from their tanks and packed into 15 cm deep flow-through trays filled with 25 l of flowing seawater and fitted with airstones for additional aeration. Spawning was induced by an intramuscular crystalline serotonin (5-hydroxytryptamine [5-HT]) injection, similar to the methods described by Gibbons & Castagna (1984) and Hirai *et al.*, (1988). After turning off seawater flows, animals were given an initial 0.4 ml injection of 2 mM solution of 5-HT (Sigma-Aldrich) in 1 µm filtered seawater (FSW) into the AAM and placed back into the aerated spawning trays. Injected animals were observed for the start of spawning and then given an additional 0.2 ml injection of 2 mM 5-HT into the AAM every 30 minutes, for up to 90 min post initial injection (up to a total of 1.0 ml injection volume) or until a good spawning was observed. Spawning was allowed to occur within the spawning trays and settled eggs were collected. Sperm were collected from spawning males immediately prior to sperm counting and fertilisation.

### Larval rearing

Eggs were pooled from five females and sperm allocated from three males. For fertilisation, the pooled eggs were mixed in 1.0 l of 0.22 µm FSW and sperm added to obtain a final concentration of $10^6$ sperm $ml^{-1}$. Fertilisation was confirmed by the breakdown of the germinal vesicle and the appearance of the fertilisation envelope and polar body. Following fertilisation, the top 70% of the beaker volume was poured off into a 5 l beaker, leaving behind any residual debris. Fertilised eggs were

further diluted with 0.22 µm FSW to a density of 15 eggs $ml^{-1}$ and split into nine 500 ml lidded jars, each filled to a volume of 450 ml.

The cultures were then reared simultaneously in the same temperature-controlled (CT) room at one of three temperature treatments, 12 ºC (± 0.5 ºC, temperature controlled water bath), 16 ºC (± 0.5 ºC, CT room ambient temperature), and 20 ºC (± 0.5 ºC, temperature-controlled water bath). Each treatment was replicated three times. Water changes and feeding were carried out every third day. Larvae were filtered and rinsed using a larval filter stack fitted with a 50 µm mesh filter. Cleaned larvae were rinsed off the filter into a jar half filled with fresh FSW and topped to a volume of 450 ml. Following the water change, larvae were fed cultured *Tetraselmis chui* at a concentration of 10,000 cells $ml^{-1}$. The algal concentration was calculated immediately before each feeding using a haemocytometer.

## Larval photography and larval morphometrics

Early development was observed frequently over the first 48 h post-fertilisation in 1 ml subsamples for the culture, then every third day. Developmental stages were photographed alive, using an Olympus BX51 compound microscope fitted with an Olympus Colorview III™ camera, controlled by AnalySIS LS Research software (Olympus Corporation). Larval shell morphometrics measurements of total shell length (TL), total shell height (TH), and shell hinge-line length (HL) were taken (Figure 3.1I) as described by Redfearn (1982), using the software ImageJ (NIH, USA). For statistical comparison with measurements made by Redfearn (1982), data contained in the published figures were digitised using the software DigitizeIt (Braunschweig, Germany).

## Scanning electron microscopy

Samples were fixed in 10% neutral buffered formalin in seawater and processed in a Bal-Tec CPD-030 critical-point drier (Bal-Tec AG), mounted on stubs and sputter coated using an Emitech K575X Peltier-cooled high resolution sputter coater (EM Technologies). Mounted samples were photographed using a JEOL 6700F FE-SEM field-emission SEM, with the lower secondary detector set to high magnification mode. All sample processing and SEM work was undertaken at the Otago Centre for Electron Microscopy (OCEM). Images were post-processed using GIMP 2.8 software (GNOME Foundation, USA) to improve contrast and black out the background.

## Statistical analyses

Significant differences in larval shell measurements (TL, TH, HL) over time and among temperature treatments were tested in a univariate repeated-measures ANOVA, using data on the days where measurements were available for all replicates (days 6, 9, 15, 18, 24, and 27). Tests for sphericity (homogeneity of variances among groups) were made using Mauchly's sphericity test ($W$) (Mauchly, 1940) and departures from the assumption of sphericity were corrected using the Greenhouse-Geisser correction ($\epsilon$). A significant interaction between temperature and day necessitated a statistical examination of the effect of temperature treatment on separate days by one-way ANOVA of the transformed data. The effect of temperature on the relationship between total larval length and larval height was examined using an analysis of covariance (ANCOVA) on $\ln(x)$ transformed measurements. All statistical analyses were undertaken using the software JMP 10.0 (SAS Institute, USA).

# Results

## Embryonic and larval development

Spawned eggs had an average diameter of 67.1 µm (range 60.1 – 75.1 µm) and before fertilisation were often pear-shaped. A thin vitelline layer of 2.0 – 2.5 µm was present, with no jelly coat visible (Figure 3.1A). The germinal vesicle was prominent, measuring 40 µm in diameter. Following fertilisation, a polar body formed within 10 – 15 min, with the first cleavage occurring within 1 h of fertilisation (Figures 3.1C, 3.2A). The cleavage plane along the animal-vegetal axis was uneven and resulted in the characteristic two-cell embryo with the larger B blastomere and the smaller A blastomere, with the polar body located at the animal end of the cleavage plane (Figures 3.1C, 3.2A). Subsequent cell divisions followed spiral cleavage as described for other bivalve embryos, with the second cleavage plane perpendicular to the first, resulting in four blastomeres (Figure 3.1D) and the third cleavage to the eight-cell stage resulting in four smaller daughter blastomeres visible when viewed from the vegetal pole (Figure 3.1E). Dexiotropic and laetropic spiral divisions gave rise to a 75 µm ciliate blastula (Figures 3.1F, 3.2B) by 15 h at 20º C. The ciliated blastula retained the polar lobe and had a sparse, uneven cover of cilia (length 18 – 20 µm) running almost equatorially around the embryo body, marking the initial development of the prototroch (Figure 3.2B). The formation of a blastopore measuring 10.5 µm across was observed in slightly larger blastulae (80 – 85 µm length), located ventral-posteriorly to the developing prototroch (Figure 3.2C). At the same time, the shell field invagination had formed dorso-posteriorly, measuring 30 µm in length with a depth of 7 – 8 µm. Early trochophores (Figure 3.2E) measuring 83 µm in length have a well-developed shell field 45 µm wide, containing a two-lobed shell pellicle of wrinkled periostracum, each lobe separated

along the axial line. Cilia surrounded the shell field, with the cilia of both prototroch and telotroch developed. During the expansion of the shell field, the blastopore was displaced ventro-posteriorly. Early trochophore larvae (Figure 3.2D) measuring 85 µm in length and had a well-developed prototroch measuring 95 – 100 µm (cilia length 23 µm), postoral cilia (cilia length 16 µm) and a telotroch (cilia length 21 µm). At the same time, the developing shell field was apparent, lying dorsal to the blastopore (Figures 3.2E). Trochophore larvae observed in samples at 15 h post-fertilisation were 102 µm in length (Figures 3.1G, 3.2F). At this stage, larvae had a well-developed prototroch with cilia 26 µm in length (Figure 3.2F). The prototroch divided the larval body into the anterior pretrochal region and the posterior post-trochal region. The apical plate of the pre-trochal region contained the apical sense organ (not clearly visible in this preparation), which gave rise to sensory apical cilia extending to 67µm (Figure 3.1G). At this stage the post-trochal region was almost covered by the dorsally-expanding shell rudiment, which tended to flatten the larval body laterally. During this expansion, the mouth was retained in a ventral position. Early veliger larvae at 22 h had a fully developed velum 110 – 120 µm in length (Figure 3.2G). Well-developed postoral cilia (21 µm long) and postanal tuft cilia (24 µm long) were present. The rudimentary shell had the characteristic D-larval form with a straight, dorsal hinge line 65 µm in length (Figures 3.1H, 3.2H). The morphology of the larval-shell hinge (Figures 3.1I, J) was as described by Redfearn (1982), with the straight-hinged D-larva having a hinge consisting of up to 25 – 30 regularly spaced teeth (Figure 3.2I), while at the umbo stage the hinge consisted of 3 evenly spaced teeth (2.2 µm × 3.5 µm) along a hinge line of 68 µm (Figure 3.2I, inset).

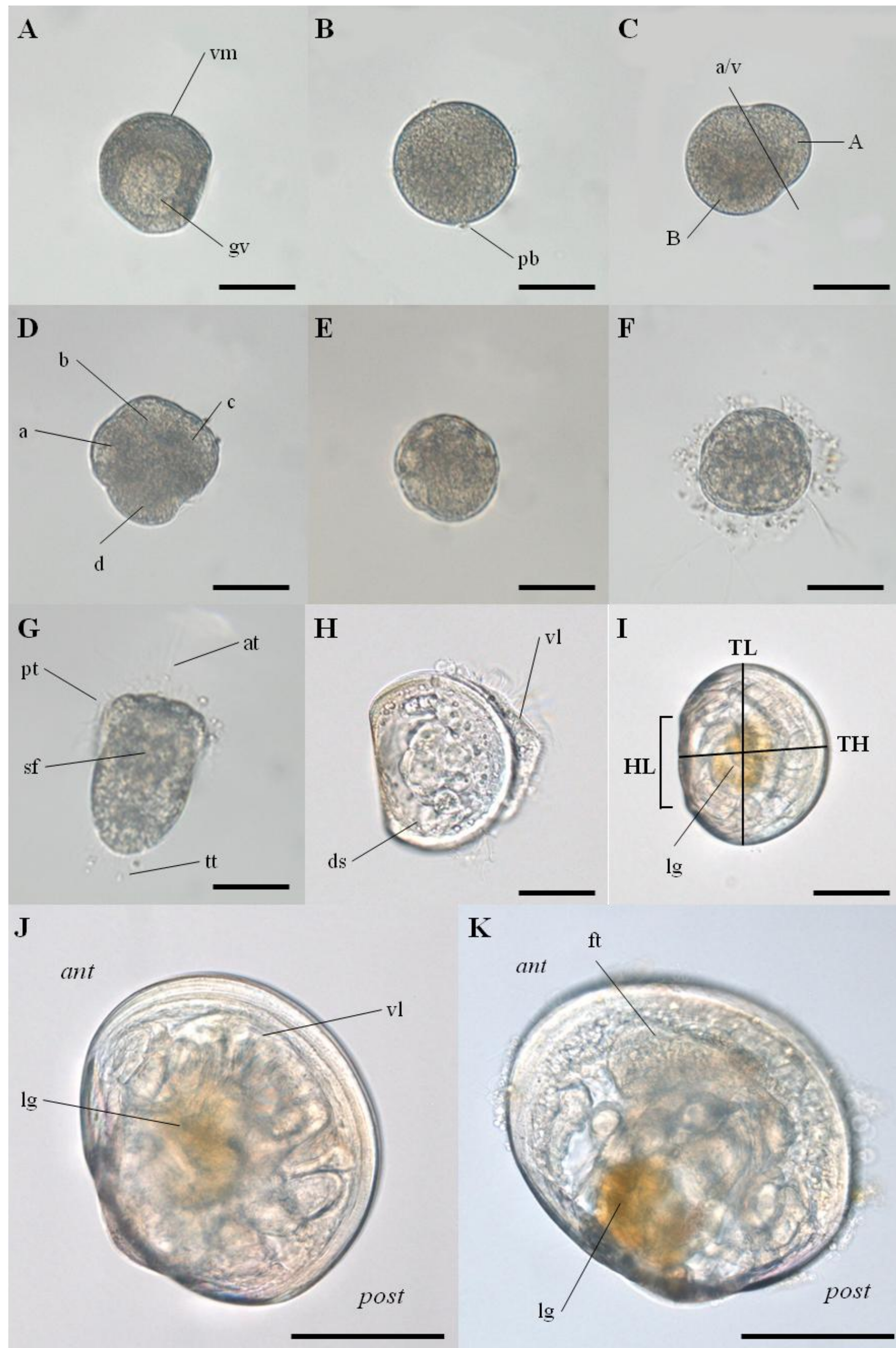



**Figure 3.1**. **Light micrographs of eggs and embryonic and larval development stages of *Paphies ventricosa.* All were reared at 16 ºC except for (J) and (K), which were reared at 20 ºC. (A) Unfertilised egg, with vitelline membrane (vm) and germinal vesicle (gv). (B) Fertilised egg at 15 min with polar body (pb). (C) Two-cell embryo at 30 min with larger B blastomere (B) and smaller A blastomere (A) dividing along animal-vegetal axis (*a/v*). (D) Four-cell embryo at 60 min with blastomeres *a* to *d* labelled. (E) Eight-cell embryo at 4 h viewed from vegetal pole. (F) Hatched blastula at 6 h. (G) Trochophore stage at 16 h, with well-developed prototroch (pt), telotroch (tt), and apical tuft (at). Region of ventral shell field (sf) is indicated. (H) Straight-hinged D-larva at 3 d with prodissoconch I (ds) and extended velum (vl). (I) Straight-hinged D-larvae at 15 d with larval gut (lg). Larval shell with shell length (TL), shell width (TH), and hinge line (HL) dimensions indicated. (J) Twenty-day umbo larva with larval gut (lg) and retracted velum (vl). Posterior (*post*) and anterior (*ant*) ends are indicated. (K) Thirty-day old pediveliger larva with foot retracted (ft). Scale bars (A-I) = 50 µm; (J, K) = 100 µm.**

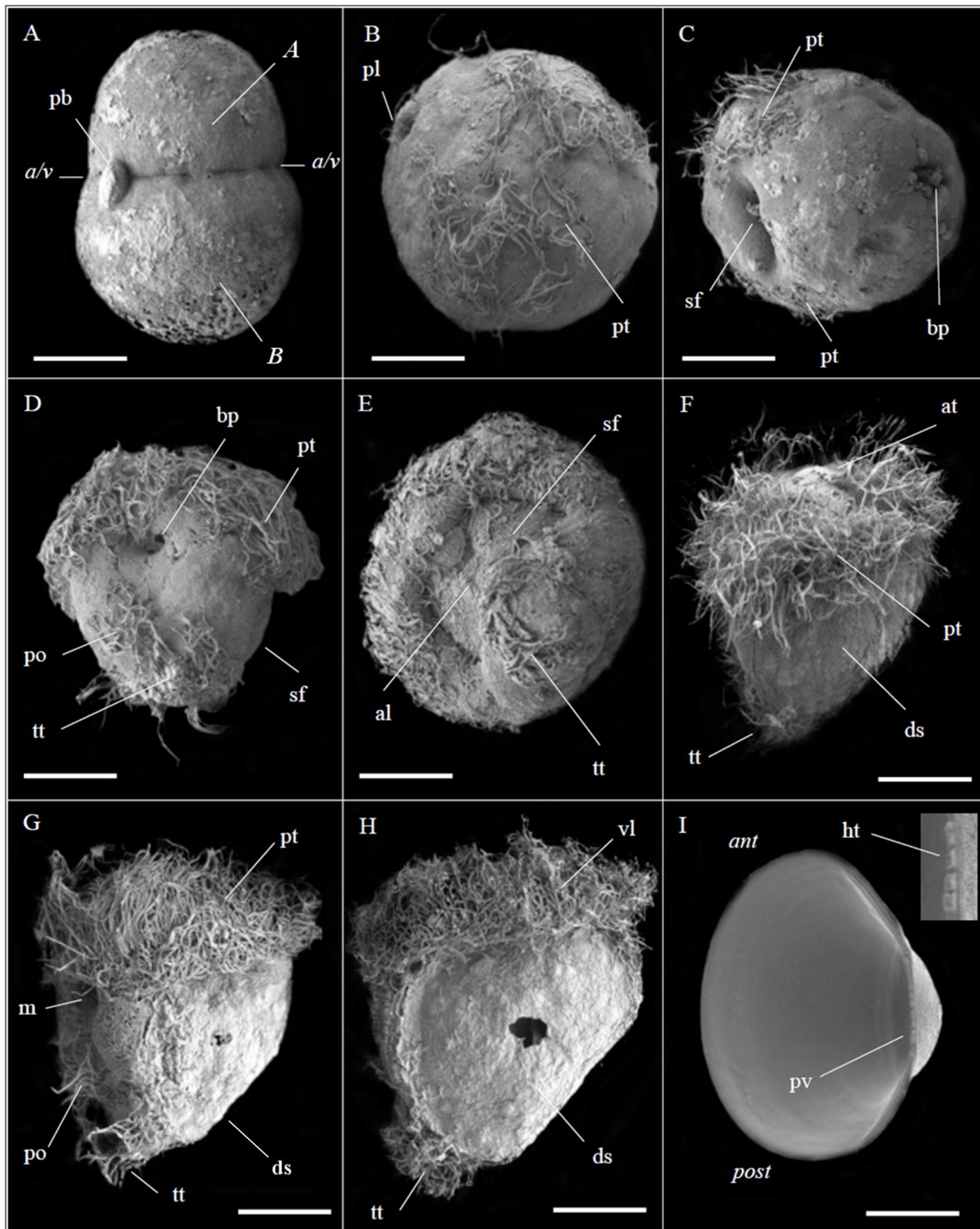



Figure 3.2. **Scanning electric micrographs of embryonic and larval developmental stages of *Paphies ventricosa*. All were reared at 16 ºC, except for (H) and (I), which were reared at 20 ºC. (A) Two-cell embryo at 1 h, with larger *B* and smaller *A*blastomere. Polar body (pb) is located on cleavage plane (animal-vegetal axis, *a/v*) at animal pole. (B) Blastula at 5 h with cilia of developing prototroch (pt) starting to cover equatorial region of embryo. Polar lobe (pl) still visible at vegetal pole. (C) Late gastrula at 8 h with blastopore (bp) located dorso-posteriorly to developing prototroch (pt), telotroch (tt), and shell field invagination (sf) ventro-posterior. (D) Early trochophore at 15 h; ventral view with well-developed prototroch (pt), telotroch (tt), and postoral (po) cilia. Shell field (sf) just visible on dorsal surface. (E) Early trochophore at 15 h, dorsal view with shell field (sf) consisting of two-lobed shell pellicle of non-calcified wrinkled periostracum, separated along axial line (al). (F) Lateral view of late trochophore with well-developed prototroch (pt) and telotroch (tt). Apical tuft (at) discernable. The expanding prodissoconch (ds) has laterally flattened the post-trochal region of larva. (G) Late trochophore at 22 h. (H) Early straight-hinged D-larva viewed ventro-laterally. (G) and (H) show well-developed velum (vl), mouth (m), postoral (po) cilia and telotroch (tt). (I) Late umbo larval shell at 3 d with provinculum (pv) and hinge with peg-like teeth (ht) (insert). Posterior (*post*) and anterior (*ant*) ends indicated. Scale bars (A, B, E) = 25µm; (C, D, F-G) = 30 µm; (I) = 50 µm.**

## Temperature and larval development

Progression through the early embryonic states was temperature dependant (Table 3.1), with blastulae observed at 15 h post-fertilisation at 20 ºC, while by the same time embryos had reached the trochophore stage at 12 and 16 ºC. D-hinge veliger larvae were first observed at 22 h post-fertilisation in the 12 and 16 ºC treatments, and at 37 h at 20 ºC. Later larvae developed more quickly in the warmer treatments, with umbo larvae observed at 21, 15, and 12 d post-fertilisation at 12, 16, and 20 ºC, respectively. Larvae reached the pediveliger stage (recognised by a protruding larval foot) after 31 d at 20 ºC, but pediveligers were not observed by 39 d in the two colder treatments.

**Table 3.1. Time to reach developmental stages for *Paphies ventricosa* larvae reared in the laboratory at three experimental temperatures. Schedule is based on the time when various larval stages were first observed during regular sampling. * Not observed in sample.**

| Stage | Time to Stage | | |
|---|---|---|---|
| | 12 ºC | 16 ºC | 20 ºC |
| Polar body formation | Not examined | 15 min | Not examined |
| Two-cell | 1 h | 1 h | 1 h |
| Four-cell | 2 h | 1 h | 1 h |
| Blastula | * | * | 15 h |
| Trochophore | 15 h | 15 h | * |
| D-hinge larvae | 22 h | 22 h | 37 h |
| Umbonate | 21 d | 15 d | 12 d |
| Pediveliger | Not reached by 39 d | Not reached by 33 d | 31 d |

Total larval TL increased from 65 μm at 2 d post-fertilisation to a maximum of 269 μm after 30 d (Figure 3.3A). Differences in larval size among temperature treatments were most apparent at day 15 when the larvae were 100 μm in length, a size at which larvae at 20 ºC progressed from straight-hinge D-larvae to umbo larvae. The maximum size of larvae reared at 12 ºC (170 μm) and 16 ºC (196 μm). A repeated-measures ANOVA of change in larval size undertaken on those days when measurements were available for all replicates (days 6, 9, 18, 21, 24, and 27) showed that, for TL, there was a significant effect of temperature ($p < 0.001$), although the effect of temperature was dependent on day (Temperature × Day, $p < 0.001$; Table 3.2A). A significant ($p < 0.05$) difference in size among temperatures was first apparent on 18 and, by day 27, larvae reared at 20 ºC were significantly larger. A similar pattern of changes in TH was observed among temperature treatments (Figure 3.3B), with those larvae reared at 20 ºC significantly larger than those at 12 and 16 ºC. Larval height increased significantly over time ($p < 0.001$; Table 3.2B), with the height significantly different among temperature treatments. The effect of temperature varied with time (Treatment × Day, $p < 0.001$) and reflected the divergence of height after day 9. Larval HL (Figure 3.3C) was significantly longer in larvae reared at 20 ºC compared with those reared at the colder temperatures ($p = 0.001$, Table 3.2C), but there was no significant difference between larvae at 12 and 16 ºC.

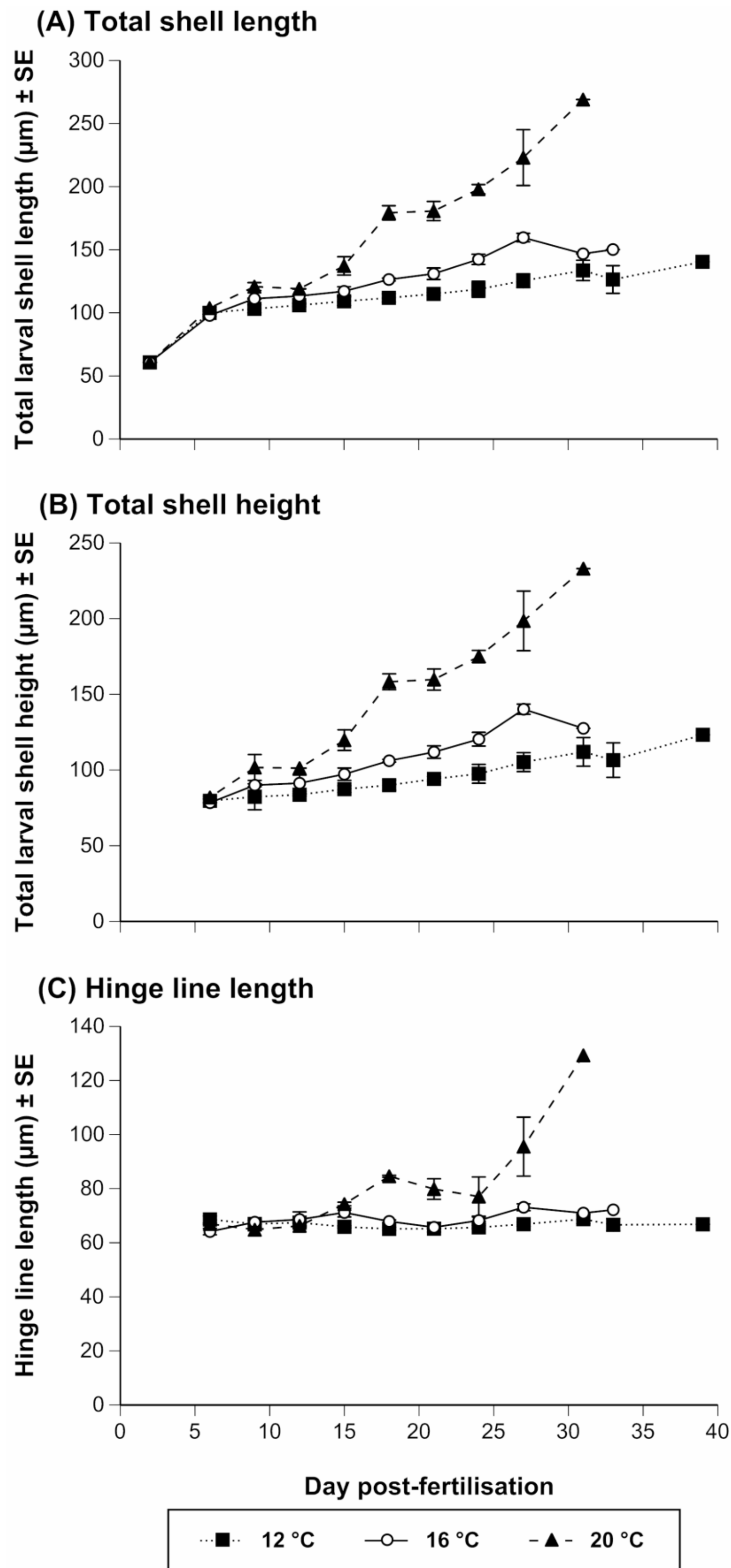



**Figure 3.3. Increase in average shell length (A), shell height (B), and hinge-line length (C) (± SE) for *Paphies ventricosa* larvae over a 39-d period at three temperatures (12, 16, and 20 ºC). *n* = average of three replicate jars, with variable number of larvae measured from each replicate at a given time point.**

**Table 3.2. Repeated-measures analysis of variance (ANOVA) of changes in larval dimensions measured on seven days (days 6, 9, 15, 18, 21, 24, and 27) for (A) total larval shell length, (B) total larval shell height, and (C) total larval hinge-length of *Paphies ventricosa* reared at three temperature treatments (12, 16, and 20 ºC). Data were ln(*x*) transformed prior to analysis and tests for sphericity were made using Mauchly's sphericity test.**

(A) Total larval shell length

| **Source** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| *Between- subjects* | | | |
| Temperature | 2, 6 | 54.47 | 0.0001 |
| *Within-subjects* | | | |
| Day | 5, 30 | 93.65 | < 0.0001 |
| Temperature * Day | 10, 30 | 11.31 | < 0.0001 |

Mauchly Criterion = 0.003, $df$ = 14, $p$ = 0.057

(B) Total larval shell height

| **Source** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| *Between-subjects* | | | |
| Temperature | 2, 6 | 55.89 | 0.0001 |
| *Within-subjects*[a] | | | |
| Day | 1.77, 10.63 | 101.89 | < 0.0001 |
| Temperature * Day | 3.54, 10.63 | 11.51 | 0.0009 |

Mauchly Criterion = 0.0003, $df$ = 14, $p$ = 0.003

[a]Degrees of freedom and $P$-value adjusted by Greenhouse-Geisser, $\epsilon$ = 0.354

(C) Larval shell hinge-length

| **Source** | ***Df*** | ***F-ratio*** | ***P*** |
|---|---|---|---|
| *Between-subjects* | | | |
| Temperature | 2, 6 | 76.66 | < 0.0001 |
| *Within-subjects*[a] | | | |
| Day | 2.95, 8.88 | 315.15 | 0.042 |
| Temperature * Day | 2.95, 8.85 | 74.04 | 0.065 |

Mauchly Criterion < 0.001, $df$ = 14, $P$ < 0.001

[a]Degrees of freedom and $p$-value adjusted by Greenhouse-Geisser, $\epsilon$ = 0.295

## Temperature and post-trochophore larval shell morphometrics

The allometric relationships between larval TL and larval TH for larvae from the three temperature treatments were generally similar (Figure 3.4), although ANCOVA indicated that the slopes of the three groups were significantly different (Temperature × TL, $F_{(2,\ 789)} = 8.712$, $p = 0.0002$; Table 3.3). A closer inspection of the data suggests that this result reflects the fact that larvae were only slightly wider for a given length as temperatures increased. This was indicated in the allometric equations of the relationship between TL ($x$) and TH ($y$), of which the slope coefficient increases with increasing temperature (i.e. 12 ºC, $y = 0.35x^{1.23}$; 16 ºC, $y = 0.44x^{1.16}$; 20 ºC, $y = 0.57x^{1.09}$). When the data are pooled for the three experimental temperatures and compared with the relationship described by Redfearn (1982) for larvae reared at 25 ºC (Figure 3.4), the slopes are also significantly different (Source × TL, $F_{(3,\ 1108)} = 12.97$, $p < 0.001$; Table 3.3). The results again reflect the greater height of larvae reared at 25 ºC, as indicated by the allometric relation $y = 0.92x^{0.99}$ for the measurements of Redfearn (1982).

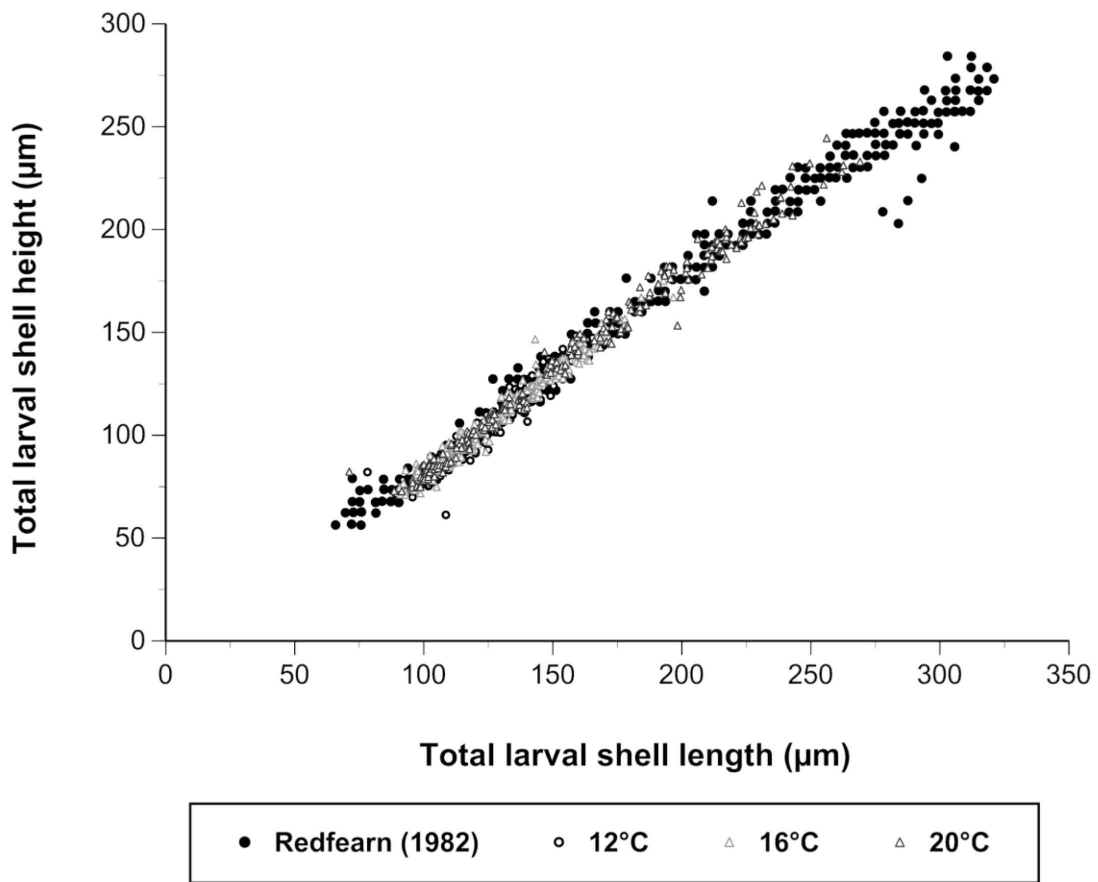



**Figure 3.4. Relationship between shell length and shell height for *Paphies ventricosa* larvae reared over a 39-d period at three experimental temperatures (12, 16, and 20 ºC) and at 25 ºC (Redfearn, 1982). Allometric relationships ($y = Ax^B$) are: 12 ºC, $y = 0.35x^{1.23}$; 16 ºC, $y = 0.44x^{1.16}$; 20 ºC, $y = 0.57x^{1.09}$; Redfearn (1982), $y = 0.92x^{0.99}$.**

**Table 3.0.3. Analysis of covariance (ANCOVA) of total larval shell length versus larval shell height for *Paphies ventricosa* larvae reared over a 39-d period at three experimental temperature treatments (12, 16, and 20º C) and at 25 ºC (Redfearn, 1982). Analyses are presented for (A) differences among the three temperatures in the present study and (B) when data of Redfearn (1982) are included. All data were ln(*x*) transformed for the analysis.**

(A) Differences among three experimental temperatures in the present study

| **Source** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| Model | 5, 784 | 6352.8 | < 0.0001 |
| Length | 1 | 21033 | < 0.001 |
| Temperature | 2 | 3.601 | 0.0152 |
| Temperature * Length | 2 | 15.375 | < 0.001 |

(B) Comparison among the present experimental treatments and Redfearn (1982)

| **Source** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| Model | 7, 1104 | 10029.3 | < 0.0001 |
| Length | 1 | 28015 | < 0.001 |
| Temperature | 3 | 3.696 | 0.011 |
| Temperature * Length | 3 | 53.112 | < 0.001 |

## Discussion

### Development of Paphies ventricosa

In this study, we have described the development of *P. ventricosa* through the early embryonic to early veliger stages. The developmental processes resemble those previously described for other members of the family Mesodesmatidae, e.g. *P. australis* (Hooker, 1997) and *P. subtriangulata* (Redfearn, 1987). *Paphies ventricosa* has a medium-sized egg diameter (67 µm), suggesting limited yolk stores, and thus falls within the range typical of other mesodesmatid species with planktotrophic larvae (60 – 73 µm). *P. subtriangulata* has relatively small eggs (56 – 61 µm; Redfearn, 1987), as does *P. australis* (56.3 µm; Hooker, 1997). For *P. australis* the four-cell and trochophore stages measure 58 and 56.81 µm, respectively (Hooker, 1997). For *P. ventricosa*, trochophores are larger (83 – 102 µm length) and this stage is first observed 15 h post-fertilisation. Development times to the D-larval stage are similar in three of the four New Zealand *Paphies* species, which reach the straight-hinged veliger stage 22 – 37 h after fertilisation in *P. ventricosa* (Redfearn, 1982; present study), 24 – 36 h post-fertilisation in *P. australis* (Hooker, 1997), and 24 – 48 h in *P. subtriangulata* (Redfearn, 1987). There are few published accounts of embryonic and larval development in other genera of Mesodesmatidae, but most, if not all, have planktotrophic larvae. Development of the Pacific species *Mesodesma donacium* has been well described; this species has a small egg (50 µm) that develops to a blastula of the same size by day 1, to a 70 µm D-larva by day 4, and to an umbo larva by day 22 (Carstensen *et al.*, 2006). *Mesodesma mactroides* from the Atlantic coast of South America releases a large number of eggs (5.3 million eggs per female) and total larval duration ranges from 2 to 3 weeks (Brazerio & Defeo, 1999). In contrast, the European intertidal species *Donacilla cornea* has

been anecdotally reported as having no planktonic stage with larvae brooded in the pallial cavity, although Whiteley *et al.*, (1997) states that the species has a planktonic larval stage of unknown duration.

Development of the pediveliger stage in *P. ventricosa* is comparatively slow compared with other bivalve species. In a review of bivalve development in 54 species across a temperature range of 5 – 26 ºC, development to pediveliger in marine species was reported to take between 6 and 40 d (Ackerman *et al.*, 1994) at 20 – 25 ºC, development of *P. ventricosa* to this stage was complete by 31 to 22 d respectively, which is slower than in commonly cultured bivalve species (e.g. 10 – 20 d in scallop species at the same temperatures; Cragg, 2006). While development rates depend on a range of variables besides temperature, phylogenetic differences will also be present and it is possible that slow to moderate development rates are a feature of the genus *Paphies* (i.e. 18 – 22 d in *P. australis*; 17 d in *P. subtriangulata*) and possibly of the Mesodesmatidae in general (> 2 – 3 weeks in *Mesodesma*).

### Early larval shell formation

While the morphology of embryonic and larval stages of *P. ventricosa* is typical for bivalves, there are several points of interest in shell formation in the early developmental stages, of which there is a limited understanding elsewhere. Aranda-Burgos *et al.* (2014) noted that in bivalve larva there is some uncertainty regarding the timing of the shell-gland formation, and whether the process occurs at the gastrula stage or later. For *P. ventricosa*, the shell-field invagination occurs at the gastrula stage, prior to the complete formation of the prototroch (Figure 4.2C). Aranda-Burgos *et al.* (2014) carefully documented the formation of the shell gland in *Ruditapes decussatus* at a similar developmental stage, as reported in *Chione cancellata* (Mouëza *et al.*, 2006) and a number of other bivalve genera (*Venus,*

*Ostrea, Crassostrea,* and *Mytilus*; see Aranda-Burgos *et al.*, 2014). In contrast, the shell field forms at the trochophore stage in *Spisula solidissima* (Eyster & Morse, 1984).

Aranda-Burgos *et al.* (2014) outlined two contrasting models of shell formation in bivalve larvae, one in which the shell-field invagination closes completely during shell production and the other in which the shell field only corresponds to the floor of the shell-field depression. The present observations are consistent with the latter process, as proposed by Mouëza *et al.* (2006) and described in *R. decussatus* by Aranda-Burgos *et al.* (2014). The expansion of the dorsal shell field in *P. ventricosa* occurs during gastrulation and early trochophore larvae have a well-developed shell field with a clearly defined axial line between the two shell lobes.

The nature of calcification in the larvae was not ascertained in the present study. In the SEM preparations of the early trochophores, the shell field had a wrinkled appearance (Figure 3.2E), similar to previous observations, which has been suggested to represent noncalcified organic periostracum (Silberfield & Gros, 2006). A wrinkled pattern in the shells of *P. ventricosa* in the straight-hinged D-larval stage was observed; similar patterns have been seen in SEM preparations of other bivalve larvae, such as *Anomalocardia brasiliana* (Mouëza *et al.*, 2006) and *R. decussatus* (Aranda-Burgos *et al.*, 2014). These wrinkles have been attributed to shrinkage artefacts associated with dehydration during SEM preparation, and not to a lack of calcification, which is likely to be initiated at the trochophore stage (Weiss *et al.*, 2002) and can occur in the prodissoconch I shell before the formation of the organic phase is complete (LaBarbera, 1974).

## Larval development and temperature

Embryonic and larval development rate in *P. ventricosa* was found to be temperature dependent within the range tested (12 – 20º C), a response that is typical of bivalve larvae (Widdows, 1991; Cragg, 2006). Examples include increased rate of development with temperature in *M. edulis* (Pechenik *et al.*, 1990), *Mytilus galloprovincialis* (Sánchez-Lazo & Martínez-Pita, 2012), *Perna viridis* (Manoj Nair & Appukuttan, 2003), *C. gigas* (Kheder *et al.*, 2010), *Mytilopsis leucophaeata* (Verween *et al.*, 2007), *Saccostrea glomerata* (Parker *et al.*, 2009) and the freshwater species *Limnoperna fortunei* (Cataldo *et al.*, 2005). While direct comparisons among species are difficult, given the range of experimental temperatures and conditions used, the magnitude of temperature-dependence observed in *P. ventricosa* is within that observed in other bivalve larvae. For example, using the temperature quotient, $Q_{10}$ (i.e. the change in growth rate across a 10 ºC temperature difference) to compare growth responses to temperature at day 27, we observed a $Q_{10}$ = 1.82 (from 12 to 16º C) and $Q_{10}$ = 2.33 (from 16 to 20º C). These values are within the $Q_{10}$ range reported for *M. galloprovincialis* (Sánchez-Lazo & Martínez-Pita, 2012: $Q_{10}$ = 0.95 from 20 to 24 ºC and $Q_{10}$ = 2.69 from 17 to 20 ºC), *M. edulis* (Sprung, 1984a: $Q_{10}$ = 1.9 from 12 to 18 ºC), *Macoma balthica* (Drent, 2002: $Q_{10}$ = 1.5 from 10 to 20 ºC) and *Donax obesulus* (Carstensen *et al.*, 2010: $Q_{10}$ = 1.19 from 17.8 to 24.6 ºC). The effect of temperature on larval development is best studied in pectinids; in a review by Cragg (2006), the relationship between time to metamorphosis and temperature was quantified using an Arrhenius plot, from which the positive response to temperature (– Ea) was remarkably constant across the family.

Temperature is thought to be second only to diet in determining larval development rates (His *et al.*, 1989; Helm *et al.*, 2004). Increases in development rate at higher

temperatures are related to higher metabolic rates (Rico-Villa *et al.*, 2009) and potentially greater assimilation efficiency of algal diets (Manoj Nair & Appukuttan, 2003). While a positive developmental response to warmer temperatures is universal, optimal development temperatures for bivalve larvae vary among species, with ranges of 15 – 20 ºC in *C. gigas* (Helm & Millican, 1982), 20 – 25 ºC in *M. galloprovincialis* (His *et al.*, 1989), 17 – 20 ºC in *M. edulis* (Hrs-Brenko & Calabrese, 1969), 22 – 26 ºC in *S. glomerata* (Parker *et al.*, 2009) and 22 ºC for *M. leucophaeata* (Verween *et al.*, 2007). The optimal temperature for larval growth in *P. ventricosa* could not be established, but TL growth over 39 days was maximal at the highest temperature treatment (20 ºC). Redfearn (1982) observed that pediveliger larvae developed after 22 d at 25 ºC, compared with 31 d at 20 ºC in the present study, suggesting an optimal temperature of at least 25 ºC.

Interestingly, while shell growth rates increased with increasing temperatures, the appearance of developmental stages among the three experimental temperatures suggested the opposite (Table 3.1), with trochophore and D-hinge larvae observed earlier at 12 and 16 ºC. It is difficult to reconcile these observations, although the faster development of preveliger stages in colder temperatures is consistent with possible acclimation to the colder temperatures experienced by the southern populations at the time of spawning (i.e. 12 and 14 ºC). While there are no examples of temperature acclimation in bivalve embryos across latitude (Widdows, 1991), examples do exist in other marine invertebrates (Pecorino *et al.*, 2013). The observations could also suggest that the thermal response of the larvae changes with developmental stage. This has been demonstrated in the larvae of *S. glomerata* (Parker *et al.*, 2009). There is also evidence for changes in the thermal tolerance of developmental stages in echinoderm larvae (Hardy *et al.*, 2014; Lamare *et al.*, 2014)

and crab zoea and megalops stages (Storch *et al.*, 2011). Indeed, changes in the response to temperature in *P. ventricosa* were noted during the development of the veliger larvae, with no apparent effect of temperature on TL increase until day 15, where there was marked divergence in larval size in the 20 ºC treatment. Day 15 broadly corresponds with the transition from the straight-hinged to umbo larva, and could indicate that developmental progression is associated with changes in temperature responses.

In terms of developmental responses to temperature in *P. ventricosa*, we note that although larval size was temperature dependent, shape was little changed (Figure 3.4). This suggests that the smaller size of larvae at colder temperatures simply reflects a slowing of larval development, rather than that physiological damage by low temperatures results in abnormal or altered development.

### Ecological implications

The response of *P. ventricosa* larvae to temperature has implications for spatial and temporal recruitment patterns. The broad latitudinal distribution of the species means that larvae develop at 12 – 16 and 18 – 25 ºC in the southern and northern populations, respectively. While other factors will affect larval development in the plankton, especially phytoplankton concentration (Pechenik, 1990; Pechenik *et al.*, 1990) and salinity (Widdows, 1991; Verween *et al.*, 2007; Carstensen *et al.*, 2010), the results suggest that spatial differences in sea temperature around New Zealand (Greig *et al.*, 1998) could drive important differences in the time to reach settlement, ranging from 22 d at 25 ºC (Redfearn, 1982) to > 39 d at 12 ºC (present study). Widdows (1991) modelled the number of bivalve larvae reaching settlement as a function of development time and noted that, at a typical larval mortality rate of $M$ = 0.15 $d^{-1}$, there would be an order of magnitude difference in the number of larvae

reaching settlement between 22 and 39 d. Recruitment of *P. ventricosa* is marked by a high degree of temporal variation (Beentjes *et al.*, 2006), and, while the specific causes are unknown, sea conditions that influence the development of the larvae will clearly be important.

# Chapter 4

# Thermal tolerance of fertilisation and early larval development in *Paphies ventricosa*

## Introduction

This study aims to increase the understanding of the temperature sensitivity of fertilisation and early larval development in toheroa, *Paphies ventricosa,* by examining thermal windows (8.0 to 30.0 °C) in the development stages of the species. Due to the broad latitudinal distribution of the species in New Zealand (see Figure 1.1) and the protracted spawning season of the species, populations of *P. ventricosa* are likely to experience contrasting temperature regimes during fertilisation and development. In this respect, at the time of spawning (spring and summer) temperatures may range from 12 to 16 ºC in southern New Zealand populations (below 45º S), to 18 to 25 ºC in northern populations (above 40º S).

Temperature is known to be one of the most important environmental factors controlling developmental rate and success in marine invertebrates, and thus may play a significant role in the recruitment dynamics of many species (Pechenik, 1987; O'Connor *et al.*, 2007), of which fertilisation and early larval development play an important role. Therefore, knowing the temperature range across which fertilisation and embryonic and larval development can occur is important for understanding the role of these processes in the geographic distribution of the species (i.e. if they may limit distributions). Quantifying developmental responses to temperature across a thermal window are also important in understanding temporal variations in

recruitment success in response to changes in sea temperature that may occur between years or over the longer term.

Previous studies have examined fertilisation and larval development in *P. ventricosa*. Redfearn (1974) reported cleavage occurring at 4 h post-fertilisation at 24 ºC in northern *P. ventricosa*. Redfearn (1982) later successfully reared northern toheroa larvae at 25 ºC to settlement in 22 days. Smith (2003) reported fertilisation and development to veliger in northern *P. ventricosa* at 19.5, 21.5 and 22 ºC. Gadomski *et al.* (2015) examined larval development in southern *P. ventricosa* at 12, 16, and 20 ºC and found a significant effect of temperature on larval development rate (in terms of shell length), with faster larval growth occurring at 20 ºC than at 12 and 16 ºC. Gadomski *et al.* (2015) also provided a detailed description of early embryonic development and larval development and morphology at the same temperatures.

There have been a few studies examining the fertilisation and larval development of other *Paphies* species (*P. australis, P. subtriangulata,* and *P. donacina*). Hooker (1997) observed fertilisation at 22 and 28 ºC and larval development through to late-stage veliger at 20 and 28 ºC in *P. australis* from the North Island, New Zealand. Grant *et al.* (1998) reported a high mean fertilisation of 95.8 and 97.5% of eggs in *P. subtriangulata* and *P. australis*, respectively, from the North Island, although experimental temperature was not reported.

While there are no published reports on fertilisation and developmental thermal windows within the Mesodesmatidae family, there have been such studies on bivalves from other families. Kennedy *et al.* (1974a) reported 100% mortality in cleaved embryos and trochophores, but not veligers, in the latitudinally widespread hard clam *Mercenaria mercenaria* at 34.9 ºC and higher. Clotteau and Dubé (1993)

found that in the temperate surf clam *Spisula solidissima*, fertilisation success fell below 50% below 6 ºC and above 24 ºC. In the subtropical Sydney rock oyster, *Saccostrea glomerata,* Parker *et al.* (2009) reported that, there was a statistically significant effect of temperature on fertilisation ($p < 0.001$) when examined at 18º, 22º, 26º, and 30º C. In this species, fertilisation rate increased from ≈ 55% at 18 ºC to ≈ 90% at 26 ºC, before falling to ≈ 75% at 30 ºC at ambient $pCO_2$. Parker *et al.* (2010) found that in the Pacific oyster, *Crassostrea gigas*, from a subtropical location, there was a statistically significant effect of temperature on fertilisation when examined between 18 and 30 ºC. While there was less variation in fertilisation rates between 18 and 26 ºC (range ≈ 85 – 93%), fertilisation decreased from ≈ 90% at 26 ºC to ≈ 75% at 30 ºC. Bylenga *et al.* (2015) found a significant effect of temperature on the fertilisation of the Antarctic bivalve, *Laternula elliptica*, when examined across the narrow thermal gradient of -1.6º to 0.4º C.

While fertilisation thermal windows are not well studied in bivalves, there have been studies on other broadcast spawning marine invertebrates. For example, Pecorino *et al.* (2013) examined fertilisation of the sea urchin *Centrostephanus rodgersii* from both Australia and New Zealand from 10 to 35 ºC and found that at 2 h post-fertilisation, there was a significant effect of temperature on fertilisation success in both the New Zealand individuals, and in the Australian individuals, with a significant drop in success occurring at ≈ 29 and 31 ºC in the New Zealand and Australian animals, respectively. Hardy *et al.* (2014) examined the tropical sand dollar, *Arachnoides placenta*, across a gradient of 14 to 37 ºC, and found that temperature had a statistically significant effect on normal development (hatching, early blastula, and mesenchyme blastula stages present) at 6 h post-fertilisation. Normal development occurred between 17 and 32 ºC, with development at 17 and 32

ºC significantly different from 19 to 31 ºC (Hardy *et al.*, 2014). Delorme and Sewell (2013) examined fertilisation in the New Zealand sea urchin *Evechinus chloroticus* (a species with a latitudinal distribution of over 13º) between 15.7 and 24.2 ºC, and found that at 2 h post-fertilisation, there was a significant effect of temperature on fertilisation success. Lamare *et al.* (2014) found that in the crown-of-thorns sea star, *Acanthaster planci*, when examined at 5 h post-fertilisation across a gradient of 19.4 to 36.5 ºC, there was a significant difference among temperature treatments, with cleaved embryos observed from 19.4º to 33.2º C. Kupriyanova and Havenhand (2005) found that temperature had a statistically significant effect on the fertilisation success in the serpulid polychaete *Galeolaria caespitose* when examined from 11 to 31 ºC. Therefore, we might expect that the thermal window of the species in terms of fertilisation and development falls within this range

These studies have generally supported the hypothesis that the latitudinal distribution of marine invertebrates is closely related to the thermal tolerance limits of adult and planktonic stages (Andronikov, 1975; Jones *et al.*, 2009; Sunday *et al.*, 2012). Adult toheroa in the northern and southern populations are known to experience temperatures within the range of 12 to 25 ºC during spawning, with extremes outside this range possible throughout the year (Gadomski & Lamare, 2015).

The present study examines the fertilisation success and early larval development across a larger thermal gradient for *P. ventricosa* to identify the thermal window of development processes, which is lacking for the species. This data was used to examine three hypotheses, namely: (1) *P. ventricosa* has a thermal window of development that is broader than the environmental temperature range of the species, and therefore the distribution of the species is not limited by the effects of temperature on fertilisation and early larval development; (2) the geographic

distribution of *P. ventricosa* reflects the thermal window of fertilisation and early larval development; and, (3) within the thermal window, temperature has a significant effect on embryonic and larval development, which may contribute to variability in recruitment associated with spatial and temporal variation in sea temperature.

## Materials and Methods

### Specimen collection and spawning

Adult toheroa were collected in October 2013 from Oreti Beach (46.4790° S, 168.2522° E), under the appropriate customary permit, and transported to the Portobello Marine Laboratory (PML) in buckets of ambient seawater. At PML, the animals were placed in circular flow-through tanks filled with sand from the study site and maintained on a diet of All-G Rich (Alltech; Appendix I) slurry (supplied at 5 g $l^{-1}$ $day^{-1}$) until needed for spawning. All-G-Rich was used instead of cultured algae, as the 10 l $day^{-1}$ required per broodstock animal (pers. comm., 2013) was beyond the production capabilities of PML. Animals were spawned in 15 cm deep trays filled with 25 l of 5 µm filtered seawater and maintained at a temperature of 15.5 °C (± 0.5 ºC). Spawning was induced by an injection of a 2 m*M* solution of crystalline serotonin (5-hydroxytryptamine, Sigma Aldrich) into the anterior adductor muscle (Mandeno, 1999). Details of spawning are further described in Gadomski *et al.* (2015; see Chapter 3).

### Thermal window of fertilisation

The thermal window of fertilisation was studied using a thermal gradient created in an aluminium heat block (791 × 172 × 66 mm) that had 48 circular wells (32 × 56

mm) milled into the block. The wells were arranged in 4 rows of 12 wells, with each row representing a replicated 12-temperature thermal range (Figure 4.1). Two JULABO F18 thermal baths fitted with JULABO PC thermostats (JULABO GmbH, Germany) controlled the heat block temperatures at each end. The baths were filled with ≈ 15 l of glycol solution (1:2 glycerine to water by weight, plus 200 ml ethanol), and set to the temperature extremes of 4 and 42° C, creating a thermal gradient within the block of temperature treatments ≈ 10.5, 12.5, 14.3, 16.0, 17.5, 19.5, 21.0, 22.5 24.3, 26.0, 28.0, and 30.0 ° C. All temperature treatments were accurate to within ± 0.5 ºC across the four replicates. The heat block was loaded with 48 glass vials (40 ml volume, 29 × 79 mm) filled with 0.22 µm filtered seawater and were allowed to come up to temperature. The seawater filled vials were loaded with unfertilised eggs (pooled from 3 females) to a final concentration of 30 eggs $ml^{-1}$, and were preconditioned at treatment temperature for 30 min prior to insemination. Eggs were then fertilised with 25 µl of sperm solution ($1.56 \times 10^6$ sperm $ml^{-1}$, from one male) to give a final sperm concentration of $10^4$ sperm $ml^{-1}$ and an egg:sperm ratio of 1:30 (see Sprung & Bayne, 1984). Samples were taken 1 h post-fertilisation and fixed to a concentration of 10% neutral buffered formalin. Fixed samples were examined using an Olympus BX51 compound light microscope fitted with an Olympus Colorview III™ camera and controlled by AnalySIS LS software (Olympus Corporation). Successful fertilisation was determined by the breakdown of the germinal vesicle, and the appearance of the fertilisation envelope and polar body (Figure 4.2). Based on appearance when viewed microscopically, individuals were classified as unfertilised, fertilised, or abnormal (Figure 4.3).

## Thermal window of development

The thermal tolerance of development was studied using the thermal gradient as described in the previous section. The water baths were set to the temperature extremes of 4 °C and 30 ºC, creating a thermal gradient consisting of 12 temperature treatments of ≈ 8.0, 9.5, 11.4, 12.9, 14.6, 16.2, 17.8, 19.2, 20.4, 22.4, 23.8, and 25.5 °C. Pooled eggs from 3 females were mixed with three drops of sperm solution ($2.7 \times 10^5$ sperm $ml^{-1}$, pooled from 5 males) and allowed to fertilise. Fertilisation rate was determined to be greater than 25%. After 1 h of fertilisation, 48 glass vials (40 ml volume, 29 × 79 mm) were loaded with eggs at a concentration of 10 fertilised eggs $ml^{-1}$, and incubated in the heat block. A reference sample was taken from each vial 1 h after the vials were placed in the heat block (2 h post-fertilisation), with additional samples taken from each vial at 15, 22, and 37 h post-fertilisation. All samples were fixed to a final concentration of 10% neutral buffered formalin in seawater. Fixed samples were examined using the same microscope set up described in the previous section. Individuals were classified into one of seven developmental stages, namely unfertilised, fertilised, embryonic (2-64 cell embryos), blastula, trochophore, veliger, and abnormal (Figure 4.3).

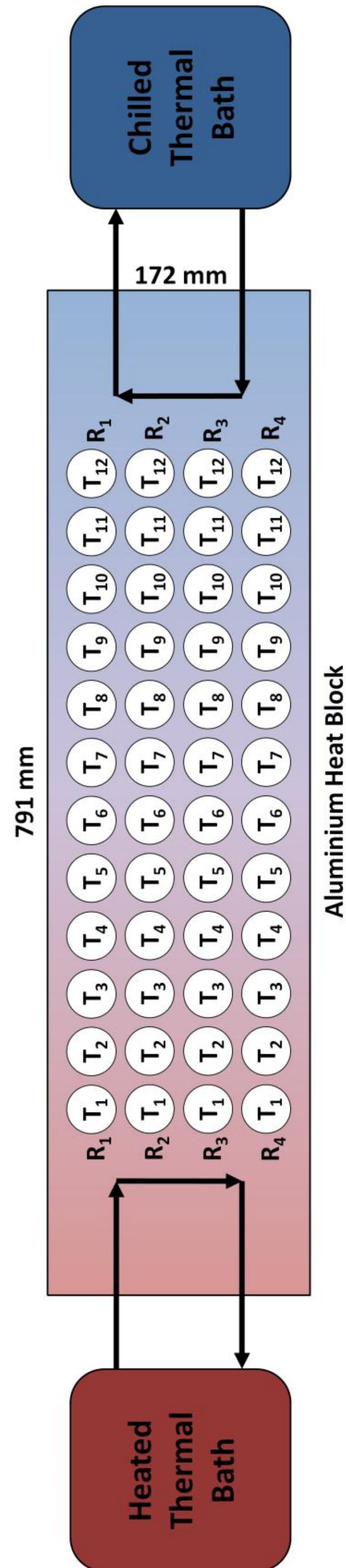



**Figure 4.1 Schematic of heat block used to create a thermal gradient for examining fertilisation and development rates, where T = temperature, and R = replicate. Arrows indicate direction of water flow. Not to scale.**

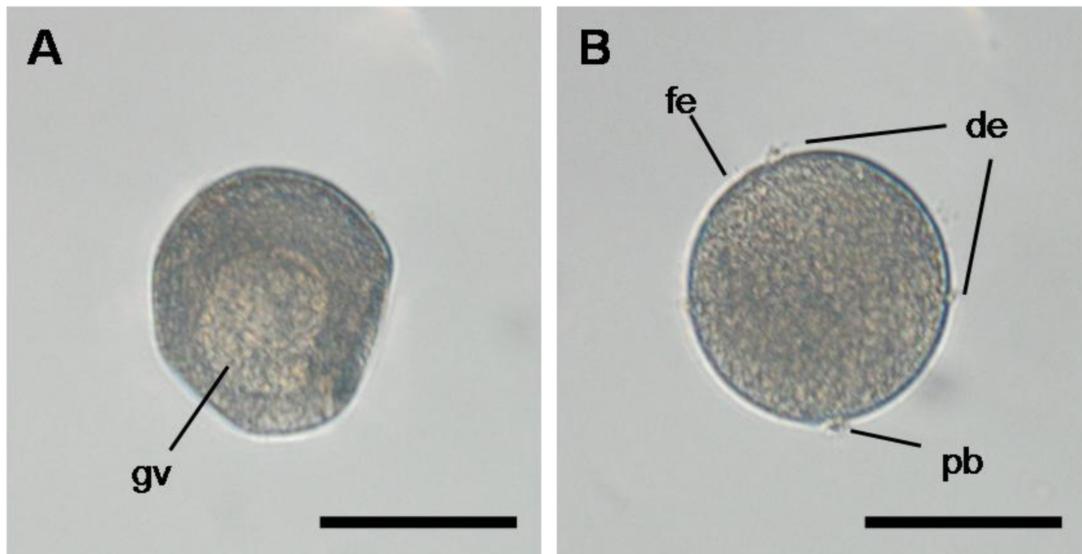



**Figure 4.2. (A) Light micrographs of an unfertilised egg showing the germinal vesicle (gm). (B) A fertilised egg, showing the polar body (pb), and the fertilisation envelope (fe). Fixation artefacts are present in the form ofdebris (de) adhering to the egg. Scale bars = 50 µm.**

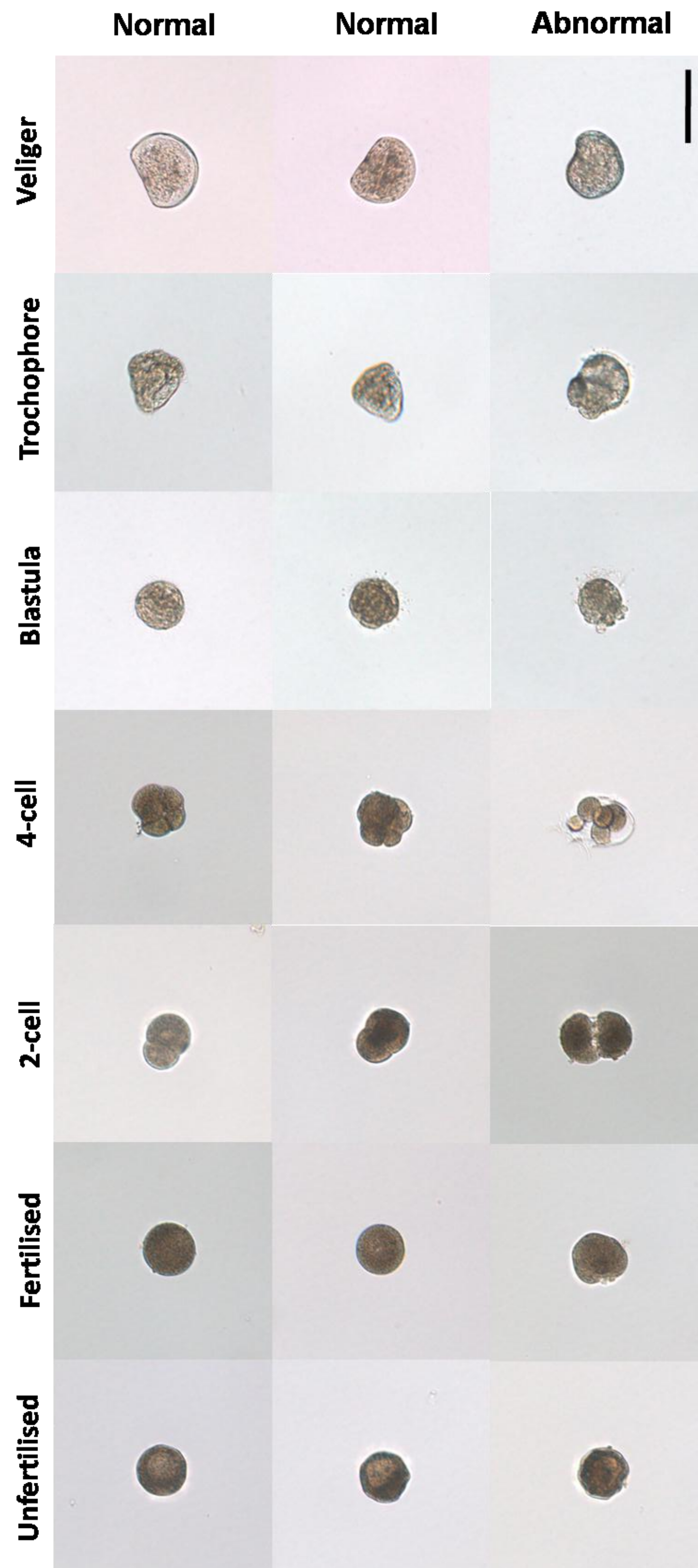



**Figure 4.3. A range of normal (top and middle row) and abnormal (bottom row) embryonic and larval development in *Paphies ventricosa*, showing the stages of unfertilised, fertilised, 2-cell embryo, 4-cell embryo, blastula, trochophore, and d-hinge veliger. Scale bar = 100 µm.**

### Statistical Analyses

Statistically significant differences ($\partial < 0.05$) in fertilisation success (fertilised and embryonic stages pooled) among temperatures at 1 h post-fertilisation were tested using one-way ANOVA. One-way ANOVA was also used to test for significant differences ($\partial < 0.05$) in the proportion of developmental stages present between twelve experimental temperatures at 2, 15, 22, and 37 h post-fertilisation. When data did not meet the assumption of homogeneity of variances, a Welch's test was used with the ANOVA (Quinn & Keough, 2002) when possible. The sample for 16.2 ºC was excluded from all analyses at 2 h post-fertilisation due to sampling error, and 37 h post-fertilisation, the sample for 8.0 ºC was excluded from analysis due to 0% recovery. Percentage data were arcsine square root transformed prior to analysis. For transformed fertilisation data, homoscedasticity was tested using Levene's test. When unequal variances were detected, the Welch's test was used. Significant differences among temperature treatments were identified using Tukey's HSD *post hoc* test. All statistical analyses were performed in JMP 12 (SAS Institute).

## Results

### Thermal window of fertilisation

One-way ANOVA indicated that there was a statistically significant effect of temperature on the fertilisation success of *Paphies ventricosa* ($F_{(10,\ 33)} = 4.017$, $p = 0.0012$) (Figure 4.4). Fertilisation success ranged from 4.6% at 28.0 ºC to 46.7% at 19.5 ºC, with fertilisation greater than 28% between 12.5 and 21.0 ºC. Fertilisation outside of this range was less than 15%. Tukey's *post hoc* test showed that 21.0,

24.3, and 26.0 ºC were statistically significantly different from 12.5 ºC. The temperature of 17.5 ºC was removed do to sampling error.

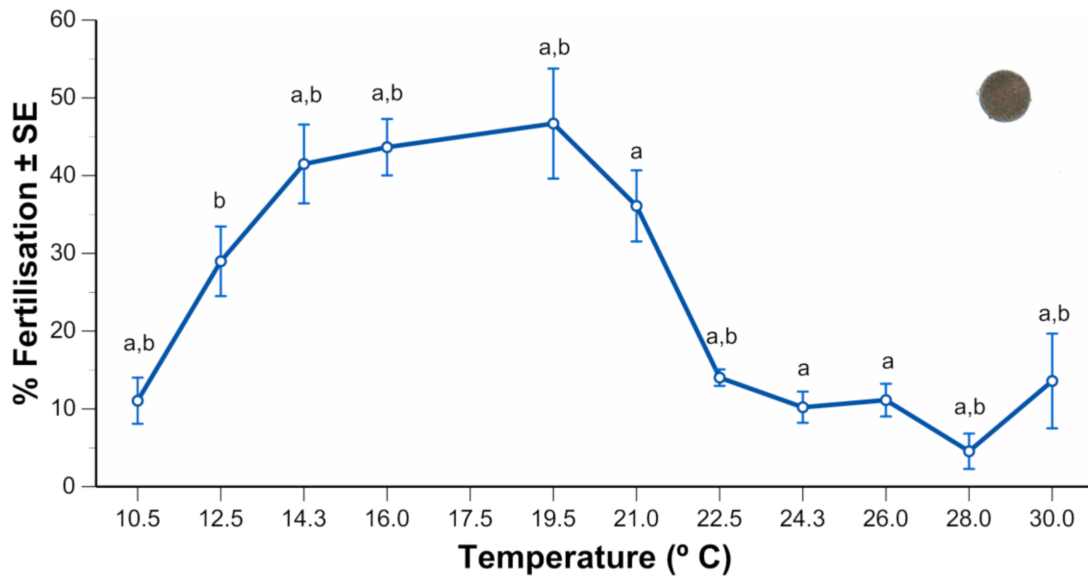



**Figure 4.4. Average fertilisation success (± SE) 1 h post-fertilisation for *Paphies ventricosa* at a range of experimental temperatures. Significant differences among temperatures are indicated by lower case lettering.. N = 4 for each data point.**

## Thermal tolerance of development stages

### *2h post-fertilisation*

At 2 h post-fertilisation (Figure 4.5), there was a statistically significant difference among temperature treatments for all stages present – unfertilised ($F_{(10, 33)} = 19.474$, $p < 0.0001$), fertilised ($F_{(10, 33)} = 21.349$, $p < 0.0001$), embryonic ($F_{(10, 13.121)} = 30.703$, $p < 0.0001$), and abnormal ($F_{(10, 33)} = 3.088$, $p = 0.007$) (Table 4.1). Unfertilised eggs ranged from 0.7 to 54.8% of the sample across the thermal gradient, but were more prevalent (> 20%) at the lower temperatures of 8.0 to 11.4 ºC (Figure 4.5). *Post hoc* tests indicate that 12.9 to 25.5 ºC are statistically different from 8.0 and 9.5 ºC, but not from 11.4 ºC; 8 and 9.5 ºC are statistically different from 12.9 to 23.8 ºC, while 9.5 ºC is also statistically similar to 11.4 ºC. Fertilised eggs ranged from 6.5 to 57.2% of the sample across the gradient and comprised > 25% of the sample from 17.8 to 25.5 ºC (Figure 4.5). *Post hoc* tests indicate that 17.8 to 25.5 ºC are statistically different from 8.0 to 14.6 ºC and that 11.4 ºC is statistically different from 8.0 and 22.4 to 25.5 ºC but similar to 9.5 and 12.9 to 20.4 ºC. Embryos were present at all temperatures, ranging from 28.4 to 78.8% of the sample, but were most prevalent (> 50%) between 12.9 and 17.8 ºC (Figure 4.5). *Post hoc* tests indicate that 12.9 and 14.6 ºC are statistically different from all other temperatures and that 8.0 and 17.8 ºC are statistically different from each other, but statistically similar to 9.5, 11.4, and 19.2 to 25.5 ºC. Abnormal development occurred across all temperatures (2.7 – 13.2%), and was higher (>10%) between 8.0 and 14.6 ºC (Figure 4.5). *Post hoc* tests indicate that 9.5 and 11.4 ºC are statistically different from 25.5 ºC, but similar to all other temperatures.

**Table 4.0.1. One-way ANOVA of the percentage of *Paphies ventricosa* developmental stages present at 2 h post-fertilisation. All data were arcsine square root transformed prior to analysis. Instances where the Welch's test was used are indicated by *.**

| Developmental Stage | *Df* | *F-ratio* | *p*-value |
|---|---|---|---|
| Unfertilised | 10, 33 | 19.474 | < 0.0001 |
| Fertilised | 10, 33 | 21.349 | < 0.0001 |
| Embryonic* | 10, 13.121 | 30.703 | < 0.0001 |
| Abnormal | 10, 33 | 3.088 | 0.007 |

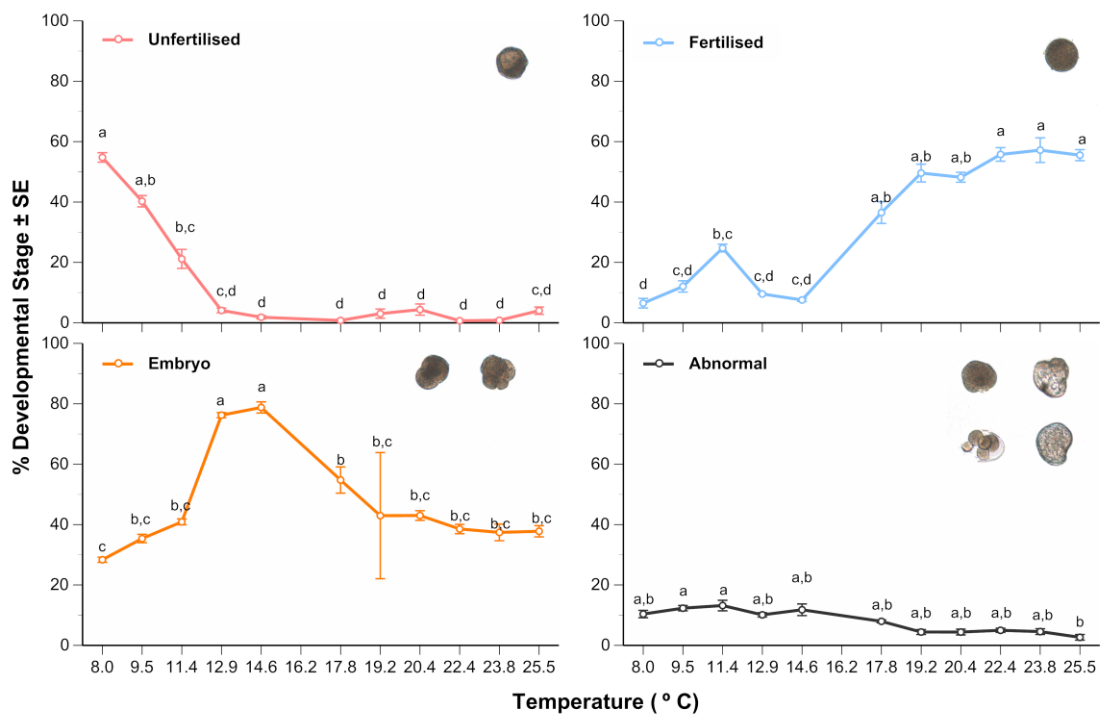



**Figure 4.5. Average percentage of developmental stages present (± SE) at 2 h post-fertilisation in *Paphies ventricosa* across a thermal gradient. Significant differences are indicated by lowercase lettering. N = 4 for each data point.**

***15 h post-fertilisation***

At 15 h post-fertilisation (Figure 4.6), there was a statistically significant difference among temperature treatments at all stages; blastula ($F_{(11, 36)} = 2.102$, $p = 0.046$); trochophore ($F_{(11, 36)} = 6.858$, $p < 0.0001$), and; abnormal ($F_{(11, 36)} = 4.555$, $p < 0.001$) (Table 4.2). Blastulae ranged from 22.5 to 83.3% of the sample, but comprised > 75% of the sample at 17.8 and from 20.4 to 23.8 ºC (Figure 4.6). *Post hoc* tests indicated that all temperatures were statistically similar to each other. Trochophores were only found between 8.0 and 19.2 ºC, where they comprised from 5.4 to 77.5% of the sample, but only made up > 75% of the sample at 14.6 ºC (Figure 4.6). *Post hoc* tests indicate that 14.6 ºC is statistically different from all temperatures, except 11.4 and 16.2 ºC, and that 20.4 to 25.5 ºC were statistically different from 11.4 and 14.6 ºC. Abnormal development was only found at 8.0, 9.5, 16.2, and 25.5 ºC, with higher percentages (> 25%) at 8.0 and 9.5 ºC than at 16.2 and 25.5 ºC (< 15%) (Figure 4.6). *Post hoc* tests indicate that 8.0 and 9.5 ºC are statistically similar to 16.2 and 25 ºC, but different from all other temperatures, and that 16.2 and 25.5 ºC are statistically similar to all temperatures.

**Table 4.0.2. One-way ANOVA of the percentage of *Paphies ventricosa* developmental stages present at 15 h post-fertilisation. All data were arcsine square root transformed prior to analysis.**

| Developmental Stage | *Df* | *F-ratio* | *p*-value |
|---|---|---|---|
| Blastula | 11, 36 | 2.102 | 0.046 |
| Trochophore | 11, 36 | 6.858 | < 0.0001 |
| Abnormal | 11, 36 | 4.555 | < 0.001 |

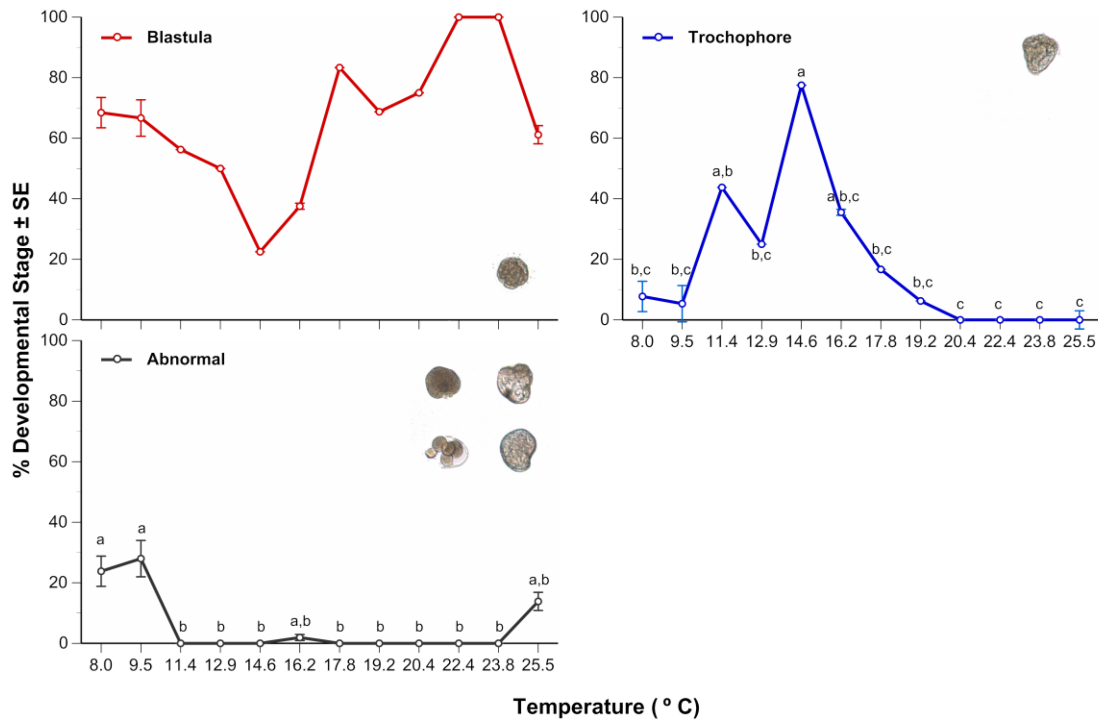



**Figure 4.6. Average percentage of developmental stages present (± SE) at 15 h post-fertilisation in *Paphies ventricosa* across a thermal gradient. Significant differences are indicated by lowercase lettering. N = 4 for each data point.**

***22 h post-fertilisation***

At 22 h post-fertilisation (Figure 4.7), there was a statistically significant difference among temperature treatments for the veliger ($F_{(11, 36)} = 8.064$, $p < 0.0001$) stage (Table 4.3). However, there was no significant difference among temperatures for the blastula ($F_{(11, 36)} = 1.307$, $p = 0.261$), trochophore ($F_{(11, 36)} = 1.544$, $p = 0.159$) and abnormally developed ($F_{(11, 36)} = 0.853$, $p = 0.591$) stages (Table 4.3). Blastulae comprised from 8.3 to 75% at all temperatures except 14.6, 17.8, and 19.2 ºC, where blastulae were not present (Figure 4.7). Blastulae comprised 50% or more of the sample at 8.0 and 25.5 ºC. Trochophores were present between the temperatures of 9.5 and 19.2 ºC (Figure 4.7). Trochophores comprised less than 15% of the sample between 9.5 and 16.2 ºC, while 17 and 19.2 ºC were comprised of over 35% of trochophores. Veligers were only present from 12.9 to 17.8 ºC, where they comprised more than 40% of the sample, except at 17.8 ºC, where they comprised less than 15% of the sample (Figure 4.7). *Post hoc* tests indicate that 14.6 ºC is statistically different from 8.0 to 11.4 and 17.8 to 25.5 ºC; 8.0 to 11.4 and 19.2 to 25.5 ºC are statistically different from 14.6 and 16.2 ºC; and that 12.9 ºC is statistically similar to all temperature treatments. Abnormal development occurred from 9.5 to12.9 ºC and at 16.2 ºC, and was highest at 9.5 and 11.4 ºC where it comprised 15% or more of the sample.

**Table 4.0.3. One-way ANOVA of the percentage of *Paphies ventricosa* developmental stages present at 22 h post-fertilisation. All data were arcsine square root transformed prior to analysis.**

| Developmental Stage | *Df* | *F-ratio* | *p*-value |
|---|---|---|---|
| Blastula | 11, 36 | 1.307 | 0.261 |
| Trochophore | 11, 36 | 1.544 | 0.159 |
| Veliger | 11, 36 | 8.064 | < 0.0001 |
| Abnormal | 11, 36 | 0.854 | 0.591 |

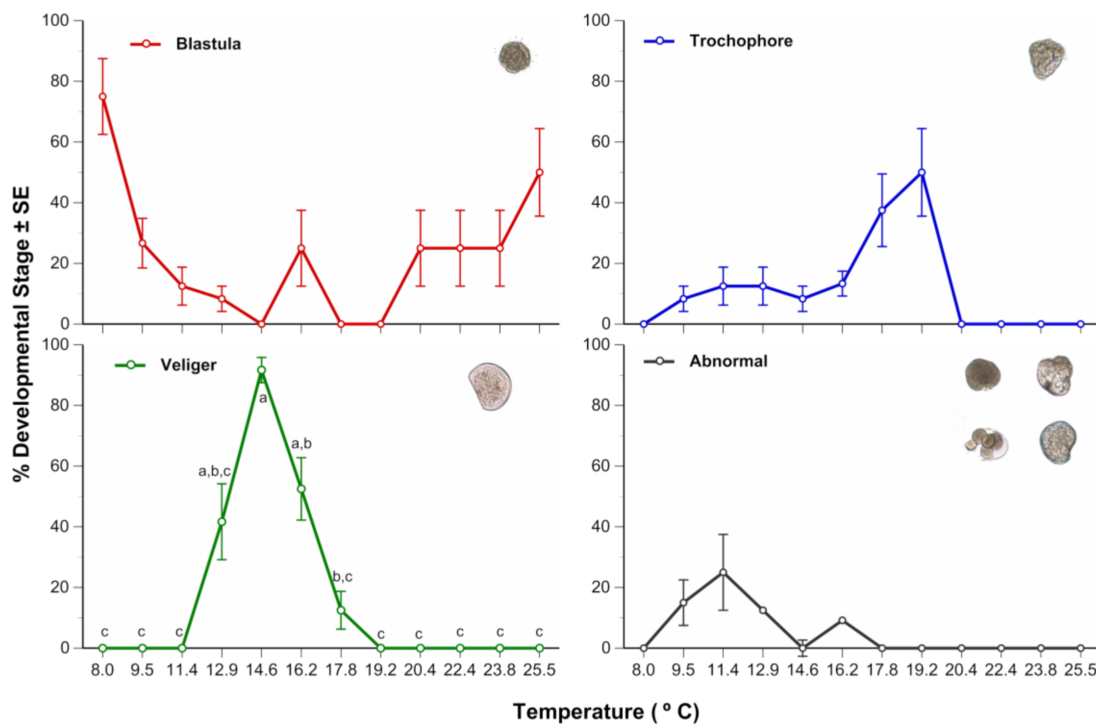



**Figure 4.7. Average percentage of developmental stages present (± SE) at 22 h post-fertilisation in *Paphies ventricosa* across a thermal gradient. Significant differences are indicated by lowercase lettering. N = 4 for each data point.**

***37 h post-fertilisation***

At 37 h post-fertilisation (Figure 4.8), there was a statistically significant difference among temperature treatments for the blastula ($F_{(10, 33)} = 2.778$, $p = 0.013$), trochophore ($F_{(10, 33)} = 3.819$, $p = 0.002$), and veliger ($F_{(10, 33)} = 5.918$, $p < 0.0001$) stages (Table 4.4). There was no statistically significant difference among temperature treatments for abnormal development ($F_{(10, 33)} = 0.920$, $p = 0.527$) (Table 4.4). Blastulae were present in the temperatures of 11.4, 16.2, and 20.4 ºC at 25% or less of the sample and at 25.5 ºC at 75% of the sample (Figure 4.8). *Post hoc* tests indicated that 25.5 ºC was statistically similar to 11.4, 16.2, and 22.4 ºC but statistically different from all other temperatures, while 11.4, 16.2, and 22.4 ºC were statistically similar to all other temperatures. Trochophores were found at 25% or more of the sample at 9.5, 22.4, and 23.8 ºC, and at a much lower percentage (2.5%) at 17.8 ºC (Figure 4.8). *Post hoc* tests indicate that 20.4 ºC is statistically similar to 9.5 and 23.8 ºC but statistically different to all other temperatures. Veligers were present between 11.4 and 22.4 ºC, comprising 75% or more of the sample from 12.9 to 19.2 ºC and 25% or less of the sample at 20.4 and 22.4 ºC (Figure 4.8). *Post hoc* tests indicate that 16.2 and 17.8 ºC are statistically similar to 12.9, 14.6, and 19.2 to 22.4 ºC and that 12.9, 14.6, and 19.2 to 22.4 ºC are statistically similar to all temperatures. Abnormal development was only observed at 22.4 and 25.5 ºC, at 12.5 and 25% of the sample, respectively (Figure 4.8).

**Table 4.0.4. One-way ANOVA of the percentage of *Paphies ventricosa* developmental stages present at 37 h post-fertilisation. All data were arcsine square root transformed prior to analysis.**

| Developmental Stage | *Df* | *F-ratio* | *p*-value |
|---|---|---|---|
| Blastula | 10, 33 | 2.778 | 0.013 |
| Trochophore | 10, 33 | 3.819 | 0.002 |
| Veliger | 10, 33 | 5.918 | < 0.0001 |
| Abnormal | 10, 33 | 0.920 | 0.527 |

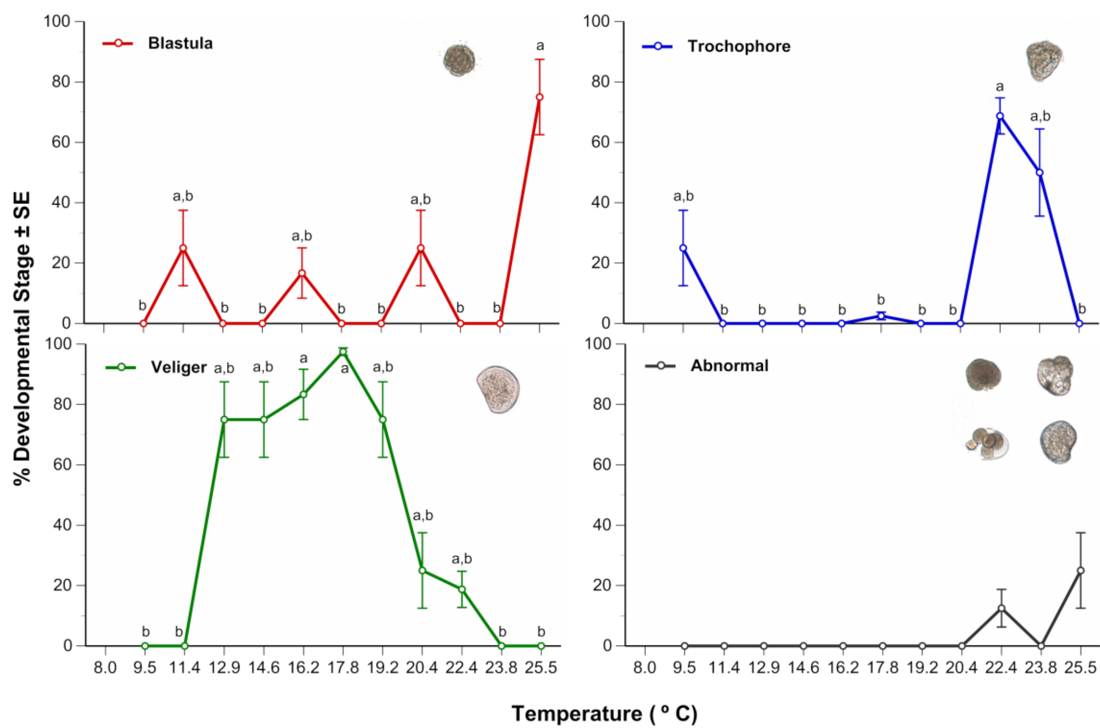



**Figure 4.8. Average, percentage of developmental stages present (± SE) at 37 h post-fertilisation in *Paphies ventricosa* across a thermal gradient. Significant differences are indicated by lowercase lettering. N = 4 for each data point.**

## Discussion

Temperature is one of the most important factors controlling larval development in marine invertebrates, thus playing an important role in the recruitment of many species (Pechenik, 1987; Pechenik *et al.*, 1990; O'Connor *et al.*, 2007). It has been suggested that the lower range for normal development does not limit distribution in some marine invertebrates (Sewell & Young, 1999). If then, the upper temperature range for normal development, including fertilisation, is the limiting factor for a species' distribution, the development and distribution of *Paphies ventricosa* could be affected by the ongoing trend of ocean warming.

### Thermal tolerance of fertilisation in *Paphies ventricosa*

In this study, the fertilisation success of *Paphies ventricosa* from southern New Zealand was examined across a thermal gradient of 19.5 ºC, (ranging from 10.5 to 30.0 ºC), the first study to do so. The detected fertilisation tolerance in *P. ventricosa* encompasses temperatures at which fertilisation has been known to occur, i.e. 12 to 25 ºC (Redfearn, 1974, 1982; Smith, 2003; Gadomski *et al.*, 2015). When compared to other *Paphies* species, such as the findings by Hooker (1997), fertilisation was successful at temperatures observed in *P. australis* (Hooker, 1997), although not at the same rate of success as *P. subtriangulata* (95.8%) and *P. australis* (97.5%) (Grant *et al.*, 1998). A possible cause for the comparatively low fertilisation rate in the present study is reduced egg quality in late season spawning – there is the possibility that broodstock naturally spawned in their tanks prior to induction, and did not have adequate time to fully recondition themselves. In some previous spawning attempts, fertilisation rates of over 80% have been achieved.

While a direct comparison to other species is difficult, the fertilisation window of thermal tolerance resembles that of bivalves from other families – in that it is most successful within the natural temperature range of a species. The surf clam, *Spisula solidissima* is common along the western Atlantic coast from the Gulf of St. Lawrence, Canada in the north, to South Carolina, USA in the south. In animals from a Canadian population from Îles de la Madeleine, Quebec, fertilisation occurred between 0 and 32 ºC, though was less than 50% successful below 6 ºC and above 24 ºC (Clotteau & Dubé, 1993). For the Gulf of St. Lawrence, open water average sea surface temperatures range from below 0 ºC in March to over 15 ºC in August and can reach summer temperatures of up to 20 ºC in coastal areas (Galbraith *et al.*, 2013). When considering that, for practical purposes, it is desirable to raise embryos at the highest possible naturally occurring temperatures for a species to accelerate development, the laboratory temperature range of 15 to 20 ºC proposed by Clotteau & Dubé (1993) as an ideal range for fertilisation and early embryonic development in northern *S. solidissima* is in line with the natural temperature range of the species (Galbraith *et al.*, 2013).

The coot clam *Mulinia lateralis*, like *Paphies ventricosa*, has a broad latitudinal distribution along the Western Atlantic, ranging from New Jersey, USA to the Yucatan Peninsula, Mexico in the Caribbean Sea. Calabrese (1969) examined the thermal window of fertilisation in *M. lateralis* from 7.5 to 34.5 ºC, and found a drastic change in fertilisation success between 10.0 and 12.5 ºC, when it jumped from 17.3% to 60.9% success, respectively. Fertilisation success was over 90% successful between 17.5 and 25.0 ºC, with the highest success rate at 20.0 ºC (Calabrese, 1969), likely regularly experienced near the southern end of its natural distribution.

The general trend of higher fertilisation success at temperatures close to a species natural range across a thermal gradient has been observed in the oysters *Saccostrea glomerata* and *Crassostrea gigas* from Australia (Parker *et al.*, 2009, 2010). *C. gigas* has both a broad, latitudinal distribution (from Japan to the top of the South Island of New Zealand) and temperature tolerance from below freezing to over 30 ºC, with spawning generally occurring above 20º C. Parker *et al.* (2010) found that *C. gigas* had optimal levels of fertilisation between 18 and 26 ºC, with levels dropping (to ≈ 75%) at 30 ºC. This thermal fertilisation window is in line with the natural temperature range of the species. *S. glomerata*, while endemic to Australia and the North Island of New Zealand, has been reported as far north as Southern Japan (Willan *et al.*, 2009), giving it a narrower latitudinal distribution than *C. gigas* in generally warmer waters. Parker *et al.* (2009) found that in *S. glomerata* fertilisation was less successful at 18 ºC (< 55%) than at 22 to 30 ºC (> 65%), with 26 ºC being the optimal temperature for fertilisation success (89%). The slight skewing of the success of thermal window of fertilisation to the right for *C. gigas* and to the left for *S. glomerata* is in line with the species respective latitudinal distributions.

The Antarctic geoduck, *Laternula elliptica*, has a circumpolar distribution with a natural temperature range of less than -1.5 ºC to 1.7 ºC (Ahn & Shim, 1998) and is sensitive to elevated temperatures – with reduced reburying capacity at 2.5 ºC, complete loss of reburying at 5 ºC, and death with prolonged exposure to temperatures exceeding 9 ºC (Bylenga *et al.*, 2015). Peak spawning in *L. elliptica* occurs from late December through February on the Antarctic Peninsula and from February to May in McMurdo Sound, Ross Sea (Bylenga *et al.*, 2015). Bylenga *et al.* (2015) found that *L. elliptica* showed a similar pattern of fertilisation success to the

present study. While at 4 h post-fertilisation, fertilisation success at -1.6 ºC was statistically significantly lower than -0.5 and 0.4 ºC, fertilisation success was not significantly different at both 24 and 48 h post-fertilisation, indicating that while delayed at lower temperatures, fertilisation success was high across a thermal window of the species natural temperature range.

This pattern of fertilisation success is also similar to that observed in other broadcast spawning marine invertebrates, including echinoderms and a polychaete. Echinoderm species exhibited thermal fertilisation windows not only at the lower end within the natural temperature range of the species, but also at the upper end beyond the natural range, including the sea urchin *Centrostephanus rodgersii* from Australia and New Zealand (Pecorino *et al.*, 2013); the sand dollar *Arachnoides placenta* from Australia (Hardy *et al.*, 2014); and, the crown-of-thorns sea star *Acanthaster planci* from the Great Barrier Reef, Australia (Lamare *et al.*, 2014). Kurpriyanova and Havenhand (2005) found that in the free-swimming polychaete, *Galeolaria caespitose* from South Australia, that peak fertilisation success (> 85%) was observed at 21 ºC, the average summer water temperature from the sampling location when spawning occurs.

While the present study has not identified the temperature limits at which fertilisation fails to occur, there is clear reduction in fertilisation success below 14.3 ºC and above 21.0 ºC, when the success rate consistently drops below 30%. Given the broad geographical distribution of *P. ventricosa* (see Figure 1.1), with adults from different populations being subjected to contrasting temperature regimes, having a wide thermal fertilisation window beyond the thermal range of a particular population is realistic (Andronikov, 1975). The maximum temperatures examined in the present study are well beyond the ambient temperatures the southern populations of *P.*

*ventricosa* would encounter *in situ*. The minimum temperatures examined are within normally encountered temperatures for the southern populations, albeit lower than normal during spawning. Based on the results of the present study, the ideal thermal window of fertilisation in *P. ventricosa* would be 14 to 21 ºC. Given the projected sea surface temperature rise for 2100 of 2 to 3 ºC for the southern populations and 3 to 4 ºC for the northern populations of *P. ventricosa* (IPCC, 2104), the fertilisation success of the southern populations will be affected less than that of the northern populations.

## Thermal tolerance of development in *Paphies ventricosa*

The current study examined early larval development across a thermal gradient of 17.5 ºC**,** from 8 to 25.5 ºC, and found that temperature had a significant effect on the presence of the early larval developmental stages to varying degrees, depending on time since fertilisation. While initial development through to the feeding veliger stage is faster between 11.4 and 20.4 ºC, once at the veliger stage, if fed the same diet, larvae at 20 and 25 ºC will grow faster than those at 12 and 16 ºC (see Chapter 3; Redfearn, 1982; Gadomski *et al.,* 2015). Hooker (1997) observed a similar timeline of development in *Paphies australis* reared at 20 ºC (± 1 ºC), as veliger larvae were first observed between 24 and 48 h post-fertilisation, compared to the 37 h for *P. ventricosa* at 20.4 ºC. Given that *Paphies australis* has been observed to develop through to late-stage veliger larvae at 28 ºC (Hooker, 1997), it is conceivable that early larval development could progress through to the veliger stage and beyond in *P. ventricosa* at temperatures greater than the maximum examined in this study, especially in the northern population.

The initial faster progression through to a more developed larval stage is important for recruitment success as veliger larvae were less likely to die from temperature

changes than trochophores and cleavage stages (Kennedy *et al.*, 1974a). Kennedy *et al.* (1974a) examined the effect of a thermal gradient (from 17.5 to 43.1 ºC) on survivorship of embryos and larvae in the hard clam *Mercenaria mercenaria* from Virginia, United States, and found that veliger larvae were more robust, followed by trochophores and cleavage stage embryos, in terms of both temperature and exposure time. A conservative estimate of early summer spawning temperatures for the population of *M. mercenaria* was 23 to 29 ºC, and cleavage stages exhibited 90% mortality at 30 min exposure to 34 ºC; trochophores exhibited 90% mortality at > 100 min exposure to 34 ºC; and veliger larvae exhibited no mortality at exposures of up to 1000 min (the length of the experiment) at 34 ºC (Kennedy *et al.*, 1974a). These results were similar to the dwarf surf clam *Mulinia lateralis*, in which the cleavage stages were more robust and the veliger stage more sensitive to temperature than *M. mercenaria* (Kennedy *et al.*, 1974b). Since the trochophore and veliger stages are more robust, they are more likely to survive environmental changes and be more likely to survive through to recruitment, making faster development through to the veliger larvae stage an advantage.

Bylenga *et al.* (2015), found a significant difference in the percentage of stages present across a thermal gradient of -1.6 to 0.4 °C in *Laternula elliptica*. At 6 h post-fertilisation, the 2-cell cleavage stage was present at significantly higher levels at -0.5 and 0.4 ºC than at -1.6 ºC; trochophores appeared at 10 d post-fertilisation at 0.4 ºC compared to 11 d for -1.6 and 0.5 ºC; and at 20 d post-fertilisation, veliger larvae were present at significantly higher levels at -0.5 and 0.4 ºC than at -1.6 ºC (Bylenga *et al.*, 2015). However, despite the faster rate of development, the rate of abnormal development increased from < 15% at 6 h post-fertilisation to > 50% at 35 d.

Parker *et al.* (2009) found that in *Saccostrea glomerata*, the percentage rate of veligers present at 24h post-fertilisation was lower at 18 ºC (≈ 30%) than at 22 to 30 ºC (≥ 60%), but that by 48 h post-fertilisation veligers were present at > 80% at 18 to 26 ºC and ≈ 60% at 30 ºC. The rate of abnormal development at 24 h post-fertilisation was higher (> 30%) at 18, 22, and 30 ºC than at 26 ºC (< 10%), while at 48 h post-fertilisation, abnormal development was < 20% at 18 to 26 ºC and ≈ 30% at 30 ºC (Parker *et al.*, 2009). Parker *et al.* (2010) found that in *Crassostrea gigas,* at both 24 and 48 h post-fertilisation, fewer larvae reached the veliger stage at 18 and 30 ºC than at 22 and 26 ºC, though were present at > 80% at all temperatures. Abnormal development rates were lower at 22 and 26 ºC (< 10%) than at 18 and 30 ºC (15 to < 25%) (Parker *et al.*, 2010). While both of these studies did note the same faster rate of development at higher temperatures (30 ºC) as expected, they both exhibited highest veliger presence and lowest abnormal development rate at 26 ºC, a peak fertilisation temperature for both *S. glomerata* (Parker *et al.*, 2009) and *C. gigas* (Parker *et al.*, 2010). These studies support the findings in other marine invertebrates in terms of development across a species' thermal range. However, the rate of abnormal development was generally much lower than in similar studies.

Calabrese (1969), extended the thermal gradient experiment previously mentioned for *Mulinia lateralis* beyond fertilisation and examined the thermal window of survival and growth rate for 8 days across the thermal gradient previously mentioned. Surprisingly, larvae had survival rates of > 80% from 7.5 to 27.5 ºC, far beyond the lower limits of the ideal fertilisation thermal gradient. The average growth rate over 8 days was only satisfactory in the range of 20 to 30 ºC, and grew the most rapidly at 27.5 ºC. There appears to be a trade off in this species, while larvae at 27.5 ºC grew the fastest (93.5% increase in mean larval length) and had a high survival rate

(88.4%) during the experimental period, they had a low fertilisation rate (43.3%). This could be a strategy so that those animals exhibiting limited fertilisation success at the southern extreme of their distribution grow faster to reach settlement and recruit to the next stage earlier than those at lower temperatures. There was no mention of abnormal development in this study.

An increased rate of development at higher temperatures has also been observed in echinoderms. Delorme and Sewell (2013) observed a similar pattern of development across a thermal gradient in the sea urchin *Evechinus chloroticus* from Hauraki Gulf, New Zealand where spawning and development of the species occurs at temperatures from 13 to 22 ºC. Across a thermal gradient from 15.7 to 24.2 ºC, blastulae were observed at 4 h post-fertilisation at temperatures ≥ 21.5 ºC, compared to 6 h post-fertilisation at lower temperatures (Delorme & Sewell, 2013). The mean number of cleavage divisions in embryos was significantly higher at temperatures ≥ 21.5 ºC than at the temperatures of 15.7 to 17.7 ºC (Delorme & Sewell, 2013). Abnormal development was greater at higher temperatures, reaching 44 and 59% in 24.2 ºC at 8 and 24 h post-fertilisation, respectively (Delorme & Sewell, 2013).

Hardy *et al.* (2014) found that in the sand dollar *Arachnoides placenta*, when examined across a thermal gradient of 14 to 37º C, at 24 h post-fertilisation the more advanced 6-armed plutei larvae were present at 27 to 32º C, ranging from 15 to 90% of the sample. There was no development (100% abnormal development) at 14, 35, and 37 C, indicating that at the higher temperatures where normal development does occur, larvae exhibited the most advanced development (Hardy *et al.*, 2014). The percentage of abnormal development was less than 10% across the development range, except for 31 and 32º C, where it reached levels of 15 and 45%, respectively,

therefore also exhibiting the pattern of both increased rate of development and increased abnormal development at higher temperatures (Hardy *et al.*, 2014).

In *Paphies ventricosa*, at 2 h post-fertilisation, the most advanced stage present, 2-64 cell embryos, occurred at the highest rates around the ideal fertilisation range (centred around 14.6º C) for the southern population, and abnormal development was < 15%. The same pattern of advanced development held true at 15 h post-fertilisation when trochophores were the most advanced stage present, though abnormal development was now much higher at the temperature extremes. At 22 h post-fertilisation, veligers, the most advanced developmental stage present, again followed the same pattern as the previous time points, though abnormal development was now highest in the lower half of the thermal window. By 37 h post-fertilisation, veligers were present at over 50% of the sample across a broader temperature range (12.9 to 19.2 ºC), with abnormal development only observed at the high extremes of the thermal window. Within the current study, it was not possible to conclude that zero recovery at any time point was due to 100% larval mortality or sampling bias. Given that the natural spawning and developmental temperature range of the species is 12 to 25 ºC, in this experiment *P. ventricosa* does not appear to follow the pattern of faster development previously identified at the higher natural range for the species (see Chapter 3; Redfearn, 1982; Gadomski *et al.*, 2015). However, when taking into consideration that this study was conducted using animals solely from the southern population, whose natural spawning temperature range is 12 to 16 ºC, the results are more representative of *in situ* conditions.

## Conclusions

Given the scope of the study, it can be concluded that *Paphies ventricosa* has a thermal window of fertilisation broader than the natural seasonal spawning and development temperature range of the species. It is likely that the thermal developmental window is also broader than the environmental range of the species, but given the limited gradient tested in the present study, the exact temperatures in which development fails to occur have not been identified. It will be important to identify especially the upper limit of the fertilisation, as this could have serious implications on a species survival given the predicted levels of ocean warming in the future (Byrne & Przeslawski, 2013). While increased temperature, and ocean pH, can have negative impacts on the development of many marine invertebrate species, this is not relevant if fertilisation fails completely (Byrne, 2010).

The ideal fertilisation and early developmental temperatures identified in the present study are in line with temperatures corresponding to spawning in the southern population of *P. ventricosa* (see Chapter 2). However, the overall geographic distribution of the species does reflect the thermal fertilisation and early larval development windows identified in this study. But successful recruitment and long-term stability of a population require that all development stages be successfully completed (Byrne, 2011), indicating an on-going need to successfully carry out future thermal window experiments across a broader thermal range, and for a longer period of time in order to successfully identify the complete thermal limits of the species.

In terms of laboratory culture of the species, there is a trade off. While higher temperatures do generally produce a faster rate of early larval development, they also

produce higher rates of abnormal development, which is detrimental to overall recruitment success. Instead, a lower temperature with a lower rate of abnormal development would be ideal in a laboratory culture setting. Additionally, based on personal observations, a lower temperature is preferred in a culture setting, as it reduces the rate of ciliate infection. Finally, within the identified thermal window, temperature does have a significant effect on development, and does contribute to the observed variability in recruitment, though other factors, such as feed availability and hydrodynamics, cannot be ruled out.

# Chapter 5

# Effect of temperature and feed concentration on the early larval development of *Paphies ventricosa*

## Introduction

This study aims to increase the understanding of the combined effect of temperature and diet, in terms of feed concentration, on the early larval development of *Paphies ventricosa* from Oreti Beach, Southland. Temperature and feed availability are two of the most important factors in the recruitment of marine invertebrate larvae (Hoegh-Guldberg & Pearse, 1995). While temperature is important, diet is often thought to be more important than temperature in overall larval development (His *et al.*, 1989; Olson & Olson, 1989; Hoegh-Guldberg, 1995; Helm *et al.*, 2004). However, both can have serious impacts on development.

While there have been previous studies on the effect of temperature on larval development in *Paphies ventricosa* (see Chapters 3 and 4; Gadomski *et al.*, 2015), there have not been any on the effects of feed concentration, let alone both temperature and feed concentration. Likewise, there have not been any additional studies on the effect of feed concentration or combined effects of feed and temperature on the early larval development of the other *Paphies* species – *P. australis, P. subtriangulata*, and *P. donacina.* While these studies are lacking for *P. ventricosa* and the *Paphies* genus, there have been studies examining diet in other bivalves.

Beiras and Pérez Camacho (1994) found that in the oyster *Ostrea edulis,* larval development rate increased with increasing feed concentration from 20,000 to 300,000 algal cells $ml^{-1}$. Rico-Villa *et al.* (2009) found that in the Pacific oyster, *Crassostrea gigas*, animals fed a 1:1 mixed cell diet of 40,000 cells $ml^{-1}$ grew faster than those at 20,000 and 12,000 cells $ml^{-1}$. Liu *et al.* (2010) examined the combined effects of feed concentration and stocking density on larval development in the basket cockle, *Clinocardium nuttalli*, and found that at all larval densities, development significantly increased with an increase in feed concentration from 0 to 50,000 cells $ml^{-1}$. Marshall *et al.* (2014) examined the combined effects of larval stocking density and feed concentration in the Pacific geoduck, *Panopea generosa,* and found that at 10 larvae $ml^{-1}$, growth rate significantly decreased with an increase in feed concentration from 5,000 to 100,000 cells $ind^{-1}$ $day^{-1}$.

Food concentration studies have also been carried out on other marine invertebrate species. In addition to the mussel *Mytilus californianus* and oyster *Crassostrea gigas,* Paulay *et al.* (1985) also examined the effects of 5,000 or 50,000 algal cells $ml^{-1}$ (species dependent) diet supplementation on larval development in the ophiuroid *Ophiopholis aculeata* and the polychaete *Serpula vermicularis*. They found that for both species, the rate of development was faster during the experimental period on the enhanced diet, though not always significantly so (Paulay *et al.*, 1985). Aldana Aranda *et al.* (1989) examined the effects of temperature and feed concentration, though not in the same experiment, on the milk conch *Strombus costatus.* They found that when fed the same algal concentration of 3,000 cells $larva^{-1}$ $day^{-1}$, larvae grew fastest at a constant 28 ºC than at a constant 24 ºC or a variable ambient temperature (28 ± 2 ºC) (Aldana Aranda *et al.*, 1989). When larvae were reared at

ambient temperature, the rate of development increased with an increase in feed concentration from 1,000 to 6,000 cells larva$^{-1}$ day$^{-1}$.

In general, studies conducted to study the combined effects of temperature and feed concentration on invertebrate larval development are lacking. Invertebrates developing at one temperature should show a range of development rates since food availability is unlikely to be constant during the development period (Hoegh-Guldberg, 1995). Thus, studies examining the combined effects can help better understand the complex dynamics driving invertebrate larval recruitment. This study aims to add to the knowledge base of larval recruitment in *Paphies ventricosa* by examining the combined effects of ecologically relevant temperatures and different mixed diet algal concentrations on early larval development in the species.

## Materials and methods

### Animal collection and conditioning

In October 2013, 40 adult toheroa (100 to128 mm shell length) were collected from the Oreti Beach intertidal, Southland (46.4790° S, 168.2522° E) under the appropriate customary permit. On the same day, animals were transported to Portobello Marine Laboratory (PML), Dunedin in seawater filled 20 l buckets. In the laboratory, animals were measured, tagged, and immediately placed into four 116 l circular flow-through tanks (43.5 x 58.5 cm, flow 3 l min$^{-1}$) fitted with airstones for additional aeration and filled to 35 cm depth with sand from Oreti Beach.

Animals were conditioned for spawning by feeding the formulated food, All-G-Rich algae (Alltech, USA). Each day, tanks were supplied with 1 l of All-G-Rich slurry (5

g $l^{-1}$ seawater, equalling 0.5 g All-G-Rich $adult^{-1}$ $day^{-1}$), which was slowly introduced to the tank via a gravity fed drip bottle system, at the rate of 0.5 l $hr^{-1}$. Aeration and water flows were left on during feeding to ensure mixing. Animal conditioning continued for over 7 weeks.

### Spawning and fertilisation

Twenty-four hours before spawning, 20 animals were placed into 15 cm deep spawning trays filled with ~25 l of 15.5 ºC (± 0.5 ºC) flowing seawater. After the acclimation period, animals were induced to spawn following the methods described in Gadomski *et al.* (2015) with the exception that after initial injection, individual animals were isolated in 1 l beakers of 5µm filtered ambient seawater. Once spawning had ceased, animals were removed from their spawning beakers, and placed back into the spawning trays.

Sperm was checked for viability (actively swimming) under a microscope. Viable sperm from 5 males was filtered through a 50 µm mesh to remove any faecal matter and sand debris, pooled, and density determined ($4.1\times 10^7$ sperm $ml^{-1}$) using a haemocytometer. Eggs were checked for quality (size, shape, and colour) under a microscope. Eggs from three females were re-suspended by stirring within their beakers and then allowed to sit for 1 min to allow faecal matter and sand debris to settle out. The top 70% of the suspended egg mixture was decanted off and pooled. Pooled egg density was determined to be 76 eggs $ml^{-1}$. Sperm solution was added to pooled eggs to a final concentration of $10^6$ sperm $ml^{-1}$. Fertilisation was confirmed under microscope by the breakdown of the germinal vesicle and the observation of the fertilisation envelope and/or a polar body.

## Larval rearing

Within 1 h post-fertilisation, the fertilised egg solution was split into 27 sterilised room temperature jars (≈ 16.0 ºC) 3 l glass jars filled with 2 l of 5 µm filtered ambient temperature (15.5 ± 0.5 ºC) sea water to a give a final concentration of 15 fertilised eggs ml$^{-1}$. The jars were then placed in to one of three temperature treatments (12, 16, and 20 ° C) (see Chapters 3 and 4; Gadomski *et al.*, 2015), maintained in separate temperature controlled rooms, where they were allowed to slowly adjust to the new environmental temperature. Jars within each temperature treatment were then split into three feed concentration treatments (1,000, 10,000, and 20,000 algal cells ml$^{-1}$) with each temperature/feed concentration combination replicated three times. Every day, each jar was fed a 1:1 mixed diet of monoculture *Tetraselmis chui* and *Isochrysis galbana* at the treatment concentration. Feed concentration was determined by verifying the monocultures' densities using a haemocytometer immediately before each feeding to calculate the volume of each algal species required to feed at the required treatment concentrations. For the first 4 days post-fertilisation, then every second day, 75% water changes were carried out prior to feeding. A 1 ml sample was taken from each treatment prior to water changes, and fixed to a final concentration of 7% neutral buffered formalin.

## Larval photography and larval morphometrics

Entire 1 ml fixed samples were examined for larvae, and when present, larvae were photographed using an Olympus BX-51 compound microscope fitted with an XC-50 camera and controlled by cellSens software (Olympus Corporation). The number of larvae found per sample ranged from 0 to 35. The larval morphometrics of total shell length (TL), total shell height (TH), and hinge line length (HL) were taken, as described by Redfearn (1982), using the software ImageJ 1.48 (NIH, USA).

## Cell-specific chlorophyll-*a* concentrations

To determine cell-specific chlorophyll *a* (chl-*a*) concentrations of *Tetraselmis chui* and *Isochrysis galbana*, algal concentrations of 250, 500, 1,000, 2,000, 5,000, 10,000, and 20,000 cells $ml^{-1}$ were extracted in 90% acetone and pigment concentrations determined spectrophotometrically following the method described by Strickland and Parsons (1972). From the relationship between chlorophyll concentration and the number of cells extracted, individual cell chl-*a* concentrations were determined to be $1.367 \times 10^{-6}$ µg chl-*a* $cell^{-1}$for *Tetraselmis chui* and $7.79 \times 10^{-7}$ µg chl-*a* $cell^{-1}$ for *Isochrysis galbana*. Therefore, at a 1:1 *Tetraselmis chui: Isochrysis galbana* feeding ratio, our feeding treatments resulted in the total chl-*a* amounts of 2.146, 21.460, and 42.920 µg chl-*a* fed to each treatment of for 1,000, 10,000, and 20,000 cells $ml^{-1}$, respectively.

## Environmental factors

Regional average chl-*a* concentrations were derived from the monthly composite Aqua MODIS chlorophyll-*a* 9 km dataset (www.oceancolor.gsfc.nasa.gov). The sea surface examined encompassed an area of 2916 $km^2$ (see Figure 2.4) and was determined to be that representing the coastal region most likely encountered by *P. ventricosa* larvae originating from Oreti Beach and living in the plankton for 30 days. When available, data was extracted for the months of September to March , the spawning period of *P. ventricosa* at Oreti Beach, from September 2002 to March 2015 (see Chapter 2; Gadomski & Lamare 2015), following the methodology detailed in Gadomski and Lamare (2015). Average sea surface temperature (SST) data for the same region was derived from the monthly composite Aqua MODIS sea surface temperature (11µ daytime) dataset for same months as the chl-*a* following the methodology detailed in Gadomski and Lamare (2015). Three month average

Oceanic Niño Index (ONI) values were obtained for the Niño 3.4 region for the years 2000 to 2014 (www.cws.ncep.noaa.gov).

### Statistical analyses

Statistical analyses were carried out on larval size (TL) data from 3, 6, and 17 days post-fertilisation. These sampling days were chosen because larvae were recovered from all treatment replicates at these times. Data were initially tested via a generalised least squares (GLS) model to find the best fit model using Akaike's Information Criterion. Initial analysis was performed using the 'gls' function from the 'nlme' package in R ver. 3.2.1 (R Core Team, 2016). The resulting best fit model had weighting to allow for non-constant variance in the data across the different combinations of temperature and time. Since the larvae were not able to be individually identified at multiple time points, it was not possible to match measurements across time and, therefore, the correlation amongst repeated measures was not able to be incorporated into the model. However, it is expected that the effects of this on the model will be relatively minor as the time separation between measurements is relatively large.

When statistically significant differences ($\partial < 0.05$) in the size of larvae (TL) were found, the interactions among sampling day, temperature, and feed concentration were examined in a three-way ANOVA. Significant interactions between treatments were identified using Tukey's HSD *post hoc* test using the 'lsmeans' packing in R ver. 3.2.1.

The effect of temperature and food concentration on the relationship between total larval shell length (TL) and larval shell height (TH) was examined using an analysis

of covariance (ANCOVA) on ln(*x*) transformed measurements. All ANCOVA analyses were performed using JMP 12 (SAS Institute, USA).

## Results

### Early larval development

#### *Development at 12 ºC*

Early larvae developed faster at 12 ºC than at 16 and 20 ºC, and within 12 ºC, larvae initially developed the fastest at 10,000 cells $ml^{-1}$ (Figure 5.1), but by the end of the experiment, were largest at 20,000 cells $ml^{-1}$. When fed 1,000 cell $ml^{-1}$, larvae grew from a size of 93.45 µm TL and 70.95 µm TH at 2-d post-fertilisation to a size of 121.63 µm TL and 100.23 µm TH at 17-d post-fertilisation for a total average growth of 1.9 µm $day^{-1}$ in TL and 2.0 µm $day^{-1}$ in TH (Figure 5.1). At 10,000 cell $ml^{-1}$, larvae grew from 89.57 µm TL and 70.53 µm TH at 2-d post-fertilisation to 153.99 µm TL and 132.43 µm TH at 17-d post-fertilisation for a total average growth of 4.3 µm $day^{-1}$ in TL and 4.1 µm $day^{-1}$ in TH (Figure 5.1). At 20,000 cell $ml^{-1}$, larvae grew from 93.54 µm TL and 72.99 µm TH at 2-d post-fertilisation to 167.25 µm TL and 147.19 µm TH at 17-d post-fertilisation for a total average growth of 4.9 µm $day^{-1}$ in TL and 4.9 µm $day^{-1}$ in TH (Figure 5.1). Larval shell growth exhibited the same pattern for both length and width, indicating normal growth (Figure 5.1). The greatest difference (46.11 µm) in the average larval size among feeding treatments was on day 17. Larvae reached the umbonate stage at all feed concentrations, but did not reach the pediveliger stage in any treatment by the end of the measurement period.

***Development at 16 ºC***

When reared at 16 ºC, early larval development was faster than 20 ºC, but slower than 12 ºC, and within the temperature, was fastest at 10,000 cells $ml^{-1}$ (Figure 5.1). When fed 1,000 cell $ml^{-1}$, larvae grew from 90.77 µm TL and 70.07 µm TH at 2-d post-fertilisation to 109.76 µm TL and 87.61 µm TH at 17-d post-fertilisation for a total average growth of 1.3 µm $day^{-1}$ in TL and 1.2 µm $day^{-1}$ in TH (Figure 5.1). At 10,000 cell $ml^{-1}$, larvae grew from 99.40 µm TL and 76.74 µm TH at 3-d post-fertilisation to 141.76 µm TL and 121.81 µm TH at 17-d post-fertilisation for a total average growth of 3.0 µm $day^{-1}$ in TL and 3.2 µm $day^{-1}$ in TH (Figure 5.1). When fed 20,000 cells $ml^{-1}$, larvae grew from 90.86 µm TL and 70.72 µm TH at 2-d post-fertilisation to 128.46 µm TL and 108.39 µm TH at 17-d post-fertilisation for a total average growth of 2.7 µm $day^{-1}$ in TL and 2.7 µm $day^{-1}$ in TH (Figure 5.1). Like 12 ºC, larval shell growth exhibited the same pattern for both length and width, indicating normal growth (Figure 5.1). The greatest difference (26.32 µm) in average larval size among feeding treatments was on day 12. Like at 12 ºC, larvae reached the umbonate stage at all feed concentrations, but did not reach the pediveliger stage by the end of the measurement period.

***Development at 20 ºC***

Early larval development was the slowest at 20 ºC, when compared to 16 and 12 ºC, but was fastest at 1,000 cells $ml^{-1}$ within the treatment (Figure 5.1). When fed 1,000 cell $ml^{-1}$, larvae grew from 100.64 µm TL and 76.62 µm TH at 3-d post-fertilisation to 126.91 µm TL and 108.39 µm TH at 17-d post-fertilisation for a total average growth of 1.9 µm $day^{-1}$ in TL and 2.7 µm $day^{-1}$ in TH (Figure 5.1). At 10,000 cell $ml^{-1}$, larvae grew from 92.85 µm TL and 70.10 µm TH at 3-d post-fertilisation to 112.01 µm TL and 91.70 µm TH at 17-d post-fertilisation for a total average growth

of 1.4 μm $day^{-1}$ in TL and 1.5 μm $day^{-1}$ in TH (Figure 5.1). At 20,000 cell $ml^{-1}$, larvae grew from 92.82 μm TL and 71.43 μm TH at 3-d post-fertilisation to 109.79 μm TL and 87.44 μm TH at 17-d post-fertilisation for a total average growth of 1.2 μm $day^{-1}$ in TL and 1.1 μm $day^{-1}$ in TH (Figure 5.1). Again, larval shell growth exhibited the same pattern for both length and width, indicating normal growth (Figure 5.1). The greatest difference (22.91 μm) in average larval size among feeding treatments was on day 12. Like both 12 and 16 ºC, larvae reached the umbonate stage at all feed concentrations, but failed to reach the pediveliger stage by the end of the measurement period.

### Statistical analyses

A GLS model using Akaike's criterion, showed that the three way interaction between sampling day, temperature, and feed concentration was the best fit model for the day, and the three-way ANOVA showed a significant difference ($p < 0.0001$) in larval shell length (Table 5.1). Within each significantly different sampling day, *post hoc* tests indicated all feed concentrations were significantly different from each other. *Post hoc* tests also indicated two-way significant interactions between temperatures within each feed concentration. For the feed concentrations of 10,000 and 20,000 cells $ml^{-1}$, each temperature treatment was significantly different from each other on each sampling day (Figure 5.2). For 1,000 cells $ml^{-1}$, at 3-d post-fertilisation, there was no significant difference between temperatures; at 6-d post-fertilisation, 12 and 16º C were significantly different from 20 º C, but no from each; and at 17-d post-fertilisation, 12 and 20 ºC were significantly different from 16 ºC, but not from each other (Figure 5.2). The means table showing the two-way interaction between temperatures within each feed concentration are presented in Appendix II.

The allometric relationships between larval length and width for larvae from the three pooled temperature treatments and three pooled feed concentration treatments visually appeared to have similar slopes. While the slopes appear very similar (Figure 5.3), ANCOVA indicated that the slopes were in fact significantly different for both temperature (Temperature × TL, $F_{(2, 1213)} = 4.608$, $p = 0.010$; Table 5.2), and feed concentration, (Feed Conc. × TH, $F_{(2, 1213)} = 16.682$, $p < 0.0001$; Table 5.2). Closer inspection, shows that whether significantly different or not, slopes increase as either temperature or feed concentration increases, when pooled respectively.

**Table 5.1. Type III analysis of variance (ANOVA) model parameters resulting from the generalised least squares (GLS) model, from which the three-way ANOVA taking into consideration sampling time (3, 6, and 17 days post-fertilisation), temperature (12, 16, and 20 ºC), and feed concentration (1,000, 10,000, and 20,000 cells ml$^{-1}$) was run. The means tables for the two-way interaction between temperature and feed concentration included in the model are presented in Appendix II.**

| **Fit Model** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| Intercept | 1, 667 | 263,122.89 | < 0.0001 |
| Temperature | 2, 667 | 350.80 | < 0.0001 |
| Feed Concentration | 2, 667 | 4.83 | < 0.0001 |
| Time | 2, 667 | 1,039.55 | 0.0082 |
| Temperature × Feed Concentration | 4, 667 | 27.08 | < 0.0001 |
| Temperature × Time | 4, 667 | 45.09 | < 0.0001 |
| Feed Concentration × Time | 4, 667 | 50.14 | < 0.0001 |
| Temperature × Feed Concentration × Time | 8, 667 | 12.74 | < 0.0001 |

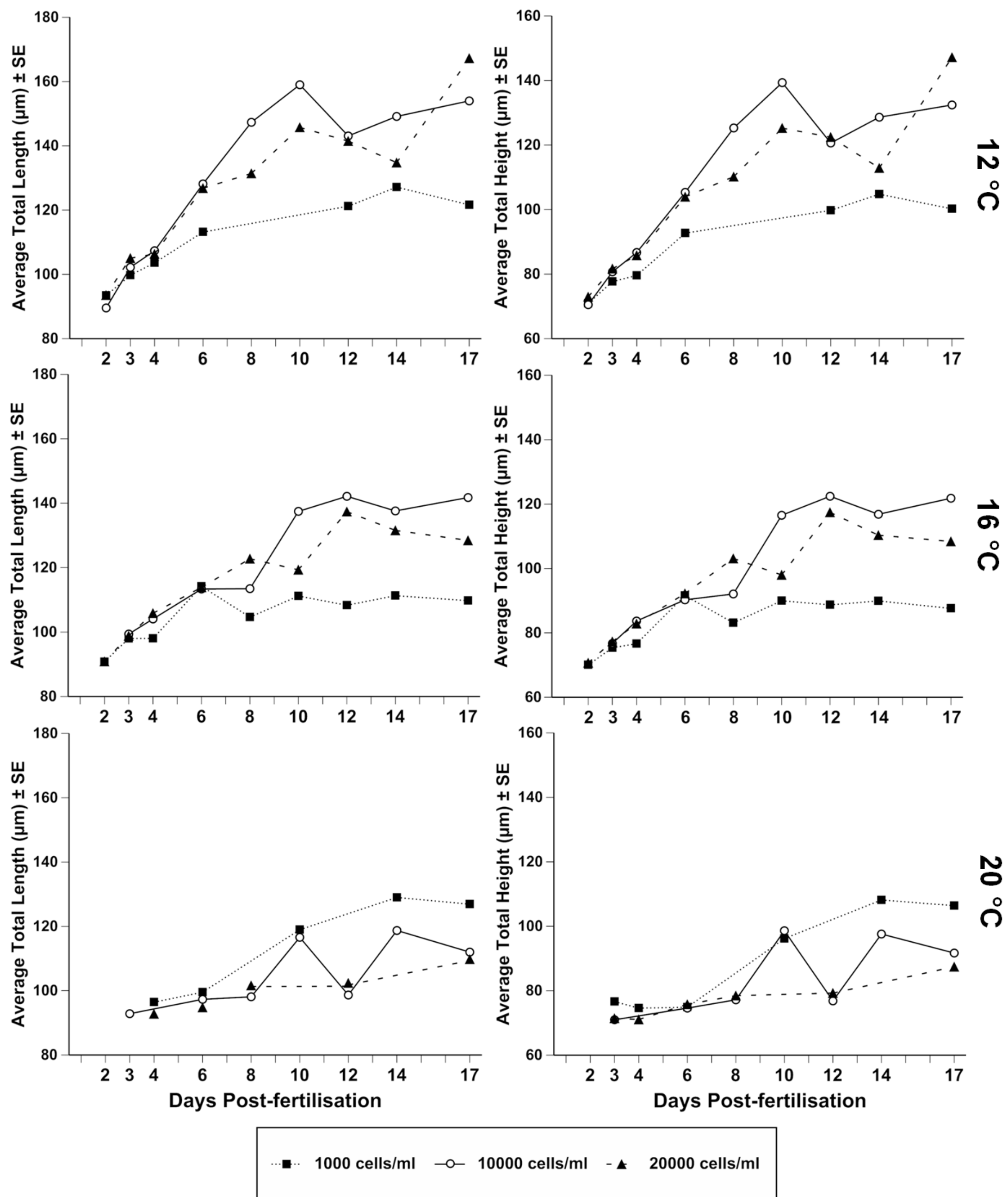



**Figure 5.1. Increase in average total shell length (± SE) and average total shell height (± SE) for *Paphies ventricosa* reared over a 17-d period at three algal feed concentrations (1,000, 10,000, and 20,000 cells ml$^{-1}$) within three different temperatures (12, 16, and 20 ºC). *n* = average of three replicate jars, with variable number of larvae measured from each replicate at given time point. When error bars are not visible, it is because the variation around the mean is too small to show on the graphs.**

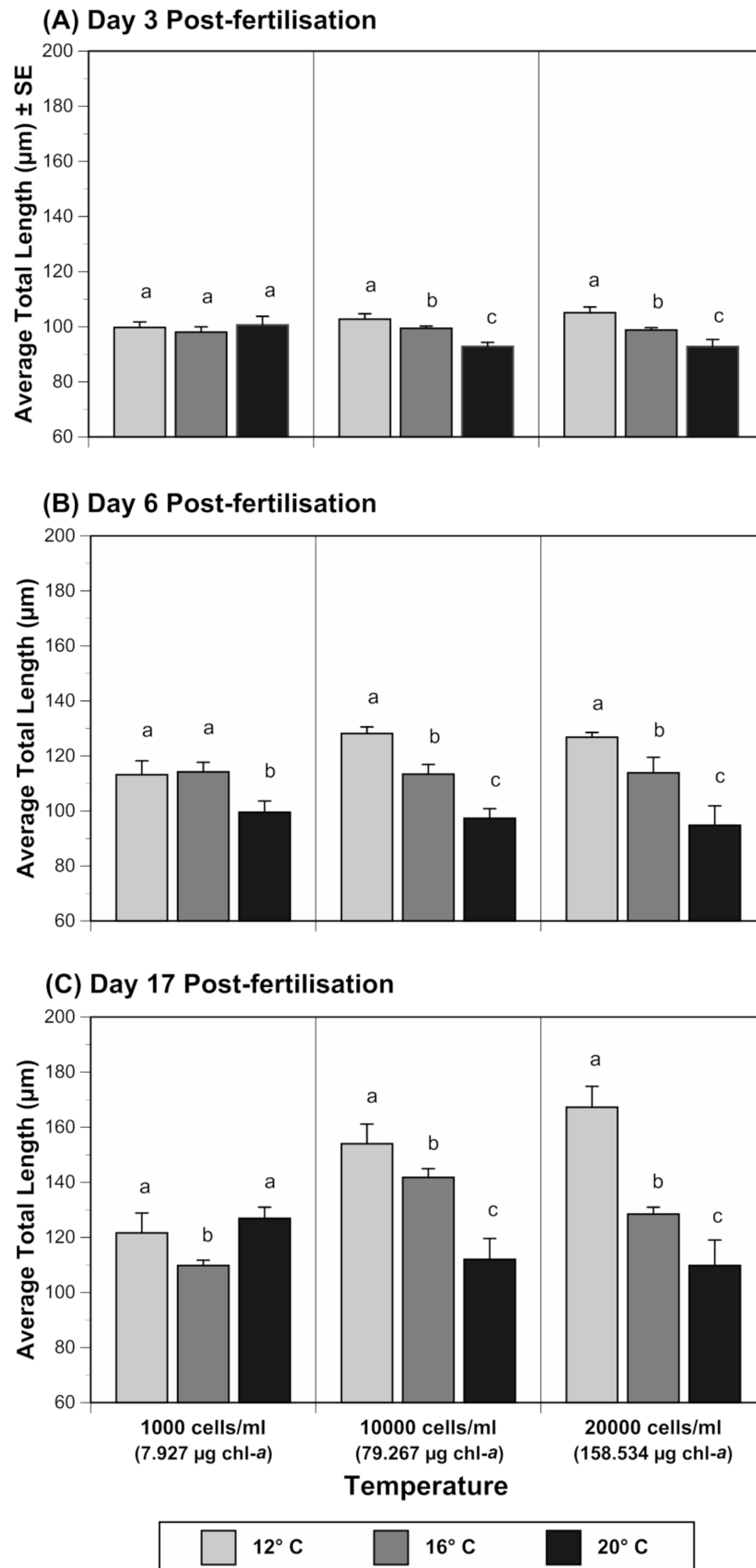



**Figure 5.2. Average total shell length of Paphies ventricosa at (A) 3, (B) 6, and (C) 17 days post-fertilisation when reared on algal feed concentrations of 1,000, 10,000, and 20,000 cells ml$^{-1}$ within the temperatures of 12, 16, and 20 ºC. Significant differences in total length among temperatures within each feed concentration are indicated by lowercase lettering. Error bars are the 95% confidence interval.**

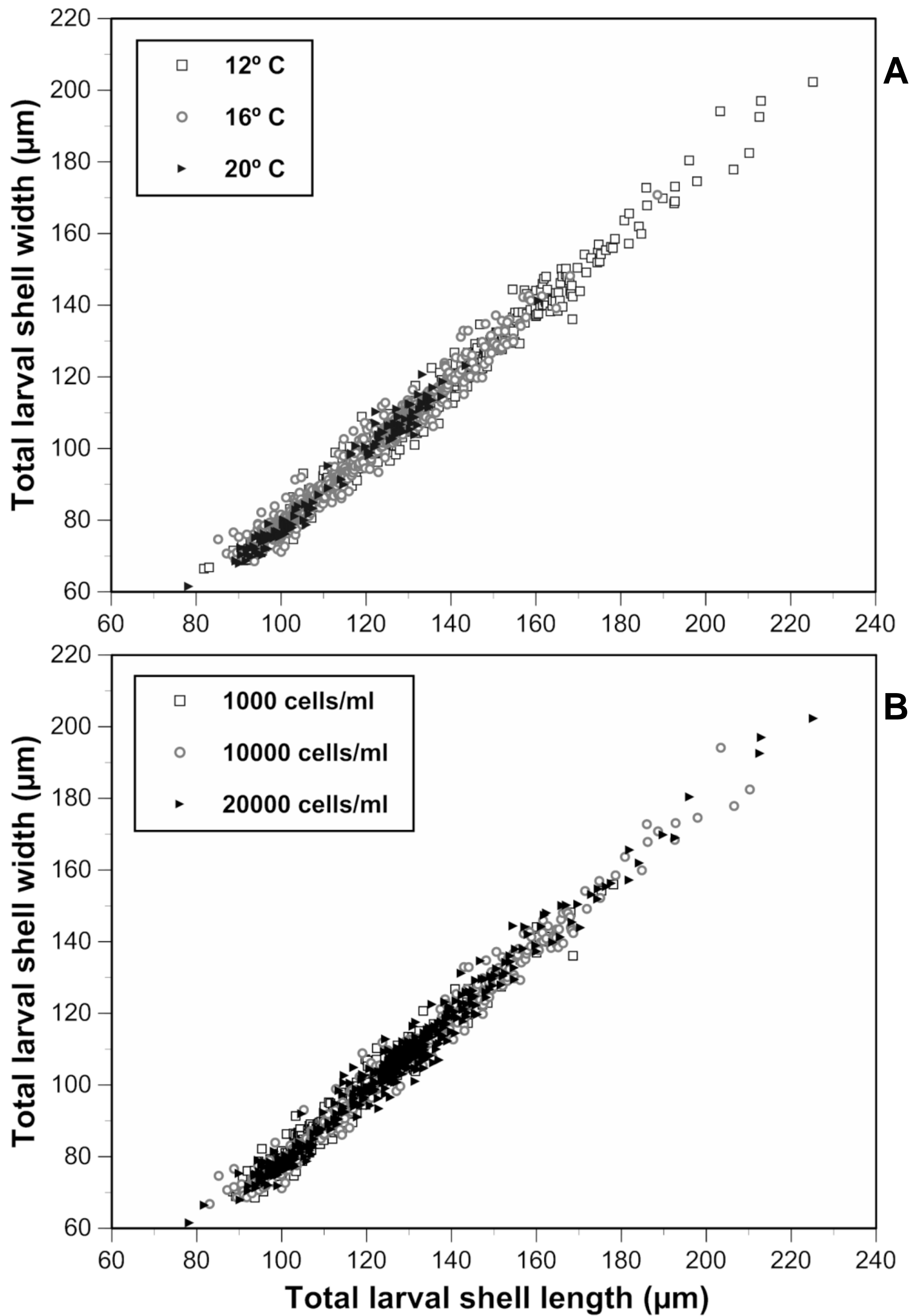



**Figure 5.3. Allometric relationship between shell length and shell width for *Paphies ventricosa* larvae reared over a 17-d period at three experimental temperatures (12, 16, and 20 ºC) and three different feed concentrations (1,000, 10,000, and 20,000 cells ml$^{-1}$. The data has been pooled into (a) temperature and (b) feed concentration.**

**Table 5.2. Analysis of covariance (ANCOVA) of total larval shell length versus larval shell height for *Paphies ventricosa* larvae reared over a 17-day period in three experimental temperatures (12, 16, and 20 °C) and at three different feed concentrations (1,000, 10,000, and 20,000 cells $ml^{-1}$). Analyses are presented for (a) differences among the three temperatures and (b) differences among the three feed concentrations,**

(a) Differences among the three experimental temperatures

| **Source** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| Model | 2, 1211 | 0.84 | 0.4314 |
| Length | 1 | 12904 | < 0.0001 |
| Temperature | 2 | 0.2223 | 0.8007 |
| Temperature × Length | 2 | 4.6079 | 0.0101 |

(b) Differences among the three experimental feed concentrations

| **Source** | ***Df*** | ***F-ratio*** | ***p*-value** |
|---|---|---|---|
| Model | 2, 1211 | 3.89 | 0.0207 |
| Length | 1 | 21758 | < 0.0001 |
| Feed Conc. | 2 | 4.5131 | 0.0111 |
| Feed Conc. × Length | 2 | 16.682 | < 0.0001 |

### *In situ* physical factors

From 2000 to 2014, average seasonal SST was within the range of 12.5 to 14.1 ºC, with maximum average monthly SST within a spawning season in the range of 14.4 to 17.2 ºC (Figure 5.4A). Average seasonal chl-*a* concentrations were in the range of 1.408 to 2.328 mg $m^3$ (Figure 5.4B), which is equivalent to approximately 158 – 284 algal cells $ml^{-1}$ of experimental mixed diet. Maximum average monthly chl-*a* within a spawning season was in the range of 2.10 to 5.47 mg $m^{-3}$.

ONI values for the years 2000 – 2014, indicate warmer El Niño events (ONI ≥ 0.5 for at least 5 consecutive months) during the 2002, 2004, 2006, 2009, and 2014 spawning seasons for *Paphies ventricosa* (Figure 5.4C). Alternately, La Niña events (ONI ≤ -0.5for at least 5 consecutive months) occurred during the 2000, 2005, 2007, 2010, 2011, and 2012 spawning seasons (Figure 5.4C).

There was a general correlation between the warmer El Niño events and increased *in situ* average chl-*a* concentration, especially noticeable during the 2006 and 2009 spawning seasons (Figure 5.4). Correspondingly, there was a general reduction in regional productivity (chl-*a*) during the colder La Niña events, such as during the 2007 and 2010 spawning seasons. The relationship between ONI Index and SST was less evident. It is expected that with El Niño events, both the regional average and maximum would be correspondingly higher, which they are not. However, it needs to be taken into consideration that the ONI Index is calculated for a much larger extent (south western Pacific Ocean), much of which is open water, and is a three month averaged value while the regional SST is calculated for a much smaller area (see Figure 2.4) comprising mostly near shore shallow waters, and is averaged over a season. The MODIS derived average seasonal SST and chl-*a* concentration did not show any correlation, either positive or negative, with each other.

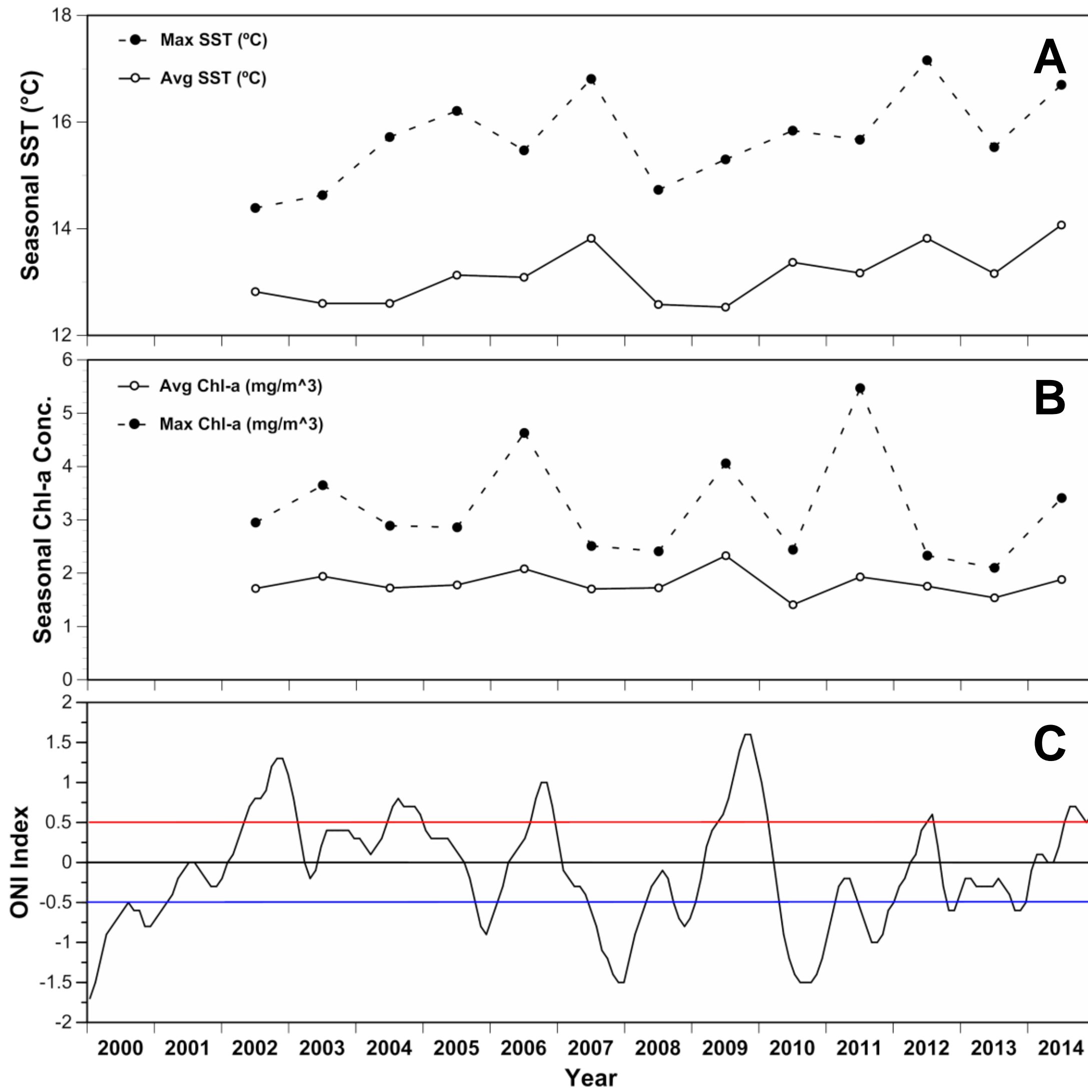



**Figure 5.4. Local environmental factors at Oreti Beach from 2000 to 2014. (A) MODIS derived SST (ºC), showing both seasonal average (Sept. – Mar.) and maximum monthly temperature during the season. (B) MODIS derived chl-*a* concentrations (mg m$^3$) showing both seasonal average (Sept. – Mar.) and maximum monthly chl-*a* during the season. Both SST and chl-*a* are only reported from 2002 onwards, as the datasets originate in 2002. (C) Three month average ONI values (Jan. – Dec.) - saasonal peaks above the red line (0.5) are warm El Niño events; seasonal peaks below the blue line (-0.5) are cool La Niña events.**

## Discussion

In this study, the effect of both temperature and feed concentration on early development of *Paphies ventricosa*, up to 17-d post-fertilisation, has been examined. The effect of temperature on the larval development of *P. ventricosa* has already been described by Gadomski *et al.* (2015) up to 39-d post-fertilisation. In the current study, the rate of development was similar to that described by Gadomski *et al.* (2015) – at 3-d post fertilisation there was little difference in TL between pooled temperature treatments in both studies. However, in the current study, TL was > 95 μm for all temperatures at 3-d post-fertilisation, while TL was reported in Chapter 3 (Gadomski *et al.*, 2015) to be ≈ 70 μm for all temperatures at 3-d post-fertilisation. This difference in early veliger TL could be the result of a number of factors.

In the current study, the period of larval rearing was shorter than that described in Gadomski *et al.* (2015) (17 days versus 39 days). This was because the larval cultures crashed after 17 days in the current experiment, likely due to biofilm, bacteria, and ciliate contamination (pers. observ.). Had the sterility been better controlled in the current experiment, it is likely the larvae would have reached pediveliger like in the experiments described in Chapter 3 (Gadomski *et al.*, 2015), which were conducted in air tight containers versus the foil covered jars utilised in the present study.

One such factor is a larger initial egg size. Increased egg size is a possible result of higher lipid content and egg quality, which could then result in a larger initial veliger (Wilson *et al.*, 1996). The possible higher egg quality can be attributed to more successful conditioning of broodstock (Utting & Millican, 1997). In the current study, broodstock were conditioned with All-G-Rich supplemental algae as opposed

to the cultured algae used in Chapter 3 (Gadomski *et al.*, 2015). Compared to a traditional cultured algae diet, All-G-Rich has higher levels of the fatty acid docosahexaenoic acid (DHA) (Appendix I). DHA diet supplementation has been shown to increase egg lipid content in scallops (Caers *et al.*, 1999; Sühnel *et al.*, 2012).

The toheroa collected in 2012 (see Chapter 3; Gadomski *et al.*, 2015) and 2013 (present study) were collected from similar *in situ* chl-*a* conditions (Figure 5.5), both times in October. At PML, they were maintained in the same tanks and for the same amount of time (≈ 7 weeks). The only major difference between the two lots of broodstock was the conditioning diet. Therefore, it is likely that the larger 3-d post fertilisation size in the current study, when compared to Chapter 3 (Gadomski *et al.*, 2015), was due to increased egg quality from the broodstock conditioning diet, as opposed to rapid larval growth (≈ 25 µm) in the first 24 to 36 h of being fed a mixed diet, as all three feed concentrations at all three temperatures had the same TL at 3-d post-fertilisation.

Another possible explanation of the difference in 3-d post-fertilisation veliger sizes between experiments relates to the offspring temperature-size rule, which is that offspring and egg size vary inversely with temperature, and that eggs are often larger at the start of the spawning season compared to the end of the spawning season (see Collin & Ochoa, 2016). From the MODIS SST regional monthly average temperature data, it is found that animals collected in October 2012 and 2013 were collected during average SSTs of 11.21 and 11.67 ºC, respectively. By the time animals were spawned in the lab after ≈ 7 weeks of conditioning, the animals used in 2012 would have exposed to higher temperatures in the lab than those in 2013 (≈ 1 ºC), which could have an effect on initial egg size, causing smaller 3-d veligers in

2012 than 2013, thus following the offspring temperature size-rule. Though, given the small temperature variance, it is more likely that the conditioning method had greater effect on initial egg, and therefore, offspring size.

In the present study, when comparing temperature treatments (feed concentrations pooled), the result is different from the anticipated result of slower growth at colder temperatures, as found for the species in Chapter 3 (Gadomski *et al.*, 2015). The present study found that when fed a 1:1 diet of *I. galbana* and *T. chui* at 10,000 cells $ml^{-1}$, larvae reared at 12 ºC grew at a faster rate than those at 16 and 20 ºC. When compared to larvae fed a single culture diet of *T. chui* at 10,000 cells $ml^{-1}$ (see Chapter 3; Gadomski *et al.*, 2015), larvae reared at 12 ºC grew at a slower rate than those at 20 and 16 ºC. While a somewhat similar pattern has been observed in the basket cockle, *Clinocardium nuttalii* (Liu *et al.*, 2010), the result of the present study is opposite of the previous findings for the species (Gadomski *et al.*, 2015). The current study's results are also opposite to the findings in many other bivalve species, such as the mussels *Mytilus edulis* (Pechenik *et al.*, 1990), *Perna virdis* (Manoj Nair & Appukuttan, 2003), *Limnoperna fortunei* (Cataldo *et al.*, 2005), *Mytilopsis leucophaeta* (Verween *et al.*, 2007), *Crassostrea gigas* (Rico-Villa *et al.*, 2009; Parker *et al.*, 2010; Kheder *et al.*, 2010), *Saccostrea glomerata* (Parker *et al.*, 2009), and *Mytilus galloprovincialis* (Sánchez-Lazo & Martínez-Pita, 2012). However, the separation in developmental rates between temperatures around 8-d post-fertilisation is similar in both *P. ventricosa* studies.

While temperature is important in determining larval development rates, it is thought to be second only to diet (His *et al.*, 1989; Helm *et al.*, 2004). Across a gradient of feed concentrations at a single temperature, one would expect growth to increase with increasing feed concentration, up to the point when growth plateaus (Sprung,

1984b; Beiras & Pérez-Camacho, 1994; Pérez-Camacho *et al.*, 1994; Rico-Villa *et al.*, 2009; Liu *et al.*, 2010). In the present study, within a temperature treatment, the effect of feed concentration became more pronounced as development progressed (Figure 5.2).

However, the chl-*a* concentrations fed during the experiment were, with the exception of 1,000 cells ml$^{-1}$ (7.927 µg chl-*a*), were far greater than concentrations any larvae is likely to encounter *in situ* at Oreti Beach, as from 2000 to 2014, the highest monthly average chl-*a* in the study area was less than 3 µg l$^{-1}$, and the maximum monthly chl-*a* concentration within a spawning season during this period was less than 6 µg l$^{-1}$. This level is still less than the lowest experimental feed concentration of 1,000 cells µg l$^{-1}$ which is a realistic, if not common, level encountered *in situ*.

## Conclusions

Of the temperature treatments examined, 12 ºC is the closest temperature southern *Paphies ventricosa* larvae would encounter most frequently *in situ* (Figure 5.4A). Thus, the results of the 12 ºC larval rearing are the most ecologically relevant to the population at Oreti Beach. Additionally, given the ideal rearing temperature of 12 ºC, and 12 ºC being close to the *in situ* conditions of the southern populations, the species may be living close to its thermal maximum for successful development, and thus larval recruitment. Given the predicted temperature increase in the southern populations' natural range by 2100 (IPCC, 2014), larval development and recruitment are likely to be negatively impacted. Based on the results of this study, for laboratory rearing of southern *Paphies ventricosa*, it is recommended that larvae

be cultured at 12 ºC and fed a diet of 20,000 cells $ml^{-1}$ daily with daily water changes with careful efforts made to maintain sterility of the larviculture set up.

At the realistic feeding rate of 1,000 cells $ml^{-1}$, by 17-d post-fertilisation, the average shell length at 12 ºC was still larger than at 16 ºC, a possible, but not probable temperature experienced *in situ*. Given that larvae usually encounter temperatures and feed concentrations at the low end of the experimental range, one would expect near constant rates of recruitment, especially given the reduced effect of ENSO on the region. However, surveys have shown that this is not the reality (Beentjes, 2010). While the temperature, chl-*a*, and ONI will influence the larval development of *P. ventricosa*, the findings of this study point to an additional factor, or series of factors, that is the primary force driving juvenile recruitment of *Paphies ventricosa* at Oreti Beach.

# Chapter 6

# General Summary and Conclusions

Reproduction in the southern population of *Paphies ventricosa* was examined in 2011 and 2012 and identified two distinct spawning events in a season – one in late spring/early summer and one in late summer/early autumn, the initial event corresponding to seasonal warming of coastal surface waters (see Chapter 2). The occurrence of two spawning events indicates that there are potentially two waves of beach recruits each year. Similar studies on the northern populations found a prolonged season of trickle spawning, with spawned individuals typically found within the population year round (Redfearn, 1974). This could be due to less temperature fluctuation experienced by the northern population compared to the southern population (Redfearn, 1974; Smith, 2003), making them less sensitive to thermal spawning cues.

For Oreti Beach, spawning appears to occur as early as September for the initial spawning event, and as late as June for the second. This difference in spawning period among populations has similarly been observed in the other *Paphies* species *P. subtriangulata* (Grant & Creese, 1995) and *P. australis* (Hooker & Creese, 1995) from different populations. Along Oreti Beach, there also appears to be a spatial component to spawning, with a tendency for individuals to be more gametogenically advanced with increasing distance north along the beach. This could be explained by the possibility of greater nutrient input from the Jacobs River Estuary and general southeast currents and longshore transport (pers. observ.; pers. comm, 2012).

Temperature may play an important role in the recruitment of *Paphies ventricosa*. This study has found that temperature has a significant effect on fertilisation across a range of temperatures (see Chapter 4). If the fertilisation window identified using individuals from the southern population holds true for individuals from the northern population, which may not be the case as thermal windows can vary among separated populations of the same species, this could help contribute to the population crashes of the past (see Chapter 1). The mid to high range of the thermal gradient examined are temperatures experienced by the northern populations during the main spawning months, when fertilisation occurs. Low fertilisation leads to low recruitment, and if combined natural mortality and harvest are greater than recruitment into a population, that population may decline. The southern populations, however, were not subjected to the same level of intense harvest as the northern populations and still suffered population crashes. So while temperature likely has an effect, it is not the primary factor driving recruitment to the larval stage in the southern populations.

Temperature also has a significant effect on embryonic and larval development. Across a thermal gradient, embryonic development (< 48 h post-fertilisation) was delayed at the low (< 11 ºC) and at the high end (> 20 ºC), the rate of abnormal development remained low (<30%) (see Chapter 4). When fed a single culture diet, reared veliger larvae grew faster at 20 ºC, followed by 16 and 12 ºC (see Chapter 3). This follows the expected pattern of accelerated growth at warmer temperatures in marine bivalve larvae (e.g. Pechenik *et al.*, 1990; Widdows, 1991; Cragg, 2006; Parker *et al.*, 2009). However, even at 20 ºC, it took a 31 d to reach the pediveliger stage, which for a temperate species is a relatively long time (Cataldo *et al.*, 2005; Aranda-Burgos *et al.*, 2014), especially when Redfearn (1982) observed pediveligers

at 22 d at 25 ºC in the northern population. When reared larvae were fed a 1:1 mixed culture diet, and again reared at 12, 16, and 20 ºC, the opposite trend was found (see Chapter 4). That is, that larval development was accelerated at lower temperatures. This further suggests that while temperature does play a role in larval recruitment, diet may have a greater role in driving recruitment in *Paphies ventricosa*.

Fertilisation in *Paphies ventricosa* across a thermal gradient shows a reduction at the lower and higher temperatures, including the natural ranges in temperature. Results from the present study suggest that the northern population of toheroa may be partially fertilisation limited. Embryological and early larval development in *Paphies ventricosa* across a natural thermal gradient is also delayed at the low and high ends. The observed fertilisation and early developmental windows for the southern population would indicate that the northern population would be more susceptible to predicted levels of sea surface temperature rise due to ocean warming by 2100 (Byrne, 2010; Byrne & Przeslawski, 2013) if they have the same thermal window, as they already experience temperatures at the high end of the examined temperature ranges, where fertilisation and developmental rates dropped and abnormal development started to increase. However, the thermal fertilisation and developmental windows have not been examined in the northern population of *P. ventricosa*, so direct comparisons may not be realistic. A study examining the thermal window of fertilisation and larval development in the northern population for comparison to the present study would help contribute to the overall understanding of recruitment in the species as a whole, and give greater insight to the sensitivity of the northern population to predicted levels of ocean warming compared to the southern population.

Another factor that could have a negative impact on fertilisation success is the observed asynchrony in gonad maturity in the population at Oreti Beach (see Chapter 2; Gadomski & Lamare, 2015). When males and females are not spawning at the same time, there is a limit to the number of sperm present to fertilise the spawned eggs. As the sex ratio did not significantly deviate from 1:1 (see Table 2.2), fertilisation is unlikely to be limited by to too few of a particular gender.

When southern *Paphies ventricosa* larvae are fed a single culture algal diet, developmental rates follow the expected pattern across a temperature range, and are in line with findings for the same diet in northern toheroa (Redfearn, 1982). However, when fed a mixed diet, the pattern deviates, which could be due to the species selected for the diet, rather than the mixed diet itself. Unfortunately, in terms of northern toheroa, there is no similar study to compare to, as there have been no larval development studies using a mixed diet on the northern population. Testing a mixed diet in the northern population and comparing them to the results found in the present study, would be helpful in further understanding recruitment in the species.

A few ideas have been put forth as to why there are observed differences between the northern and southern toheroa in terms of population stability/instability, adult size, and spawning (Various, pers. comm.). One such hypothesis is that the northern and southern populations are actually separate subspecies. Preliminary DNA analysis has ruled this false, showing that they are in fact the same species, but that the northern population is more genetically diverse than the southern population of *P. ventricosa* (Ross *et al.*, 2014). An early hypothesis for this result is that the southern population is the result of early Ngai Tahu Māori bringing back northern toheroa from the North Island and establishing the southern population, resulting in a reduced gene pool originating from a few transplanted individuals (Ross *et al.*, 2014). Another

hypothesis resulting from the preliminary DNA analysis is that the remaining genetic variant found from the southern population is the only variant that was able to adapt to the colder temperatures of Southland. Unfortunately, this was determined using only five tissue samples from the South Island, and compared to dozens from the North Island. The researchers are hoping to conduct a more in depth genetic analysis of the remaining populations in the near future (Ross *et al.*, 2014)

Another observed difference between the populations is the ease of spawning. *Paphies ventricosa* from the northern population appear to spawn more readily than the southern population. Northern toheroa were able to be induced to spawn using thermal shock and introduction of dilute sperm solution from stripped males (Redfearn, 1982), a single injection of serotonin (Smith, 2003), and by shaking transport buckets (2013, pers. comm.). Mandeno (1999) was only able to induce spawning via thermal shock after 2 h of treatment protocol. During spawning pilot studies, transport from Oreti Beach to PML, shaking of the spawning trays, a single injection of serotonin, dilute sperm solution, UV sterilisation, and thermal shock all failed to induce spawning in southern individuals (pers. observ.), hence the development of the spawning protocol described in Chapter 3 (Gadomski *et al.*, 2015). This difference in ease of spawning could be due to the fact that northern toheroa have been found to have a prolonged spawning period, and spawned individuals are found in the population nearly year round (Redfearn, 1974). A simultaneous spawning experiment after prolonged simultaneous conditioning of broodstock under the same conditions using both southern and northern *P. ventricosa* would be useful in further understanding differences in spawning.

Another critical factor affecting the success of laboratory spawning is the condition of the broodstock. Improperly or not conditioned broodstock are unlikely to spawn

successfully, if at all. Mandeno (1999) induced spawning immediately after collection from Oreti Beach and transport to PML, so the broodstock would have been wild conditioned. Smith (2003) made note of keeping wild caught animals in the lab in flowing seawater until needed, but not the amount of time they were kept or if they were fed during that time. Redfearn (1982) also kept animals in the lab in flowing seawater until needed, but again, made no mention of how long they were kept or if they were fed.

Spawning was successful using broodstock conditioned using both cultured mixed algae and the formulated feed, All-G-Rich algae (see Chapters 4 & 5). However, the high in docosahexaenoic acid (DHA) formulated feed was more successful at broodstock conditioning, and less labour intensive. The amount of 10 l cultured algae adult$^{-1}$ day$^{-1}$ needed to properly condition the broodstock (2013, pers. comm.) was not possible to produce at PML. It was possible to produce enough cultured algae to prevent the broodstock from reabsorbing their gonad tissue, but not enough to maintain healthy condition and fully ripen the gonad. With the formulated feed, additional serotonin induced spawnings were successful on animals collected in October 2013 and maintained continuously on All-G-Rich - through to February 2014 (pers. observ.). For future lab rearing of toheroa, or other bivalve species, when large-scale cultured algae production is not feasible, a formulated feed high in DHA, like All-G-Rich, is highly recommended for broodstock conditioning and laboratory maintenance.

It is interesting that since commercial harvest and open days have stopped, and harvest is tightly controlled through the implementation of customary permits, the populations of *Paphies ventricosa* have not recovered to historic levels. So what has changed? Vehicles still travel across many of the beaches en masse like they did in

the past. While it is not known how many vehicles have travelled onto the beach in the past, automated traffic counters placed at the Oreti Beach Main Entrance from 2010 to 2012 detected an average of 256 vehicles entering the beach per day (Moller *et al.*, 2014). Moller *et al.* (2014) found that vehicle traffic, which is mostly higher up the beach, was most detrimental to juvenile toheroa, which are found higher up the beach than adults. Cars were found to contribute 15% (12 month cumulative) to natural juvenile mortality rates, ute type vehicles and 4WDs adding 12%, and motorbikes adding 1% (Moller *et al.*, 2014). Vehicular juvenile mortality was up to 72% annually within 2 km north and south of the Main Entrance (Moller *et al.*, 2014), though few adults are found in this area (Beentjes *et al.*, 2009; Berkenbusch *et al.*, 2015).

As a local conservation strategy, a complete ban of driving on Oreti Beach has been suggested, but has not been well received and would bring about considerable public backlash given the culture surrounding beach usage (Various, pers. comm.), and is in fact classified as a road on maps. At Northland beaches, driving is also still allowed on toheroa beaches. In the past, seasonal closures were enforced, with up to 10 months closed out of the year (Redfearn, 1974), and some iwi currently have a complete ban on issuing permits within their jurisdiction in Northland (Various, pers. comm.). Seasonal permitting is a conservation strategy that could be locally implemented as a conservation strategy. With knowledge regarding the spatial variations in the reproduction cycle of toheroa at Oreti Beach, implementing a strategy in which permits are area specific at Oreti Beach and issued only during the winter months when mature animals are least likely to spawn. Another conservation strategy is to implement a maximum legal size in addition to the minimum legal size

(100 mm) already in place. This ensures that more sexually reproductive animals are left to contribute to the population.

It has been suggested that the damming of rivers, like the Waiau River which feeds Te Waewae Bay, Southland, cutting off sediment supply and depleting sand from the beaches, which toheroa need to survive (Various, pers. comm.), has contributed to population decline. While this could have an effect on some populations like Blue Cliffs (Beentjes *et al.*, 2006), many of the toheroa beaches have not been reported to have suffered from long term beach depletion.

An important factor of recruitment that has not yet been examined is larval transport. It is a critical first step in understanding overall larval recruitment to identify how recruitment is affected by temperature and food, but it is also very important to understand where the larvae resulting from broadcast spawning of an adult population into the surf end up as metamorphosed juveniles.

The force driving larval transport, and thus juvenile recruitment, is hydrodynamics – a combination of physical factors such as current, wind, and bathymetry. Since populations have crashed, or disappeared, it is highly plausible that the larval supply to some beaches' populations have been cut off or reduced. At present time, there has yet to be an examination of the specific hydrodynamics relevant to any toheroa population. There has also been talk of transplanting hundreds, if not thousands, of adult *P. ventricosa* from one beach to another in hopes that a population will recover or be established. An important management/conservation strategy is to protect the larval supply. Without knowing where the larvae produced by any beach will end up, management strategies and transplantations are likely to be futile. Thus hydrodynamic modelling of larval transport is the next logical step in determining

overall recruitment dynamics. Particle transport modelling can incorporate environmental factors such as local currents, wind, bathymetry, and even temperature, chl-*a* concentrations, larval size and growth rates to identify larval sources and sinks.

For the Southland populations, modelling could be very advantageous, as these toheroa live in a very dynamic environment. With the strong Southland Current that flows east through the Foveaux Strait and north along the east coast, it is likely that a number of larvae get swept up in the current and fail to recruit to a suitable habitat. Identifying the unique hydrodynamics of this area would be very advantageous to understanding local recruitment. Predictor models can take environmental data from past storm events, and recreate a particular event to estimate where larvae present in the water column at that point in time, giving a prediction of the effect on recruitment on Oreti Beach.

With the potential for large scale spat production, knowing broodstock source and juvenile sink locations would be useful from a conservation stand point, allowing for better protection of important contributing populations and the habitat of settling juveniles. Cultured spat could be introduced to areas where wild spat are known to wash ashore to help enhance a population. Additionally, a larval transport predictor model would be useful in predicting the success of adult transplantation sites before the physical efforts are made. From understanding recruitment and from a management and conservation standpoint, a hydrodynamic larval transport model should be the crucial next step undertaken in expanding the still limited *Paphies ventricosa* knowledge base.

Additional research should also be undertaken to improve the existing knowledge base, such as additional replication experiments that are adjusted to accommodate findings from the current study. Given the still limited knowledge regarding the life history of *Paphies ventricosa*, refined replication studies would be very beneficial. For example, mortality/survival rates were not actively measured in the experiments conducted in the present study, but provide valuable information regarding ideal development conditions. Repeating the studies conducted here and measuring mortality/survival rates could give greater insight into thermal windows of development and general development success. Designing experimental set ups that maximise sterility would be beneficial. While care was taken to maintain sterility in algal cultures for feeding larvae and the larval rearing containers, toheroa larvae seem to be very sensitive to bacterial and ciliate infection, and would benefit from larval experiments conducted in flow through systems. Also, finding the absolute upper and lower limits of fertilisation and development in toheroa would be beneficial in gaining greater understanding of the potential effects of ocean warming.

# Appendix I

# ALLTECH SP1

**Typical Nutrient Analysis**

***Nutritional Profile***

The following nutritional values represent the mean of several batches and though not constituting a part of the product guaranteed analysis, are consistent within normal limits (+/- 10%).

***Nutritional Profile (As received basis)***

| Moisture, % | 3.70 |
|---|---|
| Energy | |
| Acid hydrolysis fat, % | 60 |
| Acid detergent fiber, % | 14.0 |
| Carbohydrates, Calculated, % | 24.88 |
| Protein, combustion, % | 18.8 |
| Total ash, % | 3.67 |
| Major Minerals | |
| Sodium, % | 0.16 |
| Phosphorus, % | 0.30 |
| Sulfur, % | 0.74 |
| Potassium, % | 0.30 |
| Calcium, % | 0.45 |
| Trace minerals, ppm | |
| Iron | 13 |
| Copper | 1.0 |
| Zinc | 36 |
| Selenium | 0.13 |
| | |
| *Glyceride Profile, %* | |
| Diglycerides | 4.69 |
| Glycerol | < 1.0 |
| Monoglycerides | 3.81 |
| Triglycerides | 85.80 |
| | |
| *Fatty Acid Profile* | |
| *Fatty acid* | *% of Fat content* |
| Caproic Acid | <0.10% |
| Heptanoic Acid | <0.10% |
| Caprylic Acid | <0.10% |
| Nonanoic Acid | <0.10% |
| Capric Acid | <0.10% |
| Undecanoic Acid | <0.10% |
| Lauric Acid | <0.10% |
| Tridecanoic Acid | <0.10% |

| *Fatty acid profile, continued* | |
|---|---|
| *Fatty Acid* | *% of Fat Content* |
| Mystric Acid | 3.86% |
| Myristoleic Acid | 0.20% |
| Pentadecanoic Acid | <0.10% |
| Palmitic Acid | 54.69% |
| Palmitoleic Acid | <0.10% |
| Margaric Acid | 0.63% |
| Margaroleic Acid | <0.10% |
| Stearic Aid | 1.80% |
| Vaccenic Acid | <0.10% |
| Oleic Acid | <0.10% |
| Elaidic Acid | <0.10% |
| Linoleic Acid | <0.10% |
| Linolelaidic Acid | <0.10% |
| Alpha-Linolenic Acid | <0.10% |
| Gamma-Linolenic Acid | <0.10% |
| Nonadecanoic Acid | <0.10% |
| Arachidic Acid | 0.28% |
| Eicosenoic Acid | <0.10% |
| Eicosadienoic Acid | <0.10% |
| Eicosatrienoic Acid | <0.10% |
| Homo-gamma-Linolenic Acid | <0.10% |
| Arachidonic Acid | <0.10% |
| Eicosapentaenoic Acid | 0.37% |
| Heneicosanoic Acid | <0.10% |
| Behenic Acid | <0.10% |
| Erucic Acid | 0.53% |
| Docosadienoic Acid | 0.43% |
| Docosapentaenoic Acid | <0.10% |
| Docosahexaenoic Acid | 27.20% |
| Tricosanoic Acid | <0.10% |
| Lignoceric Acid | <0.10% |
| Nervonic Acid | <0.10% |
| Unknown | 0.71% |

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# Appendix II

**Appendix II.I. For 3-d post fertilisation, means tables for the two-way interaction between feed concentration and temperature, showing mean (Mean), standard error (SE), degrees of freedom (Df), lower 95% confidence limit (Lower CL), upper 95% confidence limit (Upper CL), and post hoc pairwise comparison relationship (Group) of each temperature treatment within each feed concentration.**

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **1,000 cells ml$^{-1}$** | 12 °C | 99.7288 | 1.0063 | 667 | 97.7528 | 101.7047 | a |
| | 16 °C | 98.0023 | 0.9859 | 667 | 96.0665 | 99.9362 | a |
| | 20 °C | 100.6415 | 1.5804 | 667 | 97.5383 | 103.7447 | a |

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **10,000 cells ml$^{-1}$** | 12 °C | 102.1973 | 1.0063 | 667 | 100.2214 | 104.1733 | a |
| | 16 °C | 99.3998 | 0.4142 | 667 | 98.5866 | 100.2130 | b |
| | 20 °C | 92.8512 | 0.7450 | 667 | 91.3884 | 94.3141 | b |

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **20,000 cells ml$^{-1}$** | 12 °C | 105.0601 | 1.0674 | 667 | 102.9643 | 107.1560 | a |
| | 16 °C | 98.8403 | 0.4225 | 667 | 98.01067 | 99.6699 | b |
| | 20 °C | 92.8197 | 1.2904 | 667 | 90.2859 | 95.35334 | c |

**Appendix II.II. For 6-d post fertilisation, means tables for the two-way interaction between feed concentration and temperature, showing mean (Mean), standard error (SE), degrees of freedom (Df), lower 95% confidence limit (Lower CL), upper 95% confidence limit (Upper CL), and post hoc pairwise comparison relationship (Group) of each temperature treatment within each feed concentration.**

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **1,000 cells ml$^{-1}$** | 12 °C | 113.1803 | 2.5781 | 667 | 108.1182 | 118.2423 | a |
| | 16 °C | 114.1993 | 1.7728 | 667 | 110.7184 | 117.6802 | a |
| | 20 °C | 99.5390 | 2.0789 | 667 | 95.4570 | 103.6210 | b |

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **10,000 cells ml$^{-1}$** | 12 °C | 128.1557 | 1.2013 | 667 | 125.7694 | 130.5420 | a |
| | 16 °C | 113.3581 | 1.8166 | 667 | 109.7912 | 116.9249 | b |
| | 20 °C | 97.3178 | 1.8004 | 667 | 93.7826 | 100.8529 | c |

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **20,000 cells ml$^{-1}$** | 12 °C | 126.8096 | 0.8843 | 667 | 125.0734 | 128.5459 | a |
| | 16 °C | 113.8699 | 2.8722 | 667 | 108.2302 | 119.5096 | b |
| | 20 °C | 94.7970 | 3.6008 | 667 | 87.7268 | 101.8672 | c |

**Appendix II.III. For 17-d post fertilisation, means tables for the two-way interaction between feed concentration and temperature, showing mean (Mean), standard error (SE), degrees of freedom (Df),**

**lower 95% confidence limit (Lower CL), upper 95% confidence limit (Upper CL), and post hoc pairwise comparison relationship (Group) of each temperature treatment within each feed concentration.**

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **1,000 cells ml$^{-1}$** | 12 °C | 121.6342 | 3.6733 | 667 | 114.4216 | 128.8468 | a |
| | 16 °C | 109.7559 | 0.9959 | 667 | 109.8003 | 111.7114 | b |
| | 20 °C | 126.9083 | 2.0545 | 667 | 122.8743 | 130.9424 | a |

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **10,000 cells ml$^{-1}$** | 12 °C | 153.9903 | 3.6293 | 667 | 146.8641 | 161.1165 | a |
| | 16 °C | 141.7596 | 1.6191 | 667 | 138.5804 | 144.9387 | b |
| | 20 °C | 112.0057 | 3.8436 | 667 | 104.4586 | 119.5527 | c |

| | Temperature | Mean | SE | *Df* | Lower CL | Upper CL | Group |
|---|---|---|---|---|---|---|---|
| **20,000 cells ml$^{-1}$** | 12 °C | 167.2472 | 3.8668 | 667 | 159.6547 | 174.8397 | a |
| | 16 °C | 128.4550 | 1.2366 | 667 | 126.0269 | 130.8831 | b |
| | 20 °C | 109.7885 | 4.7074 | 667 | 100.5453 | 119.0317 | c |

# References


Ackerman, J.D., Blair, S., Nichols, S.J. & Claudi, R. (1994). A review of the early life history of zebra mussels (*Dreissena polymorpha*): comparisons with marine bivalves. *Canadian Journal of Zoology*, **72**: 1169–1179.

Ahn, I.Y. and Shim, J.H. (1998). Summer metabolism of the Antarctic clam, *Laternula elliptica* (King and Broderip) in Maxwell Bay, King George Island and its implications. *Journal of Experimental Marine Biology and Ecology*, **224**(2): 253-264.

Akroyd, J.M., Walshe, K.A.R. & Millar, R.B. (2002). Abundance, distribution, and size structure of toheroa (*Paphies ventricosa*) at Ripiro Beach, Dargaville, Northland, New Zealand. *New Zealand Journal of Marine and Freshwater Research,* **36**(3): 547-553.

Aldana Aranda, D., Lucas, A., Brule, T., Salguero, E. & Rendon, F. (1989). Effects of temperature, algal good, feeding rate and density on the larval growth of the milk conch (*Strombus costatus*) in Mexico. *Aquaculture*, **76**: 361-371.

Alagarswami, K. (1966). Studies on some aspects of biology of the wedge-clam, *Donax faba* Gmelin from Mandapam coast in the Gulf of Mannar. *Journal of Marine Biological Association of India*, **8**(1): 56-75.

Andronikov, V.B. (1975) Heat resistance of gametes of marine invertebrates in relation to temperature conditions under which the species exist. *Marine Biology*, **30**: 1-11.

Ansell, A.D. (1983). The ecology of the genus *Donax*. In: Sandy beaches as ecosystems (A. McLachlan & T. Erasmus, eds), pp. 607-638. The Hague: Dr. W. Junk Publishers.

Ansell A.D. & Legardère, F. (1980). Observations on the biology of *Donax trunculus* and *D. vittatus* at Ile d'Olérom (French Atlantic Coast). *Marine Biology*, **57**: 287-300.

Aranda-Burgos, J.A., Costa, F.D., Nóvoa, S., Ojea, J. & Martínez-Patinó, D. (2014). Embryonic and larval development of *Ruditapes decussatus* (Bivalvia: Veneridae): a study of the shell differentiation process. *Journal of Molluscan Studies*, **80**: 8–16.

Arellano, S.M. & Young, C.M. (2009). Spawning, development, and the duration of larval life in a deep-sea cold-seep mussel. *Biological Bulletin*, **216**: 149–162.

Azouzi, L., Bourget, E. & Borcard, D. (2002). Spatial variation in the intertidal bivalve *Macoma balthica*: biotic variables in relation to density and abiotic factors. *Marine Ecology Progress Series*, **234**: 159-170.

Baron, J. (1992). Reproductive cycles of the bivalve molluscs *Atactodea striata* (Gmelin), *Gafrarium tumidum* Roding and *Anadara scapha* (L.) in New Caledonia. *Australian Journal of Marine and Freshwater Research*, **43**: 393-402.

Beentjes, M.P. (2010) Toheroa survey of Oreti Beach, 2009, and review of historical surveys. *New Zealand Fisheries Assessment Report*, **2010/6**. Wellington: 40p.

Beentjes, M. P., Carbides, G.D., & Willsman, A.P. (2006). Effects of beach erosion on abundance and distribution of toheroa (*Paphies ventricosa*) at Bluecliffs Beach, Southland, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, **40**(3): 439-453.

Beentjes, M.P. & Gilbert, D.J. (2006) Oreti Beach 2005 toheroa survey: yield per recruit and review of historical surveys. *New Zealand Fisheries Assessment Report*, **2006/36**. Wellington: 48p.

Beiras, R. & Pérez Camacho, A. (1994). Influence of food concentration on the physiological energetics and growth of *Ostrea edulis* larvae. *Marine Biology*, **120**: 427-435.

Berkenbusch, K., Abraham, E. & Neubauer, P. (2015). Distribution and abundance of toheroa in Southland, 2013-2014. *New Zealand Fisheries Assessment Report*, **2015/17**. Wellington: 45p.

Beu, A.G. (1971). Genera of the bivalve family Mesodesmatidae, with comments on some Australasian species. *Journal of the Malacological Society of Australia*, **2**: 113-131.

Brazerio, A. & Defeo, O. (1999). Effects of harvesting and density dependence on the demography of sandy beach populations: the yellow clam *Mesodesma mactroides* of Uruguay. *Marine Ecology Progress Series*, **182**: 127–135.

Brunton, P.M. (1978). Toheroa predation by black-backed gulls on Dargaville Beach, North Auckland, New Zealand. *Notornis*, **25**: 128-140.

Bylenga, C.H., Cummings, V.J. & Ryan, K.G. (2015). Fertilisation and larval development in an Antarctic bivalve, *Laternula, elliptica*, under reduced pH and elevated temperatures. *Marine Ecology Progress Series*, **536**: 187-201.

Byrne, M. (2010). Impact of climate change stressors on marine invertebrate life histories with a focus on the Mollusca and Echinodermata. In: Climate alert: climate change monitoring and strategy (J. Yu & A. Henderson-Sellers, eds.), pp. 142-185. Sydney: University of Sydney Press.

Byrne, M. (2011). Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology Annual Review*, **49**: 1-42.

Byrne, M. & Przeslawski, R. (2013). Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. *Integrative and Comparative Biology*, **53**(3): 582-596.

Caers, M., Coutteau, P., Cure, K., Morales, V., Gajardo., G. & Sorgeloos, P. (1999). The Chilean scallop *Argopecten purpuratus* (Lamarck, 1819): II. Manipulation of the fatty acid composition of the eggs via lipid supplementation of the broodstock diet. *Comparative Biochemistry and Physiology Part B*, **123**: 97-103.

Calabrese, A. (1969). Individual and combined effects of salinity and temperature on embryos and larvae of the coot clam, *Mulinia lateralis* (Say). *Biological Bulletin*, **137**(3): 417- 428.

Carstensen, D., Laudien, J., Sielfeld, W., Oliva, M.E. & Arntz, W.E. (2010). Early larval development of *Donax obesulus*: response to El Niño temperature and salinity conditions. *Journal of Shellfish Research*, **29**: 361–368.

Carstensen, D., Oliva, M., Sielfeld, W. & Laudien, J. (2006). Larval development of *Mesodesma donacium* (Macha). Poster presented at the XXVI Congreso de Ciencias del Mar, Iquique, Chile.

Cassie, R.M. (1951). A molluscan population with an unusual size frequency-distribution, Nature, **167**: 284-285.

Cassie, R.M. (1955). Population studies on the toheroa, *Amphidesma ventricosum* Gray (Eulamellibranchiata). *Australian Journal of Marine and Freshwater Research,* **6**: 379-382.

Cataldo, D., Boltovskoy, D., Hermosa, J.L. & Canzi, C. (2005). Temperature-dependent rates of larval development in *Limnoperna fortunei* (Bivalvia: Mytilidae). *Journal of Molluscan Studies,* **71**(1): 41-46.

Chaparro, O.R., Thompson, R.J. & Emerson, C.J. (1999). The velar ciliature in the brooded larva of the Chilean oyster *Ostrea chilensis* (Philippi, 1845). *Biological Bulletin*, **197**: 104–111.

Clotteau, G. & Dubé. (1993). Optimization of fertilization parameters for rearing surf clams (*Spisula solidissima*). *Aquaculture*, **114**: 339-353.

Collin, R. & Ochoa, I. (2016). Influence of seasonal environmental variation on the reproduction of four tropical marine gastropods. *Marine Ecology Progress Series*, **555**: 125-139.

Cragg, S.M. 2006. Development, physiology, behaviour and ecology of scallop larvae. In: Scallops: biology, ecology and aquaculture (S.E. Shumway & G.J. Parsons, eds.), pp. 45–122. Elsevier, Amsterdam.

da Costa, G., Darriba, S. & Martínez-Patiño, D. (2008). Embryonic and larval development of *Ensis arcuatus* (Jeffreys, 1865) (Bivalvia: Pharidae). *Journal of Molluscan Studies*, **74**: 103–109.

Dawson, E.W. (1959). An interpretation of the molluscan genus *Amphidesma* in New Zealand. *Journal of the Malacological Society of Australia*, **1**: 39-58.

De Villiers, G. (1975). Reproduction of the white sand mussel *Donax serra* Roeding. *Sea_Fisheries Branch Investigational Report*. H. G. v. D. Boonstra. Cape Town, South Africa, Republic of South Africa Department of Industries. **102:** 33p.

Delorme, N.J. & Sewell M.A. (2013). Temperature limits to early development of the New Zealand sea urchin *Evechinus chloroticus* (Valenciennes, 1846). *Journal of Thermal Biology*, **38**: 218-224.

Donn, T.E. Jr., Clarke, D.J., McLachlan, A. & du Toit, P. (1986). Distribution and abundance of *Donax serra* Röding (Bivalvia: Donacidae as related to beach morphology. I. Semilunar migrations. *Journal of Experimental Biology and Ecology*, **102**: 121-131.

Drent, J. (2002). Temperature responses in larvae of *Macoma balthica* from a northerly and southerly population of the European distribution range. *Journal of Experimental Marine Biology and Ecology*, **275**: 117–129.

Eyster, L.S. & Morse, M.P. (1984). Early shell formation during molluscan embryogenesis, with new studies on the surf clam, *Spisula solidissima*. *American Zoologist,* **24**: 871–882.

Gadomski, K. & Lamare, M. (2015). Spatial variation in reproduction in southern populations of the New Zealand bivalve *Paphies ventricosa* (Veneroida: Mesodesmatidae). *Invertebrate Reproduction and Development*, **59**(2): 81-95.

Gadomski, K., Moller, H., Beentjes, M., & Lamare, M. (2015). Embryonic and larval development of the New Zealand bivalve *Paphies ventricosa* Gray, 1843 (Veneroida: Mesodesmatidae) at a range of temperatures. *Journal of Molluscan Studies*, **81**: 356-364.

Gaines, S.D., White, C., Carr, M.H. & Palumbi, S.R. (2010.) Designing marine reserve networks for both conservation and fisheries management. *Proceedings of the National Academy of Science*, **107**: 18286-18293.

Galbraith, P.S., Chassé, J., Larouche, P., Gilbert, D., Brickman, D., Pettigrew, B., Devine, L., & Lafleur,, C. (2013) Physical oceanographic Conditions in the Gulf of St. Lawrence in 2012. *DFO Canadian Science Advisory Secretariat,* **2013/026**. Ottawa; 95p.

Gaspar, M. B., Ferrira, R. & Monteiro, C.C. (1999). Growth and reproductive cycle of *Donax trunculus* L., (Mollusca: Bivalvia) off Faro, southern Portugal. *Fisheries Research,* **41**: 309-316.

Gibbons, M.C. & Castagna, M. (1984). Serotonin as an inducer of spawning in six bivalve species. *Aquaculture*, **40**: 189–191.

Gosling, E.M. (2004) Bivalve molluscs: biology, ecology, culture. Oxford: Fishing New Books, 443p.

Grant, C.M. & Creese, R.G. (1995) The reproductive cycle of the tuatua – *Paphies subtriangulata* (Wood, 1828), in New Zealand. *Journal of Shellfish Research*, **14**: 287-292.

Grant, C.M., Hooker, S.H., Babcock, R.C. & Creese, R.G. (1998). Synchronous spawning and reproductive incompatibility of two bivalve species: *Paphies subtriangulata* and *Paphies australis*. *The Veliger*, **41**(2): 148-156.

Greenway, J.P.C. (1972) Further data from population surveys on toheroa (Mollusca: Eulamellibranchiata) on Northland beaches, 1962-1971. *New Zealand Marine Department Fisheries Technical Report,* **85**. Wellington; 36p.

Greig, M.J., Ridgway, N.M. & Shakespeare, B.S. (1998). Sea surface temperature variations at coastal sites around New Zealand. *New Zealand Journal of Marine and Freshwater Research*, **22**: 391–400.

Gribben, P.E. (2005) Gametogenic development and spawning of the razor clam, *Zenatia acinaces* in northeastern New Zealand. *New Zealand Journal of Marine and Freshwater Research*, **39**: 1287-1296.

Gribben, P.E., Creese, R.G., & Hooker, S.H. (2001). The reproductive cycle of the New Zealand venus clam *Ruditapes largillierti*. *Journal of Shellfish Research*, **20**: 1101-1108.

Gribben, P.E., Helson, J., & Jeffs, A.G. (2004) Reproductive cycle of the New Zealand Geoduck, *Panopea zelandica*, in two North Island populations. *Veliger*, **47**: 53-65.

Haddon, M., Wear, R.G., & Packer, H.A. (1987). Depth and density of burial by the bivalve *Paphies ventricosa* as a refuge from predation by the crab *Ovalipes catharus*. *Marine Biology*, **94**: 25-30.

Hardy, N., Lamare, M., Uthicke, S., Wolfe, K., Doo, S.S., Dworjanyn, S. & Byrne, M. (2014). Thermal tolerance of early development in tropical and temperate sea urchins: inferences for the tropicalisation of eastern Australia. *Marine Biology*, **161**: 395–409

Harvey, M. & Vincent, B. (1989). Spatial and temporal variations of the reproductive cycle and energy allocation of the bivalve *Macoma balthica* (L.) on a tidal flat. *Journal of Experimental Marine Biology and Ecology,* **129**: 199-217.

Helm, M.M., Bourne, N. & Lovatelli, A. (2004). Hatchery culture of bivalves: a practical manual. FAO Fisheries Technical Paper 471. FAO, Rome.

Helm, M.M. & Millican, P.F. (1982). Experiments in the hatchery rearing of Pacific oyster, *Crassostrea gigas* (Thunberg). *Aquaculture*, **11**: 1–12.

Herrmann, M., Alfaya, J.E.F., Lepore, M.L., Penchaszedeh, P.E. & Laudien, J. (2009). Reproductive cycle and gonad development of the Northern Argentinean *Mesodesma mactroides* (Bivalvia: Mesodesmatidae). *Helgoland Marine Research,* **63**(3): 207-218.

Hirai, S., Kishimoto, T., Kadam, A.L., Kanatani, H. & Koide, S.S. (1988). Induction of spawning and oocyte maturation by 5-hydroxytryptamine in the surf clam. *Journal of Experimental Zoology*, **245**: 318–321.

His, E., Robert, R. & Dinet, A. (1989). Combined effects of temperature and salinity on fed and starved larvae of the Mediterranean mussel *Mytilus galloprovincialis* and the Japanese oyster *Crassostrea gigas*. *Marine Biology*, **100**: 455–463.

Hoby, K. E. (1933). The anatomy of *Amphidesma ventricosum* (Gray). Marine Science. Wellington, N.Z., Victoria University of Wellington. **M.Sc.:** 116p.

Hoegh-Guldberg, O. & Pearse, J.S. (1995). Temperature, food availability, and the development of marine invertebrate larvae. *American Zoologist*, **35**: 415-425.

Hold, N., Murray, L.G., Hinz, H., Neill, S.P., Lass, S., Lo M. & Kaiser, M.J. (2013). Environmental drivers of small scale spatial variation in the reproductive schedule of a commercially important bivalve mollusc. *Marine Environmental Research*, **92**: 144-153.

Hooker, S.H. & Creese, R.G. (1995) The reproductive biology of pipi, *Paphies australis* (Gmelin, 1790) (Bivalvia: Mesodesmatidae). I. Temporal patterns of the reproductive cycle. *Journal of Shellfish Research*, **14**: 7-15.

Hooker, S. H. (1997). Larval and postlarval development of the New Zealand pipi, *Paphies australis* (Bivalvia: Mesodesmatidae). *Bulletin of Marine Science,* **61**(2): 225-240.

Hrs-Brenko, M. & Calabrese, A. (1969). The combined effect of salinity and temperature on larvae of the mussel *Mytilus edulis*. *Marine Biology*, **4**: 224–226.

IPCC. (Intergovernmental Panel on Climate Change). (2014). The fifth assessment report of the IPCC. Cambridge: Cambridge University Press, 151p.

Jones, S.J., Mieszkowska, N., and Wethey, DS. (2009) Linking thermal tolerances and biogeography: *Mytilus edulis* (L.) at its southern limit on the east coast of the United States. *Biological Bulletin*, **217**: 73-85.

Kasyanov, V.L., Kryuchkova, G.A., Kulikova, V.A. & Medvedeva, L.A. (1998). Larvae of marine bivalves and echinoderms. Smithsonian Institute Libraries, Washington, DC.

Kennedy, V.S., Roosenburg, W.H., Castagna, M. & Mihursky, J.A. (1974a). *Mercenaria mercenaria* (Mollusca: Bivalvia): Temperature-time relationships for survival of embryos and larvae. *Fishery Bulletin*, **72**(4): 1160-1166.

Kennedy, V.S., Roosenburg, W.H, Zion, H.H. & Castagna, M. (1974b). Temperature-time relationships for survival of embryos and larvae of *Mulinia lateralis* (Mollusca: Bivalvia). *Marine Biology (Berlin)*, **24**: 137-145.

Kheder, R.B., Moal, J. & Robert, R. (2010). Impact of temperature on larval development and evolution of physiological indices in *Crassostrea gigas*. *Aquaculture*, **309**: 286–289.

Kim, J.H., Son, M.H., Kang, H.-W., & Lee, K.-Y. (2013). Reproductive cycle of the surf clam, *Coecella chinensis* (Mesodesmatidae: Bivalvia). *Korean Journal of Malacology*, **29**: 113-120.

Kondo, Y. & Stace, G. (1995). Burrowing ability and life position of toheroa (*Paphies ventricosa*: Mesodesmatidae), an unusually large, deep-burrowing ocean bivalve endemic to New Zealand. *VENUS: The Japanese Journal of Malacology*, **54**(1): 67-75.

Kupriyanova, E.N. & Havenhand, J.N. (2005). Effects of temperature on sperm swimming behaviour, respiration and fertilization success in the serpulid polychaete, *Galeolaria caespitose* (Annelida: Serpulidae). *Invertebrate Reproduction and Development*, **48**: 7-17.

Labarbera, M. (1974). Calcification of the first larval shell of *Tridacna squamosa* (Tridacnidae: Bivalvia). *Marine Biology*, **23**: 233–238.

Lamare, M., Pecorino, D., Hardy, N., Liddy, M., Byrne, M. & Uthicke, S. (2014). The thermal tolerance of crown-of-thorns (*Acanthaster planci*) embryos and bipinnaria larvae: implications for spatial and temporal variation in adult populations. *Coral Reefs*, **161**:395–409.

Lambert, J.D. (2010). Development patterns in spiralian embryos. *Current Biology*, **20**: 72–77.

Laudien, J., Brey, T. & Arntz, W.E. (2003). Population structure, growth and production of the suft clam *Donax serra* (Bivalvia, Donacidae) on two Namibian sandy beaches. *Estuarine, Coastal and Shelf Science*, **58S**: 105-115.

Liu, W., Gurney-Smith, H., Beerens, A. & Pearce, C.M. (2010). Effects of stocking density, algal density, and temperature on growth and survival of larvae of the basket cockle, *Clinocardium nutallii*. *Aquaculture*, **299**: 99-105.

Mandeno, M. A. (1999). Gonadal cycle and spawning induction in toheroa (*Paphies ventricosa*) from southern New Zealand. Marine Science. Dunedin, New Zealand, University of Otago. **M.Sc.:** 54p.

Manoj Nair, R. & Appukuttan, K.K. (2003). Effect of temperature on the development, growth, survival and settlement of green mussel *Perna viridis* (Linnaeus, 1758). *Aquaculture Research,* **34**: 1037-1045.

Manzi, J.J., Bobo, M.Y. & Burrell, V.G. (1985). Gametogenesis in a population of the hard clam, *Mercenaria mercenaria* (Linnaeus) in North Santee Bay, South Carolina. *The Veliger*, **28**: 186-194.

Marsden, I. D. (1999). Reproductive cycles of the surf beach clam *Paphies donacina* (Spengler, 1793) from New Zealand. *Journal of Shellfish Research*, **18**: 539-546.

Marshall, R., Pearce, C.M. & Scott McKinley, R. (2014). Interactive effects of stocking density and algal feed ration on growth, survival, and ingestion rate of larval geoduck clams. *North American Journal of Aquaculture*, **76**(3): 265-274.

Mauchly, J.M. (1940). Significance test for sphericity of a normal n-variate distribution. *Annals of Mathematical Statistics*, **11**: 204–209.

McLachlan, A. (1996). Phyiscal factors in benthic ecology: effects of changing particle size on beach fauna. *Marine Ecology Progress Series*, **131**: 205-217.

McLachlan, A., Dugan, J., Defeo, O., Ansell, A., Hubbard, D., Jaramillo, E., & Penchaszadeh, P. (1996). Beach clam fisheries. *Oceanography and Marine Biology: An Annual Review*, **34**: 163-232.

McKinnon, S. & Olsen, D. (1994). Review of the Southland toheroa fishery. *New Zealand Fisheries Management Regional Series No. 3*. 18p.

Moller, J.A., Garden, C., Moller, S.I., Beentjes, M., Skerett, M., Scott, D., Stirling, F.F., Moller, J.S. & Moller, H. (2014). Impact of vehicles on recruitment of toheroa on Oreti Beach. *Ecosystems Consultants Report*, **2014/2**. Dunedin, New Zealand: 87p.

Mouëza, M. & Frenkiel, L. (1995). Gill ultrastructure and symbiotic bacteria in *Codakia orbicularis* (Bivalvia, Lucinidae). *Zoomorphology*, **115**: 51–61.

Mouëza, M., Gros, O. & Frenkiel, L. (1999). Embryonic, larval and postlarval development of the tropical clam, *Anomalocardia brasiliana* (Bivalvia, Veneridae). *Journal of Molluscan Studies*, **65**: 73–88.

Mouëza, M., Gros, O. & Frenkiel, L. (2006). Embryonic development and shell differentiation in *Chione cancellata* (Bivalvia, Veneridae): an ultrastructural analysis. *Invertebrate Biology*, **125**: 21–33.

Murphy, R.J., Pinkerton, M.H., Richardson, K.M., Bradford-Grieve, J.M. & Boyd, P.W. (2001). Phytoplankton distributions around New Zealand derived from SeaWiFS remotely-sensed ocean colour data. *New Zealand Journal of Marine and Freshwater Research*, **35**: 343-362.

Newell, R.I.E. & Bayne, B.L. (1980). Seasonal changes in the physiology, reproductive condition and carbohydrate content of the cockle *Cardium* (=*Cerastoderma*) *edule* (Bivalvia: Cardiidae). *Marine Biology*, **56**: 11-19.

O'Connor, M.I., Bruno, J.F., Gaines, S.D., Halpern, B.S., Lester, S.E., Kinlan, B.P., and Weiss, J.M. (2007) Temperature control of larval dispersal and the implications for marine ecology, evolution and conservation. *Proceedings of the National Academy of Science*, **104**: 1266-1271.

Olson, R.R. & Olson, M.H. (1989). Food limitation of planktotrophic marine invertebrate larvae: does it control recruitment success? *Annual Review of Ecology and Systematics*, **20**: 225-247.

Parker, L.M., Ross, P.M. & O'Connor, W.A. 2009. The effect of ocean acidification and temperature on the fertilization and embryonic development of the Sydney rock oyster *Saccostrea glomerata* (Gould 1859). *Global Change Biology*, **15**: 2123–2136.

Parker, L.M., Ross, P.M. & O'Connor, W.A. (2010). Comparing the effect of elevated $pCO_2$ and temperature on the fertilization and early development of two species of oysters. *Marine Biology*, **157**: 2435-3452.

Paulay, G., Boring, L. & Strathmann, R.R. (1985). Food limited growth and development of larval experiments with natural sea water. *Journal of Experimental Marine Biology and Ecology*, **93**: 1-10.

Pechenik, J.A. (1990). Delayed metamorphosis by larvae of benthic marine invertebrates: does it occur? Is there a price to pay? *Ophelia*, **32**: 63–94.

Pechenik, J.A., Eyster, L.S., Widdows, J. & Bayne, B. 1990. The influence of food concentration and temperature on growth and morphological differentiation of blue mussel *Mytilus edulis* L. larvae. *Journal of Experimental Marine Biology and Ecology*, **136**: 47–64.

Pechenik, J.A. (1987) Environmental influences on larval survival and development. In: *Reproduction of marine invertebrates* (A.C. Giese and J.S. Pierce, eds.). Academic Press, New York: pp 551-608.

Pecorino, D., Lamare, M., Barker, M.F. & Byrne, M. (2013). How does embryonic and larval thermal tolerance contribute to the distribution of the sea urchin *Centrostephanus rodgersii* (Diadematidae) in New Zealand? *Journal of Experimental Marine Biology and Ecology*, **445**: 120–128.

Peredo, S., Parada, E. & Valdebenito, I. (1987). Gametogenesis and reproductive cycle of the clam *Mesodesma donacium* (Lamarck, 1818) (Bivalvia: Mesodesmatidae) at Queule Beach, Southern Chile. *The Veliger*, **31**(1): 55-68.

Pérez-Camacho, A., Beiras, R. & albentosa, M. (1994). Effect of algal food concentration and body size on the ingestion rates of *Ruditapes decussates* (Bivalvia) veliger larvae. *Marine Ecology Progress Series*, **115**: 87-92.

Quinn, G.P. & Keough, M.J. (2002). Experimental Design and Data Analysis for Biologists. Cambridge University Press: Melbourne. 562p.

R Core Team. (2016). R: A language and environement for statistical computing. R Foundation for Statistical Computing: Vienna, Austria. URL https://R-project.org/.

Rapson, A.M. (1952). The toheroa, *Amphidesma ventricosum*, Fray (Eulamellibranchiata), development and growth. *Australian Journal of Marine and Freshwater Research*, **3**: 170-198.

Rapson, A.M. (1954). Feeding and control of toheroa (*Amphidesma ventricosum* Gray) (Eulamellibranchiata) populations in New Zealand. *Australian Journal of Marine and Freshwater Research*, **5**: 486-512.

Redfearn, P. (1974). Biology and distribution of the toheroa, *Paphies (Mesodesma) ventricosa* (Gray). *Fisheries Research Bulletin,* **11**: 49.

Redfearn, P. (1982). Larval shell development of the toheroa, Paphies ventricosa (Gray, 1843) (Mactracea: Mesodesmatidae). *New Zealand Journal of Marine and Freshwater Research,* **16**(1): 241-252.

Redfearn, P. (1987). Larval shell development of the northern tuatua, *Paphies subtriangulata* (Bivalvia, Mesodesmatidae). *New Zealand Journal of Marine and Freshwater Research,* **21**(1): 65-70.

Riascos, J.M., Carstensen, D., Laudien, J., Arntz, W.E., Oliva, M.E., Güntner, A. & Heilmayer, O. (2009). Thriving and declining: climate variability shaping life-history and population persistence of *Mesodesma donacium* in the Humboldt Upwelling System.

Rico-Villa, B., Pouvreau, S. & Robert, R. (2009). Influence of food density and temperature on ingestion, growth and settlement of Pacific oyster larvae, *Crassostrea gigas*. *Aquaculture,* **287**: 395-401.

Roberts, D. (1984). A comparative study of *Lasea australis, Vulsella spongiarum, Pinna bicolor* and *Donacilla cuneata* (Mollusca; Bivalvia) from Princess Royal Harbour, Western Australia. *Journal of Molluscan Studies,* **50**: 129-136.

Ropes, J.W. (1968) Reproductive cycle of the surf clam, *Spisula solidissima*, in offshore New Jersey. *Biological Bulletin*, **135**: 349-365.

Ross, P., Williams, J., Smith, S., & Hogg, I. (2014, August). Larval dispersal v. Early aquaculture: alternative explanations for the population structure and dynamics of tohera. In: Andrew Baxter (Chair), *Conservation & Biodiversity.* Symposium conducted at the New Zealand Marine Sciences Society Annual Conference, Nelson, New Zealand.

Ruiz, C., Martinez, D., Mosquera, G., Abad, M., & Sánchez, J.L. (1992). Seasonal variations in condition, reproductive activity and biochemical composition of the flat oyster, *Ostrea edulis*, from San Cibran (Galicia, Spain). *Marine Biology*, **112**: 67-74.

Sánchez-Lazo, C. & Martínez-Pita, I. 2012. Effect of temperature on survival, growth and development of *Mytilus galloprovincialis* larvae. *Aquaculture Research*, **43**: 1127–1133.

Sastry, A.N. (1979) Pelecypoda (excluding Ostreidae). In: Reproduction of marine invertebrates, Vol. 5: Mollusca: pelecypods and lesser classes (A.C. Geise & J.S. Pierce, eds.), pp. 113-292. Academic Press, New York.

Schoeman, D.S. & Richardson, A.J. (2002). Investigating biotic and abiotic factors affecting the recruitment of an intertidal clam on an exposed sandy beach using a generalized additive model. *Journal of Experimental Marine Biology and Ecology*, **276**: 67-81.

Sewell, M.A. & Young, C.M. (1999). Temperature limits to fertilization and early development in the tropical sea urchin *Echinometra lucunter*. *Journal of Experimental Marine Biology and Ecology*, **236**: 291-305.

Silberfield, T. and O. Gros (2006). Embryonic development of the tropical bivalve *Tivela mactroides* (Born, 1778) (Veneridae: subfamily Meretricinae): a SEM study. *Cahier de Marine Biologie,* **47**: 243-251.

Smith, S. (2003). The reproduction and recruitment of toheroa (*Paphies ventricosa*). Marine Science. Auckland, N.Z., University of Auckland. **M.Sc.:** 106p.

Sprung, M. (1984a). Physiological energetics of mussel larvae (*Mytilus edulis*). I. Shell growth and biomass. *Marine Ecology Progress Series*, **17**: 283–293.

Sprung, M. (1984b). Physiological energetic of mussel larvae (*Mytilus edulis*). II. Food uptake. *Marine Ecology Progress Series*, **17**: 295-305.

Sprung, M. & Bayne, B.L. (1984). Some practical aspects of fertilizing the eggs of the mussel *Mytilus edulis* L. *Journal du Conseil/Conseil Permanent International pour l'Exploration de la Mer*, **41**: 125-128.

Stace, G. (1991). The elusive toheroa. *New Zealand Geographic*, **9**: 18-34.

Strickland, J.D.H. & Parsons, T.R. (1972). A practical handbook of seawater analysis. *Bulletin of the Fisheries Research Board of Canada*, **167**. 331p.

Storch, D., Fernández, M., Navarrete, A. & Pörtner, H.-O. (2011). Thermal tolerance of larval stages of the Chilean kelp crab *Taliepus dentatus*. *Marine Ecology Progress Series*, **429**: 157–167.

Sunday, J.M., Bates, A.E., and Dulvy, N.K. (2012) Thermal tolerance and the global redistribution of animals. *Nature Climate Change*, **2**: 686-690.

Sünhel, S., Lagreze, F., Zanette, G., Magalhães, A.R.M., & Ferreria., J.F. (2012). Effect of the fatty acid EP and DHA in the conditioning of the scallop *Nodipecten nodosus* (Linné, 1758). *Aquaculture*, **330-333**: 167-171.

Uribe, R.A., Oliva, M.E., Aguilar, S., Yamashiro, C. & Riascos, J.M. (2012). Latitudinal variation in the reproductive cycle of two bivalves with contrasting biogeographical origin along the Humboldt Current Upwelling Ecosystem. *Scientia Marina,* **76**(4): 713-720.

Utting, S.D. & Millican, P.F. (1997). Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. *Aquaculture*, **155**: 45-54.

Verween, A., Vincx, M. & Degraer, S. (2007). The effect of temperature and salinity on the survival of *Mytilopsis leucophaeata* larvae (Mollusca, Bivalvia): the search for environmental limits. *Journal of Experimental Marine Biology and Ecology*, **348**: 111–120.

Waller, T.R. (1981). Functional morphology and development of veliger larvae of the European oyster, *Ostrea edulis* Linné. *Smithsonian Contributions to Zoology*, **328**: 1–70.

Waugh, G. Duncan & Greenway, J.P. (1967). Further evidence for the existence of sub-littoral populations of toheroa *Amphidesma ventricosum* Gray (eulamelli-branchiata), off the west coast of New Zealand. *New Zealand Journal of Marine and Freshwater Research*, **1**: 407-411.

Weiss, I.M., Tuross, N., Addadi, L. & Weiner, S. (2002). Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite. *Journal of Experimental Zoology*, **293**: 478–491.

Whiteley, D.A.A., Owen, D.F. & Smith, D.A.S. (1997). Massive polymorphism and natural selection in *Donacilla cornea* (Poli, 1791) (Bivalvia: Mesodesmatidae). *Biological Journal of the Linnean Society*, **62**: 475–494.

Widdows, J. 1991. Physiological ecology of mussel larvae. *Aquaculture,* **94**: 147–163.

Willan, R.C., de C. Cook, S., Spencer, H.G., Creese, R.G., O'Shea, S., and Jackson, G.D. (2009). Phylum Mollusca. In: *New Zealand Coastal Invertebrates, Volume One* (S. de C. Cook ed.), pp. 295-566. Canterbury University Press, Christchurch, New Zealand.

Wiliams, J.R., Sim-Smith, C., & Peterson, C. (2013). Review of factors affecting the abundance of toheroa (*Paphies ventricosa*). *New Zealand Aquatic Environment and Biodiversity Report No. 114*. 79p.

Wilson, B. R. & Hodgkin, E.P. (1967). A comparative account of the reproductive cycles of five species of marine mussels (Bivalvia: Mytilidae) in the vicinity of Freemantle, Western Australia. *Australian Journal of Marine and Freshwater Research,* **18**: 175-203.

Zaidman, P.C., Kroeck, M.A., Oehrens Kissner, E.M. & Morsan, E.M. (2012). Reproductive pattern of southern geoduck, *Panopea abbreviata*, at El Sotano (San Matias Gulf, Patagonia, Argentina). *Marine Biology Research,* **8**(2): 172-181.

Zardus, J.D. & Morse, M.P. 1998. Embryogenesis, morphology and ultrastructure of the pericalymma larva of *Acila castrensis* (Bivalvia: Protobranchia: Nuculoida). *Invertebrate Biology*, **117**: 221–244.