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Sperm performance and fertilisation in the sea urchin *Evechinus chloroticus* under increasing $p\text{CO}_2$

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A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy in Biological Sciences

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Abstract

Ocean acidification (OA) is resulting from the large-scale changes in seawater carbonate chemistry caused by increasing atmospheric CO₂. The biological implications include decrease in diversity, biomass, and trophic complexity, as well as a wide range of both positive and negative species responses. The physiological mechanisms underlying changes in species responses remain uncertain. Limited mechanisms to defend cellular homeostasis mean the early life stages of marine broadcast spawners are considered the most vulnerable to increasing pCO₂. Here I examine the responses of sperm and fertilisation of a broadcast spawning sea urchin (*Evechinus chloroticus*) to OA.

Sperm of New Zealand populations of four echinoids analysed by transmission electron microscopy conformed to the general echinoid sperm morphology with ultrastructure showing among species differences and *E. chloroticus* morphology to be the most variable. Evaluating sensitivity of sperm performance used paired analysis of internal pH (pHᵢ) and swimming performance in near- to far-future pCO₂ level waters (bubbled with pre-mixed gases to 1000 and 1800 µatm respectively) as predicted under Representative Concentration Pathway (RCP) 8.5. With decreasing seawater pH (-0.32, -0.58) a respective stepwise decrease of *E. chloroticus* sperm pHᵢ (-0.17, -0.21) corresponded with a lower % motility (-9, -19), a faster curvi-linear velocity swimming speed (VCL = 3.17, 2.82 µm.s⁻¹), but lower straight line velocity (VSL = -20.16, -29.12 µm.s⁻¹). Fertilisation trials across a ten-step gradient between control and far-future pCO₂ levels precisely described the FS response of individual male:female crosses, informing on within-population variability, while nested half-sibling crosses examined gamete effects on variation of FS response. Beyond an overall general tolerance of FS response to near-future OA, proceeded by a slow population level decline to far-future OA (-13.4 and -28 % respectively), among-cross variability ranged from a rapid decline to no effect. Both males and females contributed to FS variability.

This study shows OA is impacting *E. chloroticus* sperm performance and regulation of pHᵢ in a non-linear way. Evidence of pCO₂ induced sperm narcosis provides a plausible mechanism for lower FS. Variability in performance of sperm and FS response to OA indicate the possible adaptive potential to OA, with implications for long term species survival in the face of environmental change.
This thesis is dedicated to my mother,

Janet Cameron
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Chapter 1. General introduction

1.1 Ocean acidification

The onset of the Industrial Revolution and a dramatic rise in anthropogenic CO₂ emissions have led to atmospheric concentrations increasing from 280 parts per million (ppm) to new peaks of over 400 ppm at Mauna Loa (in 2014 to 2016). These current atmospheric concentrations have not been seen for over 800,000 years and, perhaps, for more than 25 million (summary of studies in: Pearson & Palmer, 2000; Lüthi et al., 2008). Initially the uptake of CO₂ by the oceans was perceived as a convenience due to the amelioration of CO₂ build-up in the atmosphere; the majority of the CO₂ taken up by the oceans has remained in the surface waters (Chen & Millero, 1979; Sabine et al., 2004). The oceans are estimated to have absorbed up to 50% of global CO₂ emissions (Sabine et al., 2004; Wanninkhof et al., 2013), and are projected to continue to absorb CO₂ as atmospheric concentrations carry on rising (IPCC, 2013).

As a non-ideal gas, the dissolving of CO₂ in seawater results in a chemical change of an increase in bicarbonate and hydrogen ions, along with a corresponding decrease in carbonate concentrations. The balance of these different changes is driven through a series of thermodynamic equilibrium reactions starting with CO₂ dissolving and reacting with seawater to form carbonic acid (square brackets denote concentration):

$$[\text{CO}_2]_{\text{aqueous}} + [\text{H}_2\text{O}] \rightleftharpoons [\text{H}_2\text{CO}_3]$$

Followed rapidly by the dissociation of carbonic acid to bicarbonate and hydrogen ions resulting in lower pH:

$$[\text{H}_2\text{CO}_3] \rightleftharpoons [\text{H}^+] + [\text{HCO}_3^-]$$

The ocean has an inherent buffering capacity that is involved with the mitigation of the increasing hydrogen ion concentration [H⁺] through a reaction between hydrogen ions and carbonate to form more bicarbonate:

$$[\text{H}^+] + [\text{CO}_3^{2-}] \rightleftharpoons [\text{HCO}_3^-]$$
This decrease in carbonate concentration changes the carbonate bioavailability to marine calcifiers to mineralize their skeletons; at some point becoming low enough to cause calcium carbonate dissolution as saturation state reaches one or less:

\[ \text{CaCO}_3 \rightleftharpoons \text{Ca}^{2+} + \text{CO}_3^{2-} \]

Ocean acidification (OA) refers to the decrease in the pH as a direct consequence of elevated levels of hydrogen ions in the seawater. The change in carbonate concentration is not the only direct chemical effect of increasing \([H^+]\); other impacts include changes in toxicity and availability of metal ions, driven by changes in \([OH^-]\) and metal speciation (reviewed in: Millero et al., 2009).

Since the Industrial Revolution, OA has resulted in a decrease in global average surface pH by around 0.1 units with further decreases expected into the future (Meehl et al., 2007). The projected atmospheric CO₂ concentrations based on the Intergovernmental Panel on Climate Change (IPCC) Representative Concentration Pathway (RCP) 8.5, emission scenario for the end of this century (near-future) are just under 1000 ppm with further increases to under 2000 ppm by the year 2300 (far-future) (Meinshausen et al., 2011; Hartin et al., 2015). These changes are associated with a corresponding decrease in ocean pH of ~0.4 and 0.7 respectively as well as a shoaling of the aragonite and calcite saturation horizons (Caldeira & Wickett, 2005; Hönisch et al., 2012).

According to reconstructions of past climate conditions, the current and projected levels of atmospheric CO₂ and OA conditions have occurred before (Hönisch et al., 2012); however, there are two primary concerns: Firstly, current changes are occurring after a relatively long period of stable, low concentrations (Lüthi et al., 2008). Secondly, and perhaps more important, the current rate of increase is unprecedented and has no analogue, raising significant concern for the impact on marine ecosystems (Guinotte & Fabry, 2008; Hönisch et al., 2012; Turley & Gattuso, 2012).

1.2 Biological impacts

Ocean acidification has been acknowledged as one of the major challenges faced by marine ecosystems (Raven et al., 2005; Doney et al., 2012; IPCC, 2014; Andersson et al., 2015; Yates et al., 2015). Observational studies in locations of naturally high CO₂ illustrate some of these processes, and have shown community responses can be quite...
varied. Natural CO₂ seeps located in the temperate Mediterranean and tropical Papua New Guinea (PNG) provide a gradient of increasing acidity that have resulted in changes to community composition and species interactions (Hall-Spencer et al., 2008; Fabricius et al., 2011). Combined with a drop in diversity of calcifying species at these sites, there is a community shift to seagrass habitat. In contrast, a fjord in the Baltic Sea experiences high seasonal fluctuations of pCO₂ where, despite summer peaks well above levels predicted for the end of this century, calcifiers are still abundant (Thomsen et al., 2009).

Other studies using laboratory simulated conditions have shown that although not all species will be vulnerable to increasing OA (Dupont & Pörtner, 2013), a wide diversity of marine species will be negatively impacted (Kroeker et al., 2013). Some of the most sensitive to OA are the calcifiers (Dupont & Pörtner, 2013) due to the direct impact of altered seawater chemistry on calcification processes and calcium carbonate dissolution (Jury et al., 2010; Ries, 2011a; Ries, 2011b; Rodolfo-Metalpa et al., 2011; Tambutté et al., 2011). A range of other negative impacts have been found across all life stages from a wide range of taxa including annelids (Lane et al., 2012; Lewis et al., 2013), cnidarians (Kaniewska et al., 2012; Suggett et al., 2012), crustaceans (Dissanayake et al., 2010; Pansch et al., 2013), echinoderms (O'Donnell et al., 2010; Moulin et al., 2011; Wood et al., 2011; Uthicke et al., 2013a), fish (Bignami et al., 2013; Couturier et al., 2013), foraminifera (Bernhard et al., 2009), and molluscs (Bamber, 1990; Beesley et al., 2008; Tomanek et al., 2011; Bylenga et al., 2015). The range of responses and relative sensitivities are also illustrated in changes in community structure, such as meiofaunal community (Sarmento et al., 2015), and meta-analyses (Dupont et al., 2010a; Kroeker et al., 2010; Harvey et al., 2013).

Despite the wide ranging effects of OA discussed above, the early life stages of marine invertebrates may be among the most sensitive to increasing OA (Kurihara, 2008; Melzner et al., 2009). Some of the key aspects of larval development that are impacted by OA include size, biomineralisation, survival and recruitment (Findlay et al., 2009; Albright et al., 2010; O'Donnell et al., 2010; Barros et al., 2013). However, changes in physiological responses to OA are not always evident in impacts to morphology or development. Applying modern techniques to look beyond the physical traits to allelic expression, transcriptomics or metabolomics indicate that the effects of increasing pCO₂ can occur through changes that underpin physiological processes such as biomineralisation, lipid metabolism and internal pH (pHi) regulation (Evans et al., 2013;
Chapter 1. General introduction

Pespeni *et al.*, 2013b). To what extent changes in physiological responses apply to changes in gamete performance under increasing OA is relatively poorly understood beyond measures of fertilisation success.

**1.3 Impacts on sea urchin fertilisation and early cleavage**

The gametes of broadcast spawning marine invertebrates are released into the surrounding seawater where they are subjected to any perturbation in environmental conditions. Across a species distribution, the gametes are generally equipped with mechanisms to defend optimal cellular conditions and enable tolerance to natural fluctuations and natural extremes in environmental conditions (Hamdoun & Epel, 2007; Helmuth, 2009). Together, the scale of any environmental perturbation and the inherent tolerance of gametes will impact the response of fertilisation and early cleavage success to OA.

The effect of OA on fertilisation success varies across different marine invertebrates with some taxa, such as crustaceans, being more resilient than others (Kurihara, 2008; Harvey *et al.*, 2013; Wittmann & Pörtner, 2013). In general, while fertilisation and early cleavage in sea urchins appears to be relatively resilient to near-future OA, most impacts occur with further increases in $p$CO$_2$ towards far-future conditions (e.g.: Kurihara & Shirayama, 2004; Byrne *et al.*, 2010a; Morita *et al.*, 2010; Byrne, 2011; Martin *et al.*, 2011; Moulin *et al.*, 2011; Dorey *et al.*, 2013; Bögner *et al.*, 2014; Frieder, 2014; Sewell *et al.*, 2014). Some exceptions to this general resilience do occur, with both higher and lower levels of sensitivity to OA reported (Martin *et al.*, 2011; Frieder, 2014; Sung *et al.*, 2014). A growing number of studies also show that intra-specific differences underlie the general population level tolerance to increasing OA (Evans & Marshall, 2005; Schlegel *et al.*, 2012; Sewell *et al.*, 2014; Kavousi *et al.*, 2015).

One of the main sources of within-species variation in fertilisation and early cleavage comes from differences in gamete traits. The influence of both eggs and sperm to fertilisation success are well described in sea urchins (Levitan *et al.*, 1991; Levitan, 1996; Evans *et al.*, 2007) including in response to environmental change (Foo *et al.*, 2012; Kelly *et al.*, 2013; Sung *et al.*, 2014). Gametes are also limited in their ability to compensate for the large-scale rapid environmental change expected with OA (Hamdoun & Epel, 2007; Tadros & Lipshitz, 2009). A key component to predicting the impact of OA on
fertilisation and early cleavage rates is a better understanding of how the underlying performance of gametes will be impacted.

1.4 Sea urchin gamete activation & mitosis

Sea urchin gametes are stored in a quiescent state in the gonads under conditions that are low in O₂, high in CO₂, and have a low pH. Activation of both eggs and sperm involves multiple processes that are dependent on changes in O₂ tension, CO₂, pH; and other cations including calcium (reviewed in: Nakazawa et al., 1970; Chia & Bickell, 1983; Epel, 1990). Complex choreographies of cellular process and signal transduction pathways, including a rise in the pHi, are initiated on activation of both eggs and sperm. The eggs are activated with the fusion of a sperm with the egg membrane (Johnson et al., 1976; Epel, 1978, 1990; Foltz, 1995; Swann & Jones, 2002; Morgan, 2011; Vacquier, 2012; Swann & Lai, 2015), and the sperm activated on spawning and dilution in seawater (Johnson et al., 1983; Trimmer & Vacquier, 1986). Ocean acidification is suggested to disrupt the cellular processes involved with the activation of gametes, including the pH_i-dependent ion balance machinery, protein and enzyme functions, energetic pathways and signal transduction pathways (reviewed in: Bevensee & Boron, 2008; Place & Smith, 2012).

1.4.1 Internal pH

Internal pH plays a fundamental role in the regulation of a myriad of cellular processes that are key to the maintenance of normal cell activity (Busa & Nuccitelli, 1984; Bevensee & Boron, 2008). With the activation of sea urchin gametes, pH_i increases to a level that allows optimal cellular activity; whether that be for sperm swimming or mitosis in the fertilised egg (the zygote). Under OA conditions, the rise in pH of the surrounding seawater is expected to have knock-on impacts for defence of the optimal cellular conditions. In part this is through the limitations of ion pumps, such as the rate limitation of those shifting protons (H⁺) out of the cells to regulate pH_i (Gatti & Christen, 1985; Darszon et al., 1994). The increased energetic cost of higher levels of pH_i regulation (Busa & Nuccitelli, 1984; Place & Smith, 2012) are also likely to have knock-on impacts through to other energy dependent processes (Sokolova, 2013). The energetic cost of OA is exemplified in developing sea urchins with the increase in ATP allocation from 40% to 84% for protein synthesis and ion transport (Pan et al., 2015); a decrease of available ATP
of this magnitude, for example, will impact the energy available for ATP dependent sperm swimming, egg jelly induced acrosome reaction (Christen et al., 1986) and stable binding of sperm to eggs (Hirohashi & Lennarz, 1998).

1.4.2 OA impacts on mitosis

Once fertilised, OA has consistently been shown to impact cleavage rates of the sea urchin zygote (Kurihara, 2008; Moulin et al., 2011; Foo et al., 2012; Place & Smith, 2012; Cohen-Rengifo et al., 2013). So far, only a few studies have looked at mechanisms underlying this OA induced delay in mitosis; these have included a change in pH\textsubscript{i} of the unfertilised egg (Bögner et al., 2014) and potential increased energetic cost of defending homeostasis (Place & Smith, 2012). One of the possible outcomes of any change to pH\textsubscript{i} is through the pH\textsubscript{i}-dependent cellular processes involved in the first mitotic divisions, such as the architectural rearrangement of the zygote or increased DNA and protein synthesis (Spray et al., 1981; Busa & Nuccitelli, 1984; Peracchia, 1987; Schatten & Schatten, 1989; Epel, 1990; Chandler, 1991; Watanabe et al., 1997, 1998; Miller & Epel, 1999; Hamaguchi & Hamaguchi, 2001; Place & Smith, 2012; Ciapa & Philippe, 2013; Bögner, 2016).

1.4.3 OA impacts on sperm

Important components of the activation of sea urchin sperm involve the alkalinisation of pH\textsubscript{i} and the release of CO\textsubscript{2} (Johnson et al., 1983; Trimmer & Vacquier, 1986). Due to the pH-dependence of many cellular processes, the usual increase of pH\textsubscript{i} from < 7 to approximately pH 7.5 results in an increase in swimming through pH throttled cellular processes. The increased $p$CO\textsubscript{2} and decreased pH of OA are suggested to have a narcotic effect on sea urchin sperm through an impaired ability to regulate pH\textsubscript{i} (Havenhand et al., 2008; Byrne, 2011; Reuter et al., 2011), and the associated impact on pH-dependent processes such as the activation of the flagellar motors, energy transfer to the motors, ATP generation, mitochondrial substrate supply and more (Cardin & Meara, 1953; Mohri & Yasumasa, 1963; Gibbons & Fronk, 1972; Christen et al., 1982; Christen et al., 1986; Brokaw, 1987; Golding et al., 1995; Ellington, 2001; Neill & Vacquier, 2004; Nomura et al., 2005; Nishigaki et al., 2014; Vacquier et al., 2014). Although numerous studies suggest OA induced sperm narcosis as a mechanism underlying reduced sperm performance, this has yet to be empirically investigated.

Measures of sperm performance can include the proportion of sperm swimming (% motility) and sperm swimming speeds; both of these have key roles in fertilisation kinetics.
models (Vogel et al., 1982; Levitan et al., 1991; Levitan, 2000). Under OA conditions both negative and positive effects on sperm motility and swimming speed are evident across a range of marine invertebrates including bivalves (Havenhand & Schlegel, 2009; Vihtakari et al., 2013), cnidarians (Morita et al., 2010; Nakamura & Morita, 2012), polychaetes (Lewis et al., 2013; Schlegel et al., 2014), asteroids (Uthicke et al., 2013a), holothurians (Morita et al., 2010), as well as echinoids (Havenhand et al., 2008; Caldwell et al., 2011; Schlegel et al., 2012; Graham et al., 2015). Computer assisted sperm analysis (CASA) is commonly used to measure changes in swimming characteristics, including % motility and speed parameters such as the through-the-water and progressive straight line speed.

Declines in % motility and speed can negatively impact on fertilisation (Havenhand et al., 2008) by in-effect resulting in sperm limitation (Reuter et al., 2011). From the handful of studies exploring these ideas under OA conditions, the actual responses appear to be species-specific. Although the most common mechanism proposed to underpin the change in echinoid sperm performance under OA conditions is a change in sperm pH, recently, decreased motility has also been linked to lower mitochondrial membrane potential (Schlegel et al., 2015). Certainly within the echinoids, both the positive and negative impacts of OA to swimming speed and % motility, along with their varied corresponding impacts to fertilisation, questions the suggested narcosis effect.

1.5 Variation and resilience

The growing body of literature describing the effects of OA on the sensitivity or resilience of fertilisation in broadcast spawners has developed from the initial population level responses using pooled gametes from multiple individuals (Kurihara & Shirayama, 2004; Byrne et al., 2010b; Ericson et al., 2010), to the individual male:female cross level that is providing insight into the variability in trait responses to OA (Sunday et al., 2011; Foo et al., 2014; Sewell et al., 2014). If there is a genetic basis for the variability in resilience traits, it is possible that selective pressure of OA and transgenerational effects will lead to longer term adaptation (Agrawal et al., 1999; Kelly et al., 2013; Thor & Dupont, 2014). Determining the level of inter-individual variation is identified as an important area for further work and is crucial for developing more accurate predictions of the impacts and evolutionary potential of a species under the pressure of increasing OA
Chapter 1. General introduction

(Kurihara, 2008; Sunday et al., 2011; Kelly et al., 2013; Sewell et al., 2014; Vihtakari et al., 2016).

1.6 Study species

The endemic echinoid *Evechinus chloroticus* is of cultural and economic importance through fisheries, as well as ecological importance as a habitat modifier through algal grazing (Miller & Abraham, 2011). *Evechinus chloroticus* is found in coastal waters throughout New Zealand with a distribution extending from the Three Kings Islands in the north (Schiel et al., 1986) to the sub-Antarctic waters around The Snares Islands (Fenwick & Horning, 1980) and also eastward to the Chatham Islands (Dix, 1970a); although unsubstantiated reports have previously suggested the range extends to the sub-tropical Kermadec Islands (Pawson, 1961; Barker, 2007). Average summer sea surface temperatures (SST) across this distribution range from 13 ºC in the south to 20 ºC in the north (Garner, 1969). *Evechinus chloroticus* is most abundant in waters less than 12 m deep (Dix, 1970a; Barker, 2007), although the depth range includes from the intertidal to a depth of 60 m (Barker, 2007).

The preferred habitat of *E. chloroticus* varies across the distribution but is associated primarily with laminarian kelps (*Ecklonia radiata* or *Macrocystis pyrifera*) and other algae (*Carpophyllum maschalocarpum, Carpophyllum angustifolium, Sargassum sinclairii,* and *Lessonia variegata*) (Barker, 2007). Habitat suitability also depends upon physical conditions with *E. chloroticus* favouring moderate wave exposure, but only rarely found in areas of highest wave action (Choat & Schiel, 1982; Barker, 2007). Decreased water movement and increasing sediment often result in decreased juvenile and adult densities (Phillips & Shima, 2006; Walker, 2007). The distribution is normally patchy within habitats (Andrew & Choat, 1982) with *E. chloroticus* considered to play an important role in community structure through impacts of algal herbivory on habitat suitability for grazing molluscs and fish (Andrew & Choat, 1982; Andrew, 1988).

Within a generalised reproductive season from November to March, spawning timing in *E. chloroticus* differs between populations across the latitudinal distribution (Brewin et al., 2000) and also within populations (Walker, 1984). The annual reproductive cycle of *E. chloroticus* examined through gonad indices (Dix, 1970b; Walker, 1982; McShane et al., 1996; Brewin et al., 2000; Lamare et al., 2002; Wing et al., 2003) shows
that across the latitudinal distribution, the start and duration of the reproductive season varies (Barker, 2007). The reproductive output of *E. chloroticus* varies both temporally and spatially throughout the latitudinal range depending upon factors such as diet and population density (Andrew, 1988; Barker *et al.*, 1998; Brewin *et al.*, 2000).

As a broadcast spawner gametes of the dioecious *E. chloroticus* are released into the water column from gonopores on the aboral surface. Spawning usually occurs around the higher SST of the austral summer and shows a high degree of synchrony within populations (Lamare & Stewart, 1998; Lamare & Barker, 1999; Barker, 2007). At a finer scale, within the Hauraki Gulf temporal differences in spawning occurred between small localised populations with only a 10 km separation. In that study, despite spawning being synchronous within locations, spawning events were separated by up to 2 months between locations (Walker, 1982).

Previous published work on the potential effects of OA on *E. chloroticus* is limited to two studies. First, Clark *et al.* (2009) studied the negative impacts of OA to larval development and biomineralisation of skeletal elements. Second, Hurd *et al.* (2011) determined that in adults, a steep pH gradient occurs from the area between the spines of adults to the surrounding ambient seawater conditions. Further work is needed to expand the understanding of how species such as *E. chloroticus* will respond to OA.

1.7 Aims of research

The overall objective of this research was to characterise the effects of increasing ocean acidification on sperm performance and fertilisation of the broadcast spawning sea urchin *E. chloroticus*. The main research questions addressed in this thesis are: firstly, is *E. chloroticus* sperm swimming performance impacted by OA and is sperm narcosis, lower % motility and lower swimming speed, driven by changes in pH? Secondly, how will OA impact fertilisation success in the ecologically important *E. chloroticus*? Thirdly, are there intraspecific differences in fertilisation success that suggest possible adaptation of tolerance to OA?
Chapter 1. General introduction

1.8 Thesis structure

This thesis is comprised of four main empirical data chapters that present details of the research and findings towards addressing these research objectives.

1.8.1 Chapter 2

Ultrastructure of the sperm of four New Zealand populations of echinoids was examined with transmission electron microscopy to confirm and compare morphological arrangement, as well as determine the cell volume for calculation of ion concentration in Chapter 3. This chapter presents the first ultrastructural study of sperm from New Zealand populations of endemic, regional and cosmopolitan species.

1.8.2 Chapter 3

To examine pH\textsubscript{i} in sea urchin sperm cells, protocols for the fluorescent dye analysis of pH\textsubscript{i} were developed. In particular, this chapter presents the incubation protocols and calibration for Carboxy SNARF-1; the latter utilising cell volume to accurately determine a species-specific internal potassium concentration for correct buffer formulation.

1.8.3 Chapter 4

Sperm performance and quality were assessed under increasing OA conditions using computer assisted sperm analysis (CASA) along with paired pH\textsubscript{i} analysis on individual \textit{E. chloroticus} males. This chapter presents the first study to directly test the hypothesis of OA induced sperm narcosis driven by changes in pH\textsubscript{i}.

1.8.4 Chapter 5

Fertilisation and early cleavage were assessed under increasing OA conditions at the level of individual male:female crosses in \textit{E. chloroticus}. In addition nested half-sibling crosses were used to further assess the contribution of males and females to the underlying intraspecific variability in tolerance of fertilisation and early cleavage. This chapter presents the first study to describe the effects of OA on fertilisation in \textit{E. chloroticus}, and adds to the growing understanding around individual variation in marine species.

1.8.5 Chapter 6

Chapter 6 presents a summary and discussion of the findings of this thesis, and discusses further research directions to build on this work.
Chapter 2. Comparative ultrastructure of spermatozoa from two regular and two irregular New Zealand echinoids

The morphology of echinoderm spermatozoa has been studied for almost 170 years, with the first simple descriptions presented by R. Wagner in 1841, and the subsequent development of descriptive terminology through comparative ultrastructural studies (reviewed in: Chia & Bickell, 1983). Broadly categorised as “primitive,” spermatozoa with a spherical or conical head, mid-region, and long flagellum are typical of species with external fertilisation (Rothschild, 1956; Bernstein, 1962; Chia & Bickell, 1983; Trimmer & Vacquier, 1986). There are of course exceptions to this basic pattern, including that of the holothuroid *Rhabdomolgus ruber*, which has a highly derived sperm morphology and unusual spawning behaviour (Eckelbarger & Riser, 2013).

In contrast to those of asteroids, crinoids, holothuroids, and ophiuroids, echinoid spermatozoa have distinctly conical heads (e.g.: Jamieson, 1991). In echinoids, DNA is enclosed in a densely packed conical nucleus at the anterior region of the sperm; anterior to the nucleus, the sperm is capped with a membrane-enclosed acrosome complex (Afzelius, 1955; Summers et al., 1975). In the mid-region of the sperm cell, the single ring-shaped mitochondrion provides ATP from a primarily lipid-based energy store (Mita & Yasumasu, 1983). ATP powers the high energy demand of not only locomotion, but also the acrosome reaction and binding with the egg (Hirohashi & Lennarz, 1998). The posterior section of the sperm cell is the flagellum, which, through an axoneme whose microtubule arrangement is highly conserved across kingdoms (Lin et al., 2012), provides locomotion as well as the chemotactic response to egg jelly peptides in proximity to the egg (Darszon et al., 2008).

Beyond the phylogenetic grouping indicated by the conical shape of the echinoid sperm head, further relationships can be inferred through fine ultrastructural details as well as biochemical characteristics of spermatozoa. Groupings within the echinoids have been made, in part, through comparative ultrastructural work that shows species-specific characteristics (Chia et al., 1975; Eckelbarger et al., 1989a). Researchers have correlated head length to development mode (Raff et al., 1990), and have mapped ultrastructure and energy metabolism to phylogeny (Mita & Nakamura, 1998). Additionally, spermatozoa
from irregular and regular urchins have been suggested to have characteristic features based on acrosome complex and mitochondrion morphology (Drozdov & Vinnikova, 2010).

The ultrastructure of echinoid spermatozoa from New Zealand (NZ) populations has not previously been described. Here, we describe the ultrastructure of four species found in NZ. Two of these are irregular urchins: the endemic clypeasteroid sand dollar *Fellaster zelandiae* (Gray 1855), and the widespread spatangoid heart urchin *Echinocardium cordatum* (Pennant 1777) [previously *Echinocardium australis* Gray 1851], whose spermatozoa have been described in non-NZ populations (Afzelius, 1955; Jessen et al., 1973; Drozdov & Vinnikova, 2010). The other two are regular urchins: the endemic camarodont, kina, *Evechinus chloroticus* (Valenciennes 1846), and the recently arrived (Pecorino et al., 2012) diadematoid black urchin *Centrostephanus rodgersii* (Agassiz 1863). The irregular echinoids form a monophyletic clade sister to the camarodonts; together, these form a clade sister to the diadematoids (Figure 2-1). We describe comparative morphological characteristics in relation to previously suggested groupings within the echinoids, and also describe sperm characteristics relevant to metabolic and physiological studies, such as mitochondrial arrangement and cell volumes.

**Figure 2-1:** Phylogenetic relationships of the echinoid families studied here. Families in bold represent irregular urchins. The phylogenetic tree is modified from Smith and Kroh (2013), and is based on molecular and morphological data.

### 2.1 Methods

#### 2.1.1 Collection

Adult echinoids were collected from their natural habitats around the greater Hauraki Gulf during their respective breeding seasons in 2011 and 2012. The heart urchin (*Echinocardium cordatum*) and kina (*Evechinus chloroticus*) spawn through the austral summer, while black urchins (*Centrostephanus rodgersii*) are winter spawners, and sand dollars (*Fellaster zelandiae*) are suggested to be year-round spawners (Nipper et al., 1997;
Chapter 2. Sperm ultrastructure

Hall & Golding, 1998). The four species have distinctive morphological features that allow confident and repeatable identification (Barraclough Fell, 1948; Mills et al., 2014). We use the taxonomy of Kroh and Mooi (2011). Adults of *E. chloroticus* were collected by snorkel from a depth of < 3 m from the rocky reef of Mathesons Bay (36°18’6.58” S; 174°48’0.71” E); *E. cordatum* and *F. zelandiae* from depths of < 0.5 m from the intertidal soft sediment of Matakia Bay, Whangaparaoa Peninsula (36°37’36.25″S; 174°46’33.84″E); and *C. rodgersii* were collected by SCUBA from depths of < 10 m from the Mokohinau Islands (35°54’33.11″ S; 175°6’22.18″ E). Collected individuals of the last species were spawned immediately while all other species were transported in 20 L buckets with seawater to the seawater facilities at the University of Auckland and kept in 50 L aquaria until spawning. The aquaria for the burrowing sand dollar and heart urchin were flat and shallow (1000x650x150 mm) with a 25-50 mm layer of sediment collected from the same location as the animals.

### 2.1.2 Spawning

All specimens were spawned within a week of collection by an injection of up to 5 mL 0.55 M KCl through the peristomal membrane into the coelomic space; the volume varied among species due to their different internal volumes. Injected animals were lightly agitated to help mix the coelomic fluids. In the regular urchins, “dry” (undiluted) spermatozoa were collected with a Pasteur pipette from the gonopores on the aboral surface; dry spermatozoa were kept on ice until dilution. In the irregular urchins, individuals were inverted over containers of chilled (on ice) 1 µm filtered sea water with the gonopores immersed, and allowed to spawn. Spermatozoa were then concentrated by centrifugation (15 minutes at 500 rpm using an Eppendorf centrifuge 5810R refrigerated centrifuge at 4 °C). Sperm samples were activated in seawater prior to preservation to ensure sperm morphology represented a standardised non-quiescent state in both regular and irregular urchins.

### 2.1.3 Sample preparation and microscopy

Sample fixation for transmission electron microscopy (TEM) was performed within 1 hr of spawning using seawater buffers for primary fixation (Cosson & Gulik, 1982; Raff et al., 1990). In brief, sperm samples were diluted in 0.22 µm filtered seawater (FSW). Glutaraldehyde was then added to a final concentration of 2.5%, and samples were left overnight at 4 °C. After each of three 10 minutes washes in 0.22 µm FSW, samples were centrifuged (3000 rpm in an Eppendorf minispin for 4 minutes) and the supernatant
removed. For secondary fixation, samples were incubated in a 1% solution of osmium tetroxide in FSW for 1 hr, centrifuged as above, and the supernatant removed. Samples were then subjected to an eight step ethanol dehydration series at 10 minutes intervals, with a final wash with 100% ethanol followed by acetone. Samples were centrifuged as above between each step. Once in acetone, samples were initially left in dilute resin mix (1:1 acetone to epoxy resin) for 1 hour before being transferred to 100% resin overnight. After replacing with fresh resin, sperm samples stood for a further 8 hours before being embedded in resin-filled mold cups and placed in a 60 °C oven for 48 hours. Ultra-thin sections were prepared with a diamond (Diatome) knife using a Leica EM UC6 ultratome. Sections were transferred to 200 mesh copper grids, then stained with 2% aqueous uranyl acetate for 30 minutes and Reynolds lead citrate for 3 minutes. Mounted sections were observed and photographed using a Phillips CM12 TEM operating at 120 kV and from 9800 to 54000x magnification.

2.1.4 Analysis

Identification of sperm morphological characteristics was based on standard terminology used in the echinoderm literature (Chia & Bickell, 1983; Koch & Lambert, 1990; Jamieson, 2000), with additional specialised terminology for the flagellar necklace (Kleve & Clark, 1980; Cosson & Gulik, 1982; Drozdov & Vinnikova, 2010).

The image processing package Fiji (Fiji Is Just ImageJ; Schindelin et al., 2012) was used to estimate dimensions of spermatozoa, using the scale bar on each TEM image to calibrate measurements. Spermatozoa from at least three males were collected for each species, and measurements from 1 to 14 spermatozoa for each parameter were obtained from multiple grids for each male. Measurements of each parameter were initially averaged for each individual, before calculating species averages. Careful interpretation of images was required to avoid underestimations of maximum sizes by ensuring longitudinal sections were through the widest and most central part of the spermatozoa; this was assessed through surrounding morphological details such as acrosome and flagellum morphology, as well as the general symmetry of the arrangement of the ultrastructural details. Sperm head volumes were calculated by taking the head as a simplified cone. Mitochondrial volumes were calculated using Pappus’s centroid theorem for volume \( V \) of a torus, with modifications to account for the irregular mitochondrial shape through averaged cross sectional area of the mitochondrion \( M \), using the equation \( V = 2\pi R x M \), where \( R \) describes the distance from the centre of the tube to the centre of the torus.
For all measurements, significant differences between species were determined using ANOVA with post hoc groupings given by Tukey’s HSD test ($\alpha = 0.05$) using JMP (version 11.02.0). Assumptions of homoscedasticity (Levene or Brown-Forsyth test) and normality (Shapiro–Wilk test) were also checked, although the power of these tests was limited by small sample size.

### 2.2 Results

#### 2.2.1 Gamete morphology

In general, sagittal sections from all four species showed that spermatozoa were composed of an extended conical head consisting of the electron-dense nucleus tipped anteriorly with the acrosomal complex, a single mitochondrion in the mid-region, and a single long flagellum (Figure 2-2a–d). Morphometric data on spermatozoa of each species are shown in Table 2-1. We first present descriptions of sperm morphology in each species (Figure 2-3a–y), and then consider comparative ultrastructure in light of the phylogenetic relationships described above.

![Image 1](image1.png)

Figure 2-2: Ultrastructure of the spermatozoa of four New Zealand echinoids. a. *Fellaster zelandiae* (Fz). b. *Echinocardium cordatum* (Eco). c. *Evechinus chloroticus* (Ech). d. *Centrostephanus rodgersii* (Cr). All scale bars = 0.5 $\mu$m. av, acrosomal vesicle; f, flagellum; m, mitochondrion; n, nucleus.
Table 2-1: Ultrastructure of spermatozoa of the New Zealand echinoids *Fellaster zelandiae*, *Echinocardium cordatum*, *Evechinus chloroticus*, and *Centrostephanus rodgersii*. Linear dimensions are in µm and volumes are in femtolitres. Values are means ±SE, with brackets denoting sample sizes greater than three (sample size = number of individual male urchins; unreported technical replication for each trait varied between 1-14 spermatozoa per individual). Volumes are calculated from averages per individual. Letters represent Tukey HSD groupings (\( \alpha = 0.05 \)). Significance represented by ** p < 0.0001, * p < 0.001.

<table>
<thead>
<tr>
<th></th>
<th>Irregular</th>
<th>Regular</th>
<th>ANOVA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><em>F. zelandiae</em></td>
<td><em>E. cordatum</em></td>
<td><em>E. chloroticus</em></td>
</tr>
<tr>
<td>Acrosome complex length</td>
<td>1.14±0.07 A</td>
<td>1.37±0.15 A</td>
<td>0.45±0.01 (4) B</td>
</tr>
<tr>
<td>Head length (acrosome and nucleus)</td>
<td>2.66±0.06 A</td>
<td>3.58±0.10 B</td>
<td>4.44±0.21 (5) C</td>
</tr>
<tr>
<td>Nucleus diameter</td>
<td>1.06±0.09 BC</td>
<td>1.36±0.00 A</td>
<td>0.91±0.03 (7) C</td>
</tr>
<tr>
<td>Overall length of sperm head &amp; mitochondrion</td>
<td>3.09±0.07 A</td>
<td>4.02±0.06 B</td>
<td>4.92±0.12 (5) C</td>
</tr>
<tr>
<td>Mitochondrion diameter</td>
<td>1.14±0.03 B</td>
<td>1.32±0.04 A</td>
<td>0.91±0.03 (7) B</td>
</tr>
<tr>
<td>Mitochondrion volume</td>
<td>0.39±0.08 B</td>
<td>0.59±0.03 A</td>
<td>0.27±0.01 (5) B</td>
</tr>
<tr>
<td>Total volume per sperm cell</td>
<td>1.18±0.21 B</td>
<td>2.32±0.08 A</td>
<td>1.29±0.06 (5) B</td>
</tr>
<tr>
<td>Ratio (overall length:nuclear diameter)</td>
<td>2.93</td>
<td>2.97</td>
<td>5.29</td>
</tr>
</tbody>
</table>

### 2.2.1.1 *Fellaster zelandiae*

The irregular urchin *Fellaster zelandiae* had the shortest conical sperm head of the four species, with an overall head length of 3.09 µm on average. Nucleus diameter was 1.06 µm, and the total volume of the sperm cell was 1.18 femtolitre (fL) (Figure 2-2a; Table 2-1). The anteriorly located acrosomal complex was 1.14 µm long and comprised of a conical- to round-shaped acrosomal vesicle with a flattened basal area, under a double membrane (Figure 2-3a). The long subacrosomal material (or periacrosomal material) extended into a deep and voluminous nuclear fossa (or subacrosomal fossa) (Figure 2-3a). At the posterior end of the nucleus the adjoining ring-shaped mitochondrion (1.14 µm in diameter) with tightly folded cristae surrounded the parallel distal and short proximal centrioles, as in *Centrostephanus rodgersii* (Figure 2-3e,m). The proximal centriole was located close to the nucleus and separate from the distal centriole (Figure 2-3m,p), while the more central distal centriole (or basal body) extended as the flagellum (Figure 2-3e,i).
The microtubule fibril arrangement changed in the transition region from the hollow-centred distal centriole to give the characteristic nine external doublet and central single pair, or 9+2, arrangement of the flagellum (Figure 2-3i,r). The anterior end of the distal centriole extended out from the very shallow nuclear invagination of the centriolar fossa, and was tipped with a plug of electron-dense material (the dense cap, Figure 2-3i). Part of the elaborate flagellum to mid-region attachment was apparent in the fine detail of the electron-dense peri-centriolar complex (flagellar necklace) and the electron-dense membrane between the flagellum and surrounding the mitochondrion (Figure 2-3i). This flagellar necklace attached the flagellum at the posterior end of the distal centriole to the mid-region at the posterior end of the mitochondrion (Figure 2-3i). Lipid droplets were located around the flagellum in an apparent radial arrangement, posterior to the mitochondrion (Figure 2-3n).
Chapter 2. Sperm ultrastructure
Chapter 2. Sperm ultrastructure

Figure 2-3: Details of the ultrastructure of the spermatozoa of four New Zealand echinoids. In a-u, each column represents a species (as labelled in the top row: *Fellaster zelandiae* (Fz), *Echinocardium cordatum* (Eco), *Evechinus chloroticus* (Ech), and *Centrostephanus rodgersii* (Cr)). a-d. Longitudinal sections through the acrosomal vesicle and nuclear fossa. e-h. Cross sections of mitochondria and parallel centrioles. i-m. Longitudinal sections through the flagella, mitochondria, and nucleus in the mid-region. n-q. Mid-region sections (n, cross; o-q, longitudinal) showing lipid drop arrangements. r-u. Cross sections of flagella. v-y. Cross sections of heads of Ech and Fz. v. Anterior nucleus (Ech). w. Posterior end of nuclear fossa (Fz). x. Nuclear fossa including subacrosomal material (Fz). y. Acrosomal vesicle (Fz). All scale bars = 0.2 µm. av, acrosomal vesicle; cf, centriolar fossa; dc, distal centriole; f, flagellum; ld, lipid drop; n, nucleus; ne, flagellar necklace; nf, nuclear fossa; m, mitochondrion; md, microtubule doublet; pc, proximal centriole; pr, postacrosomal rod; r, radial spoke; sm, subacrosomal material; sp, single pair; tr, transition region.

### 2.2.1.2 *Echinocardium cordatum*

The conical sperm head of *Echinocardium cordatum* had an overall length of 4.02 µm and a nucleus diameter of 1.36 µm (Figure 2-2b; Table 2-1). The sperm cell of this species had the largest sagittal section area and the largest volume (2.32 fL) of all four species. The nucleus was tipped with an acrosomal complex 1.37 µm long comprising a rounded acrosome vesicle with a flattened basal area. The acrosome vesicle was extended anteriorly on the postacrosomal rod from a very deep nuclear fossa (Figure 2-3b). The fixation of the postacrosomal rod was not sufficient to preserve the organisation of the filamentous fibres. At the posterior end of the nucleus the distal centriole extended out from a very shallow indentation of the centriolar fossa, and ran parallel to the short proximal centriole (Figure 2-3j) as shown in *C. rodgersii* (Figure 2-3m,q). The distal centriole formed the 9+2 microtubule arrangement of the flagellum through the transition region (Figure 2-3j,s). At the posterior end of the distal centriole, the elaborate flagellar neck-lace connected the flagellum close to the posterior end of the ring mitochondrion (Figure 2-3j,f), which was 1.32 µm in diameter. The lipid droplets were usually located in pairs at the posterior end of the mitochondrion (Figure 2-3o).

### 2.2.1.3 *Evechinus chloroticus*

With the greatest intra- and inter-male variability in sperm cell morphology, sample sizes were increased for *Evechinus chloroticus* to better estimate mean sizes. The long (4.44 µm) and narrow (0.91 µm) sperm head of *E. chloroticus* had a relatively small volume (1.29 fL; Figure 2-2c; Table 2-1). The short acrosomal complex (0.45 µm; Figure 2-2c) included a rounded acrosome vesicle with a flattened basal area and short subacrosomal material (Figure 2-3c). A deep centriolar fossa enclosed the entire distal
centriole, forming the flagellum just posterior to the nucleus (Figure 2-3k). Running parallel to the flagellum was the short proximal centriole, often found between the mitochondrion and the nucleus (Figure 2-3g,k); the proximal centriole was also seen in a variety of other orientations in spermatozoa with non-symmetrical mitochondria. In this same area, the flagellar necklace complex attached the flagellum to the anterior end of the 0.91 µm diameter mitochondrion (Figure 2-3k). The fine detail of the flagellum cross section showed the electron-dense radial spokes (r) linking the doublets to the single central pair of the 9+2 microtubule arrangement (Figure 2-3t). No evidence of lipid droplets attached to the posterior end of the mitochondrion was found; however, non- to lightly-staining membrane-bound areas within the mitochondrion might potentially have contained lipid, and have been labelled as such in Figure 2-3p. Alternatively, as in some other echinoid species (e.g.: Marks et al., 2008), lipid droplets may be absent in the spermatozoa of *E. chloroticus*.

### 2.2.1.4 Centrostephanus rodgersii

The sperm head of the regular urchin *C. rodgersii* had an overall length of 4.18 µm, with an average nucleus width of 1.24 µm and cell volume of 2.03 fL (Figure 2-2d; Table 2-1). A short acrosomal complex (0.45 µm) at the tip of the nucleus was comprised of a rounded acrosome vesicle and short subacrosomal material in a shallow nuclear fossa (Figure 2-3d). Extending out from a relatively deep centriolar fossa, the distal centriole ran parallel to the short proximal centriole (Figure 2-3h,m,q) and formed the 9+2 microtubule arrangement of the flagellum in the transition region (Figure 2-3m,u). At the posterior end of the distal centriole, the flagellum was attached to the mid-region by the flagellar necklace close to the posterior end of the 1.24 µm diameter mitochondrion (Figure 2-3m). Lipid drops were observed attached to the mitochondrion as singles (Figure 2-3q) and occasionally pairs.

### 2.2.2 Comparative ultrastructure

As well as statistically significant differences in sperm morphology found between species (Table 2-1), three morphological characteristics clearly distinguished the two broad groupings of irregular and regular echinoids, despite the latter not being monophyletic. First, sperm head length:width ratios were < 3 in the irregular urchins, and > 3 in the regular urchins (Table 2-1; Table 2-2). Second, deep nuclear fossa in the acrosomal complex of the irregular urchins were significantly longer than those from the regular urchins (Figure 2-3; Table 2-1). Third, a very small invagination of the centriolar fossa at
the posterior end of the nucleus of irregular urchins contrasted with the larger invagination found in the regular urchins; this invagination was deepest in *E. chloroticus* (Figure 2-2; Figure 2-3; Table 2-2).

Table 2-2: Comparative features of spermatozoa from four New Zealand echinoids: *Fellaster zelandiae*, *Echinocardium cordatum*, *Evechinus chloroticus*, and *Centrostephanus rodgersii*

<table>
<thead>
<tr>
<th>Description</th>
<th>Irregular</th>
<th>Regular</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Length:width ratio &gt; 3</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2. Deep nuclear fossa</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>3. Very shallow centriolar fossa invagination</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4. Lipid drops posterior on mitochondrion</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>5. Radial arrangement of lipid drops</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>6. Flagellar necklace anterior on mitochondrion</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>7. Parallel centriolar pair in the mid region</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>8. Single 9+2 flagellum</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Morphological features shared by spermatozoa of all four species included a parallel pair of centrioles in the mid-region, and a 9+2 arrangement in the flagella (Table 2-2). In addition, although there were species-specific differences in mitochondrial diameter and volume (Table 2-1), generally mitochondria were fairly uniform in shape and size across all four species examined, except for the slightly smaller mitochondria of *E. chloroticus* (Table 2-1). Interestingly, despite the use of consistent fixation protocols, in comparison to the majority of “normal” mitochondrial sections, a wide variety of morphological variants were only found for *E. chloroticus*. Variants included elongate mitochondria as well as mitochondria with varying degrees of asymmetry (Figure 2-3k,p; Figure 2-4).
The arrangement of the acrosome complex was also shared across all four species, although the morphological details and sizes of this complex varied among species. Comparisons between adjacent cross sections from the acrosome vesicle toward the anterior part of the nucleus of both *E. chloroticus* and *F. zelandiae* showed broad differences between the acrosome vesicle, nuclear fossa with subacrosomal material, nuclear fossa without subacrosomal material, and cross section of the nucleus. These differences were evident between cross sections of all four species, but were most visible in *F. zelandiae* with its voluminous nuclear fossa.

Other sperm morphological features such as the location and arrangement of the lipid drops or the location of the flagellar necklace showed no clear pattern that differentiated irregular and regular echinoids (Table 2-1).

### 2.3 Discussion

All four species examined had spermatozoa with small conical heads, with average lengths ranging from 3.09 to 4.92 µm. These lengths are consistent with measurements reported for echinoid species with planktotrophic larval development (average 4.84 µm; Raff *et al.*, 1990) and contrast with the longer sperm heads found in species with direct development (averaging 10.23 µm). However, the length to width ratio of the sperm heads of *Evechinus chloroticus* fell outside of the ratios for other species with planktotrophic development, being more similar (long and narrow) to those in direct developing species.
(Raff et al., 1990). Based on the comparative ultrastructure reported here, spermatozoa of all four species show statistically significant, species-specific characteristics as well as some characteristics that differentiate the regular and irregular sea urchins.

Comparisons of sperm traits across the geographic distribution of widespread species can reveal high levels of among population variability, and, to a lesser degree, within population variability. For example, in *Strongylocentrotus droebachiensis* there are differences in fine ultrastructural detail (amount and location of filamentous actin) within populations, and on a broader geographic scale, differences in sperm length between Atlantic and Pacific populations (Manier & Palumbi, 2008; Marks et al., 2008). Similarly, there are distinct morphological differences between spermatozoa of *Echinocardium cordatum* from the Baltic Sea (Jessen et al., 1973) and the Sea of Japan (Drozdov & Vinnikova, 2010) (note, however, that these populations are in different clades of the *E. cordatum* “species complex”: Chenuil & Féral, 2001; Chenuil et al., 2008). The spermatozoa of *E. cordatum* from New Zealand share more characteristics with those from the Sea of Japan than those from the Baltic, reflecting the distinct Pacific clade proposed by Chenuil et al. (2008).

Grouping the four families into their regular and irregular categories was reflected in the three morphological traits of sperm head length:width ratio, depth of the centriolar fossa, and depth of the nuclear fossa. Although irregular urchin species have previously been described as having long sperm heads and shallow nuclear fossa (Summers & Hylander, 1974; Drozdov et al., 2012b), the opposite is described here. The relatively short and wide sperm heads of the irregular urchins *Fellaster zelandiae* and *E. cordatum* (length:width ratio < 3) contrasted with the longer and narrower sperm heads of the regular urchins *Centrostephanus rodgersii* and *E. chloroticus* (length:width ratio > 3). The nuclear fossa of the acrosome complex was also much deeper in the irregular urchins. In *E. cordatum* the depth is thought to be a function of housing the posterior end of the long and anteriorly extended postacrosomal rod (Jamieson, 2000). However, the acrosomal vesicle was not extended on the postacrosomal rod in spermatozoa of *F. zelandiae*; instead, it was similar to the acrosome complex of the regular urchin *S. droebachiensis* (Marks et al., 2008). Although these traits may not hold as generalisations across all irregular and regular species, they do appear to separate the four NZ species into their respective regular and irregular groups.
Similarly, the depth of the centriolar fossa can also be used to separate the NZ echinoids into regular and irregular groups. However, the arrangement of the associated flagellar necklace was not as useful in distinguishing regular from irregular urchins (Table 2-2). As with the two regular urchins from NZ, a deep centriolar fossa is described in other regular urchins such as *Arbacia punctulata* (Franklin, 1965), *S. droebachiensis* (Afzelius & Murray, 1957) and the diadematoid *Diadema setosum* (Mita et al., 1995). In comparison, the shallow centriolar fossa in the irregular urchins *F. zelandiae* and *E. cordatum* is a characteristic shared with other irregular species such as *Clypeaster japonicus* (Mita & Nakamura, 1998) and *Echinarchnius parma* (Summers & Hylander, 1974). However, this shallow arrangement has also been described for some regular urchins, including a few deep-sea echinothuriid species (Eckelbarger et al., 1989a). The deeper centriolar fossa, like that found in the regular urchin *E. chloroticus*, is suggested to strengthen the attachment between the flagellum and long conical head (Chia & Bickell, 1983) and, in part, reflects the suggested location of the flagellar necklace in the four NZ echinoids. Compared to other echinoderm classes and in particular the holothuroids, the echinoid flagellar necklace is not as well described (Jamieson, 2000). The presence of the necklace is described in both freeze fracture work (Cosson & Gulik, 1982) and through the presence of actin around the axonemal centriole (Baccetti & Burrini, 1983). The apparent location of the flagellar necklace in these NZ species reflects that reported and illustrated by Drozdov and Vinnikova (2010), with the most anterior position occurring in the regular urchin *E. chloroticus*, while the flagellae of spermatozoa of the irregular urchin *F. zelandiae* are attached close to the posterior edge of the mitochondrion.

As described for echinoderms in general, the single flagellum originates from the distal centriole of the mid-region centriolar pair (Chia & Bickell, 1983). In echinoids the members of the centriolar pair are usually found to be parallel; however, a nonparallel arrangement has also been described (Baccetti & Burrini, 1983). Similarly, an exception to the usual single flagellum is seen in *Phrissocystis multispina*, where individuals produce both uni- and bipolar-tailed spermatozoa (Eckelbarger et al., 1989b). Consistent with echinoid spermatozoa being generally considered as morphologically conservative (e.g.: Chia & Bickell, 1983), the four species examined here had a mid-region with a parallel centriolar pair and a distal centriole extended as a single flagellum, surrounded at the anterior end by the ring-shaped mitochondrion.
As with the location of the flagellar necklace, no comparative groupings are evident in the mitochondrial cross sections, with spermatozoa from all four species sharing similar morphology overall. However, a number of *E. chloroticus* mitochondria exhibited asymmetry along with disparate shapes and sizes, despite the use of identical preservation protocols for all four species. Deviations from the symmetrical ring-shaped mitochondria are not uncommon, with asymmetrically disposed mitochondria found in species such as the regular urchin *Heliocidaris erythrogramma* (Raff et al., 1990) and the irregular urchin *E. parma* (Summers & Hylander, 1974). Deviations in symmetry have even been suggested to be the most prevalent state in echinoid species (reviewed in: Chia & Bickell, 1983), and interspecific differences in mitochondrion morphology proposed as a taxonomic feature along with head shape (Drozdov et al., 2012a). On the other hand, asymmetrical mitochondria have sometimes been described as being abnormal (Afzelius, 1955), and intraspecific differences in mitochondrion morphology are sometimes evident (e.g.: Summers & Hylander, 1974). Mitochondria in general are dynamic (Bereiterhahn & Voth 1994) and the occurrence of abnormal mitochondria in the spermatozoa of *E. chloroticus* may result from a higher sensitivity to the handling and preservation techniques despite identical methods used across all four species examined. Abnormal specimens were excluded from morphological measurements.

Lipid drops were found on the posterior end of the mitochondria in *F. zelandiae*, *E. cordatum*, and *C. rodgersii*. In contrast, in *E. chloroticus* lipid reserves may be located between the flagellum and the surrounding mitochondria (although their presence requires further investigation), as described for the closely related Echinometra species (Mita et al., 2004) and *Hemicentrotus pulcherrimus* (Mita & Nakamura, 2001). Like the deep water echinothuroid *Phormosoma placenta* (Eckelbarger et al., 1989a) and the spatangoid *Brissopsis lyrifera* (Afzelius & Mohri, 1966), the lipid droplets in *F. zelandiae* appear to be arranged in a radial pattern around the flagellum. This provides endogenous energy storage far in excess of that observed in the other species examined here (Eckelbarger et al., 1989a), thereby providing the potential for extended sperm longevity.

The external environment into which spermatozoa of broadcast spawners are released plays a critical role in their capacity to maintain activity and ultimately achieve fertilisation (Cabrita et al., 2008). On spawning, dilution of echinoderm spermatozoa in seawater results in characteristic helical and planar swimming motion (Woolley & Vernon, 2001) through the alkalinisation of internal pH (pHi) followed by the initiation of flagellar
bending (Johnson et al., 1983). At the same time, the metabolism of the lipid energy reserves is controlled in part through pH sensitive lipase and phospholipase A2 activity (Mita et al., 1990; Mita, 1991). With the cell volume values presented here, absolute ion values in marine invertebrate spermatozoa obtained from techniques such as ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) can now be converted into concentrations to provide more appropriate estimates of osmotic and ionic cellular environments, and a better understanding of sperm performance and quality (Cabrita et al., 2008; Fauvel et al., 2010).

This study provides the first description of sperm ultrastructure from four New Zealand echinoids from four different families. Although species-specific variations exist in several of the attributes described here, some key features reflect the irregular and regular groupings within the echinoids. The addition of sperm head volume measurements provides critical information for use in future physiological studies. Combined, these observations can contribute to more accurate assessments of sperm performance and quality of broadcast spawning echinoids under different conditions, including those resulting from global environmental change.
Chapter 3. Methods for the analysis of internal pH in sea urchin sperm

3.1 Introduction

Ocean acidification (OA) is resulting in a physiological challenge for marine organisms with potential impacts on cellular processes such as acid-base homeostasis and regulation of internal pH (pHi). If the sperm of sea urchins lack the capacity to defend an optimal pHi under the effects of OA we might expect impacts on pHi-dependent cellular processes involved with sperm activation, motility and ultimately fertilisation (Nishigaki et al., 2014). This is the most commonly suggested mechanism for the OA induced reduction in both sperm performance (% motility and swimming speeds) and levels of fertilisation reported for sea urchins (Havenhand et al., 2008; Byrne, 2011; Reuter et al., 2011). Protocol development based on previous sperm analyses using a combination of fluorescent dye and flow cytometry (e.g.: Gillan et al., 2005; Hossain et al., 2011; Robles & Martinez-Pastor, 2013) will allow the characterisation of pHi of *Evechinus chloroticus* sperm cells, and the effect of increasing OA.

Fluorescent dyes can be used to show fine scale changes in echinoderm sperm pHi, with the two most popular dyes being BCECF (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein) (Guerrero & Darszon, 1989; Seifert et al., 2015) and SNARF-1 (5-(and-6)-carboxy SNARF®-1) (Sase et al., 1995; Nakajima et al., 2005). Compared to the dual excitation single emission characteristics of BCECF, the single excitation dual emission of SNARF-1 is an advantage for analysis in single laser flow cytometers.

The excitation of SNARF-1 with a 488 nm argon laser and the associated isobestic point of ~610 nm enables the quantitative determination of pHi through the calibrated pH-dependent shift in emission spectra (Figure 3-1) (Molecular Probes, 2010). Additionally the pKa of pH 7.5 (although see Buckler & Vaughan-Jones, 1990 for a suggested value of between 7.6 to 7.8 in mammalian cells) and a physiologically relevant range from pH 7 to 8 make SNARF-1 ideal for analysing pHi in sea urchin sperm with a pHi of around 7.5 when activated (Christen et al., 1982; Christen et al., 1983; Johnson et al., 1983; Lee et al., 1983).
Calibration of SNARF-1 fluorescence measurements using the polyether antibiotic nigericin (Bond & Varley, 2005) allows quantification of pH_i through the equalisation of ion concentrations across the cellular membrane. Using a series of pH buffers across the expected pH_i range, the nigericin acts as an ionophore to equalise hydrogen ion concentrations [H^+], therefore effectively setting the pH_i to the buffer pH (Thomas et al., 1979; Hamidinia et al., 2004). However, nigericin also equalises other cations including sodium and potassium, therefore perturbing the cellular ionic environment with consequences for SNARF-1 emissions (Pressman, 1976; Riddell et al., 1988; Negulescu & Machen, 1990). Therefore, correct formulation of buffers is important when measuring pH_i to minimise perturbations of the SNARF-1 fluorescence signals.

SNARF-1 and nigericin calibrations have been used in assessment of sperm pH_i for a number of different species including boars (Kamp et al., 2003), starfish (Nakajima et al., 2005) and marine shrimp (Lindsay & Clark, 1992); with each study using a unique set of protocols. This chapter outlines the optimisation of protocols for using calibrated SNARF-1 analysis of pH_i in sperm of the sea urchin *Evechinus chloroticus*. This information will be used in Chapter 4 for the characterisation of the effect of OA on pH_i.
3.2 Methods

The development and optimisation of SNARF-1 incubation and analysis protocols involves three main methodological issues: (1) The incubation time and concentration of SNARF-1 needs to be determined to optimise the fluorescence signal and minimise compartmentalisation; (2) The parameters of the flow cytometer to be used in measurement need to be optimised for SNARF-1 excitation and emission acquisition; and (3) The correct buffer formulation as well as time and concentration of nigericin incubation needs to be determined to produce accurate values for pHᵢ calibration (Figure 3-2). Protocol development was performed using fresh *Evechinus chloroticus* sperm during the summer reproductive season, and out of season work performed using either cryopreserved *E. chloroticus* sperm (method from Serean Adams, Cawthron Institute, modified from: Adams *et al.*, 2008) or fresh sperm from the echinoid *Fellaster zelandiae*.

![Carboxy SNARF-1 incubation parameters](image)

![Flow cytometer setup](image)

![pHᵢ calibration](image)

Figure 3-2: Key steps in development of SNARF-1 incubation and analysis protocol: Incubation time and concentration for optimised fluorescence signal; flow cytometer parameterisation for excitation of SNARF-1 and emission acquisition; pHᵢ calibration accuracy dependent on correct buffer formulation and nigericin incubation conditions.

3.2.1 Sea urchin collection and spawning

*Evechinus chloroticus* were collected from the shallow subtidal (1-3 m depth) at Mathesons Bay (36°18’6.58” S; 174°48’0.71” E) within the Hauraki Gulf, New Zealand, during the austral summers of 2012-2014. Adults were transported to the seawater
facilities at the University of Auckland within 2 hours of collection and maintained in environmentally controlled conditions (18 °C, 12:12 dark:light cycle). Aerated aquaria (80 L) were stocked with a minimum of 2 L of seawater per urchin with 50-75% water changed every 2-3 days to minimise faecal matter and debris build up. Urchins were fed to satiation on seaweed (*Ecklonia radiata* and *Carpophyllum* spp.) and spawned within 2 weeks of collection. Standard procedures for inducing spawning involved a 1-3 mL injection of 0.5 M KCl into the coelomic space and sperm collected dry from the aboral surface and stored in 1.5 mL Eppendorf tubes in iced water.

### 3.2.2 SNARF-1 preparation

Carboxy SNARF®-1, acetoxymethyl ester, acetate (Invitrogen, Molecular Probes C1272, Lot # 1151593) is supplied as 20 x 50 µg vials of a lyophilized solid that is prone to hydrolysis, and should be stored at -20 °C protected from moisture and light (Molecular_Probes, 2010). To ensure optimum dye performance, a stock solution of 1.25 mM SNARF-1 was prepared in a supplied vial with anhydrous dimethyl sulfoxide (DMSO, from Sigma-Aldrich) and kept in the dark at -18 °C between uses.

### 3.2.3 Incubation protocol

To maintain performance characteristics of the sperm (activation, motility and viability) the duration of incubation needed to be within in a 3-hour window post spawning (Ohtake, 1976; Ohtake *et al.*, 1996; Adams *et al.*, 2003).

A standard 4 µL aliquot of dry sperm was added to 76 µL of 0.22 micron filtered seawater (FSW) containing SNARF-1 to give the target final dye concentration of between 0.05 and 130 µM, as well as a control without SNARF-1. Incubations were prepared in brown coloured 1.5 mL Eppendorf tubes and mixed gently by hand (vortexing for homogenous suspension was avoided as this rapidly results in removing flagella from sperm cells; pers. obs. and Brokaw (1986)) and left in ice-water for the required incubation period (30 minutes to 9 hours) in the dark to avoid photobleaching (Venn *et al.*, 2009).

The incubation duration and concentration can have consequences for compartmentalisation in the different regions (acrosome, head, mitochondria and flagellum) of the sperm cell (Kamp *et al.*, 2003; Han & Burgess, 2009). A relatively short incubation period (1.5 hour) had previously been identified to minimise compartmentalisation in mitochondria (Venn *et al.*, 2009). Incubation protocol
Chapter 3. SNARF-1 methods

development targeted the lowest concentration and shortest duration that showed repeatable and stable SNARF-1 emissions.

3.2.4 Fluorescence acquisition – flow cytometer

Fluorescent signals from sperm cells were obtained using a predetermined target sperm concentration of 1 million cells/mL and analysed immediately in a single laser flow cytometer (BD Biosciences FACS Calibur) optimised for E. chloroticus sperm cells using a 488 nm argon laser and proprietary BD CellQuestPro software. A combination of forward and side scatter (FSC & SSC respectively) were used to identify the sperm cells. The SNARF-1 emission fluorescence was collected through detectors FL2 and FL3 (585/42 nm and 670 nm long-pass respectively) with voltages adjusted for autofluorescence and position on the axes of the unstained sperm population. Only partial compensation was performed for SNARF-1 due to emission characteristics and detection range of FL2 and FL3, but also due to the rapid loss of dye under the lowest pH calibration buffers; this was most pronounced in the cryopreserved sperm with calibration buffers of < 7.75 pH. Once event rate had fallen to between 350 to 150 events per second, a single consolidated population of sperm cells was identifiable by FSC and SSC, at which point data from approximately 5000 events were collected from each sample. The SNARF-1 fluorescence ratio (R) calculated from FL3/FL2 is used in further calculations (see Section 3.2.8 for more details). The high throughput rates and rapid acquisition of the flow cytometer provide a high level of sampling repeatability that averages emissions across the cell (Martínez-Pastor et al., 2010; Hossain et al., 2011).

3.2.5 Sperm viability

A visual comparison of unstained and SNARF-1 stained sperm viability was made at 400x mag on a microscope slide using bright field microscopy to confirm retention of high levels of motility in stained cells. Only sperm samples exhibiting high levels of motility were used in protocol development.

Sperm viability was also checked after the SNARF-1 incubation period in parallel control incubations (no SNARF-1 staining) with a 1 µM propidium iodide (PI) incubation using the flow cytometer set up as described above. Propidium iodide fluorescence was detected in FL2 (on the FACS Calibur flow cytometer) increasing 20- to 30-fold when bound with nucleic acids in DNA and RNA. As PI is cell membrane impermeant, only cells with intact membranes do not fluoresce and are considered viable.
3.2.6 Calibration

Calibration of SNARF-1 fluorescence measurements using the nigericin method allows quantification of pH$_i$ (Bond & Varley, 2005). Nigericin was added to SNARF-1 stained sperm mixtures which were then analysed through the flow cytometer across a range of nigericin concentrations and incubation times. As nigericin acts as an ionophore for a range of cations including sodium and potassium (Pressman, 1976; Riddell et al., 1988), with implications for SNARF-1 emissions (Negulescu & Machen, 1990), correct buffer formulation is required to limit the effect of nigericin primarily to [H$^+$]. Using a buffer series across a range of pH values enables calibration of pH-dependent shifts in SNARF-1 fluorescent ratio for further sample analysis.

3.2.6.1 Considerations for calibration buffer formulation

Minimising changes in [Na$^+$] and [Ca$^{2+}$]$_i$ is achieved through replacing sodium chloride with choline chloride, as choline has been shown to block nigericin-induced across-membrane movement of Na$^+$ and Ca$^{2+}$ (Pressman, 1976; García-Soto et al., 1987; Riddell et al., 1988).

3.2.7 *Internal potassium*

The internal potassium ion concentrations reported in the literature do not satisfactorily support a particular $[K^+]_i$ value on which to base buffer $[K^+]$ in this study. Values of $[K^+]_i$ for marine organisms in general range from 480 mM in cnidarian tentacles (Herrera *et al.*, 1989) to 105 mM in puffer fish sperm (Takai & Morisawa, 1995). Therefore, multiple analytical techniques were used to examine $[K^+]_i$ in sperm cells of *E. chloroticus*, with calculations based on average sperm volume (Chapter 2), average sperm counts and average sample $K^+$ content from the following methods:

**Method 1: ICP-OES** (inductively coupled plasma – optical emission spectroscopy). To obtain values of $[K^+]_i$ of activated sperm, four replicate mixtures of 50 µl of dry sperm were first diluted in 50 mL of 20 °C, FSW in 50 mL centrifuge tubes before being centrifuged at 4000 rpm for 30 minutes (Eppendorf 5810-R machine). After the supernatant was removed, the remaining four pellets of sperm were combined in a 2 mL tube and vortexed to create a homogenous suspension. Parallel replicate dilutions were prepared for haemocytometer counts and a set of samples sent to Hills Analytical Laboratories (Hamilton) for $[K^+]$ analysis. The latter prepared the samples with nitric and hydrochloric acid micro digestions at 85 °C for 1 hour before analysis.

**Method 2: Blood gas analysis.** Samples were prepared in either MOPS (3-(N-morpholino)propanesulfonic acid) buffer (without $K^+$) or de-ionised water (DIW) for analysis on a GEM Premier 3500 with iQM (Instrumentation Laboratory) running internal quality control tests alongside external standards and blanks. The Gem Premier 3500 blood gas analyser is used to measure multiple parameters of blood samples, for the purposes of this study only values of $[K^+]$ were used. Sperm samples were prepared in MOPS or DIW and were put on ice in a sonicating waterbath (Unisonics FXP-8D) for 15 minutes. Replicates and serial dilutions were used to test quality and reliability of this machine for this application.

**Method 3: SNARF-1 null-point measurements using nigericin.** Using SNARF-1 and the flow cytometer for sample analysis, incubated *E. chloroticus* sperm were examined in a calibration buffer series (described in Section 3.2.6) with $[K^+]$ ranging from 100 to 550 mM. To balance osmolality the $K^+$ from KCl was balanced with CoCl to the same final concentration. The pH of the calibration buffers was set to 7.5, a figure between the 7.6 suggested by NMR at a 10x sperm dilution (Johnson *et al.*, 1983) and 7.4 as reviewed by
(Darszon et al., 2002) to be the pH$_i$ of activated sea urchin sperm. Therefore it is assumed that with the addition of nigericin, any shift in fluorescence ratio from sperm in FSW can be attributed to the perturbation from unequal [K$^+$], not a perturbation of the pH$_i$ (Babcock, 1983).

**3.2.8 Flow cytometry analysis with FlowJo**

The FACS Calibur output data-files (.fcs) were processed in FlowJo (version 10.0.8) by gating the single population of sperm cells using FSC & SSC. The SNARF-1 stained cells of this gated population were isolated by setting additional active gates on both FL2 and FL3 histograms based on the unstained control. A small proportion (average for FL2 and FL3 for each sample < 5%) of the samples were determined to be unstained and excluded from further analysis. Using the mean fluorescence intensities of the gated populations, the fluorescence ratio (R) was calculated from FL3/FL2 and used in further calculations of pH$_i$.

**3.3 Results & discussion**

**3.3.1 SNARF-1 optimisation for internal pH**

The SNARF-1 incubation series was performed to determine optimal dye concentration and incubation time. The wide range of concentrations spanning 0.05 through to 130 µM attempted to identify any concentrations that appeared toxic to the sperm cells (Figure 3-4). The lower concentrations of 0.05 to 7.5 µM were unsuitable due to only a low proportion of sperm (0.52 to 50%) being gated, indicating low level of staining. SNARF-1 concentrations at or above 10 µM resulted in a high number of gated sperm cells (> 95%). The relatively stable fluorescent ratio with increasing dye concentrations of 10 µM or greater suggests limited toxicity and dye-dye interaction as discussed in Babcock (1983). Therefore, the lowest stable concentration of 10 µM SNARF-1 was used in further experiments with *E. chloroticus* sperm. The range of incubation times showed 1 hour to be too short with a low fluorescence; however, the 1.5 hr incubation gave a much stronger fluorescence signal with > 95% gating and low variation (Figure 3-4). A potential benefit of a longer incubation bringing higher temporal stability to the value of R, was counteracted by the need to minimise the degradation of dry sperm over time (even when kept on ice); therefore, the incubation period was set at 1.5 hr. The shorter duration and low concentration also minimised any time- or concentration-
dependent compartmentalisation of the SNARF-1; any differences in pH\textsubscript{i} among compartments were averaged using flow cytometry (Hamamah & Gatti, 1998).

![SNARF-1 fluorescence ratio](image)

Figure 3-4: SNARF-1 fluorescence ratio for different incubation conditions (time and concentration) n = 3, error bars = standard error, time of incubation is given in the legend.

### 3.3.2 Calibration – Nigericin

In *E. chloroticus* sperm a low nigericin concentration of 0.8 µM led to a rapid increase in SNARF-1 emission ratio (R) that was maintained for 10 minutes; however, a 3.2 µM incubation led to a significant decline in R over the same period (Figure 3-5 A). A decrease in number of gated cells also occurred between 0.8 and 3.2 µM incubations (up to -36.4% in FSW). The duration of incubation made no significant improvement in R across the buffer range tested, although an increased R was evident at the higher pH of 8.2 (pH Figure 3-5 B); however, the longer incubation resulted in R being calculated from a lower proportion of the sample, as gated cell numbers decreased by up to 92.5%. Although SNARF-1 signal loss did occur at low pH with less cells being gated, a lack of signal from PI led to the conclusion that this could not be attributed to cell death. A final concentration of 1 µM nigericin was determined for *E. chloroticus* sperm cells, along with a 1 minute incubation, to give a relatively consistent and stable separation in R across the buffer series.
to be used (6.8, 7.5 and 8.2; Figure 3-5 B). Despite incubations of up to 30 minutes being used in a range of different cell types (Thomas et al., 1979; Negulescu & Machen, 1990), *E. chloroticus* sperm cells were more consistent with those cells exhibiting a rapid to immediate equalisation (Guffanti et al., 1979; Rotin et al., 1987; Shapiro, 2005).

![Figure 3-5: Nigericin-induced shift in carboxyl SNARF-1 fluorescence ratio (R) from *E. chloroticus* sperm cells after 1 and 10 minutes nigericin incubations for (A) different nigericin concentrations, and (B) different buffer pH at 1 µM nigericin. Data points are offset for clarity (n = 3).](image)

### 3.3.3 Internal potassium

As the nigericin used in calibration facilitates the movement of K⁺ across cellular membranes, unequal buffer [K⁺] and sperm cell [K⁺] impacts the fluorescence signal of SNARF-1. Therefore analyses of *E. chloroticus* sperm [K⁺] provided values for the formulation of calibration buffers with a target [K⁺] value of 466 mM.

ICP-OES analysis was performed after acid digests at the commercial facility of Hills Laboratories to give an average [K⁺] of 498 mM with close agreement between the 3 individuals analysed (Table 3-1; Appendix 1). The values from the blood gas analysis were the lowest at 436 mM, with larger variation evident between individual preparations than dilution factors (Figure 3-6; Table 3-1). The more *in vivo* analysis of [K⁺] for *E.
chloroticus sperm looked at the change in fluorescent ratio over a potassium gradient and the SNARF-1 fluorescence emission ratio (R) compared with the control sperm in normal seawater. The [K⁺]i of *E. chloroticus* sperm is approximated to be between 450 to 475 mM, where R equals zero (Figure 3-7).

Figure 3-6: Internal potassium concentration across different sample dilutions from blood gas analyser (n = 6, with technical replication at each sample dilution from 1 to 3); different individuals in different colours; dashed line = average of individuals [436 mM]).
Figure 3-7: SNARF-1 emission ratios (R) across a [K⁺] gradient at buffer pH 7.5 in sperm cells of *Evechinus chloroticus*. Differences calculated from R of fresh sperm. Internal [K⁺] equalised with buffer [K⁺] using nigericin (n = 2), relationship described with third order polynomial regression line.

Table 3-1: Summary of internal potassium concentration [K⁺]ᵢ of *E. chloroticus* sperm. ICP-OES = inductively coupled plasma – optical absorption spectroscopy. Values presented as: average ± standard error; replication indicated in brackets; with null-point K⁺ value from linear regression calculation.

<table>
<thead>
<tr>
<th>Method</th>
<th>[K⁺]ᵢ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: ICP-OES</td>
<td>498±34 (n = 3)</td>
</tr>
<tr>
<td>2: Blood gas analysis</td>
<td>436±33 (n = 6)</td>
</tr>
<tr>
<td>3: Null-point measurements</td>
<td>463.6 (n = 2)</td>
</tr>
</tbody>
</table>

Both ICP-OES and blood gas analysis indicated that *E. chloroticus* sperm [K⁺]ᵢ level was well above the mammalian 150 mM [K⁺], with values possibly as high as 498 mM (Table 3-1). Due to the acid digests used in ICP-OES sample preparation, these analyses may have resulted in higher [K⁺]ᵢ values due to dissociation of potassium otherwise not freely available to contribute to [K⁺] of the sperm cell. The final value for buffer formulation targeted the average of the three methods with a [K⁺] value of 466 mM, a value close to that from the SNARF-1 null-point measurements method. The high level of variability evident between individuals and analytical techniques does not provide strong support for a particular value; however, it does suggest that using levels much below 450 mM will likely result in [K⁺] induced shifts in SNARF-1 emissions leading to over-estimations of pHᵢ (Negulescu & Machen, 1990).

The literature suggests that a high level of [K⁺]ᵢ is plausible due to factors including the presence of concentrating mechanisms, and [K⁺] is considered to be the most common osmotically active intracellular cation making an efficient contribution to ionic balance (Measures, 1975; Bowlus & Somero, 1979; Robertson, 1980; Yancey *et al.*, 1982; Kirschner, 1991; Oren, 2011). Internal potassium has been reported for tissues and cells from a wide range of organisms including mammals, fish, coccolithophores and marine invertebrates (Table 3-2). With a [K⁺]ᵢ of generally between 100-150 mM, mammals have concentrations that are considerably less variable than reported for tissues and sperm of marine invertebrates; that range from 120 to 480 mM (Table 3-2). Levels for sea urchin sperm range from 125 mM (Babcock *et al.*, 1992) to > 300 mM (Lee *et al.*, 1983), whereas sperm from freshwater carp are ~63 mM on activation (Krasznai *et al.*, 2003) (Table 3-2).
Some studies adopted [K⁺]i values derived for species from different phyla; however, the applicability of this is questionable between distant taxa (Table 3-3), particularly those from different osmotic environments. For example, the questionable use of mammalian (bovine) sperm values as a suitable value to be used for the study of marine invertebrate (starfish) sperm (Table 3-3). In this study of *E. chloroticus* sperm, the 466 mM [K⁺]i determined for use in final pH buffers is higher than previously determined for sea urchin sperm.

Table 3-2: Experimentally derived values of internal potassium (K⁺) and the analytical techniques used in different studies, for a range of organisms and a variety of tissue and cell types. Nigericin values are included where stated. Concentrations listed in descending order for different taxa. n/a = not applicable.

<table>
<thead>
<tr>
<th>mM K⁺</th>
<th>Organism</th>
<th>Nigericin (µg/ml)</th>
<th>Analysis technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>480</td>
<td>Cnidarian tentacle</td>
<td>n/a</td>
<td>“intracellular ion contents obtained by subtracting the extracellular ion content from total tissue content”</td>
<td>Herrera <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>301</td>
<td>Cnidarian body wall</td>
<td>n/a</td>
<td>Micro electrode</td>
<td>Shen and Sui (1989)</td>
</tr>
<tr>
<td>215</td>
<td>Sea urchin - eggs, (hypotonically swollen)</td>
<td>n/a</td>
<td>Nigericin null-point 9AA uptake ratio</td>
<td>Lee <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>125-175</td>
<td>Sea urchin - sperm</td>
<td>n/a</td>
<td>Swollen sperm – BCECF null-point with nigericin</td>
<td>Babcock <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>111.5</td>
<td><em>Evechinus chloroticus</em> muscle tissue</td>
<td>n/a</td>
<td>FAAS</td>
<td>Emily Frost (personal communication)</td>
</tr>
<tr>
<td>147.8</td>
<td><em>E. chloroticus</em> gut &amp; nerve tissue</td>
<td>n/a</td>
<td>FAAS</td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>Puffer Fish – sperm</td>
<td>20</td>
<td>Methodology undetermined</td>
<td>Takai and Morisawa (1995)</td>
</tr>
<tr>
<td>120</td>
<td>Bovine - sperm</td>
<td>2 nmol</td>
<td>Fluorescein Null-point with nigericin</td>
<td>Babcock (1983)</td>
</tr>
<tr>
<td>120</td>
<td>Hamster - ovary</td>
<td>5</td>
<td>third-order polynomial</td>
<td>Wieder <em>et al.</em> (1993)</td>
</tr>
<tr>
<td>137</td>
<td>Human lymphocytes</td>
<td>3</td>
<td>BCECF null-point with nigericin</td>
<td>Balkay <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
<td>K+ measurement</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td>----------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>Human - U266</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>Human - JY</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>Human - HUT-78</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>Mammal</td>
<td>20</td>
<td>Not stated</td>
<td>Cody et al. (1993)</td>
</tr>
<tr>
<td>146</td>
<td>Mouse - Balb/c-3T3</td>
<td>5</td>
<td>FAAS</td>
<td>Giuliano and Gillies (1987)</td>
</tr>
<tr>
<td>139</td>
<td>Mouse lymphocytes</td>
<td>3</td>
<td>BCECF null-point with nigericin</td>
<td>Balkay et al. (1997)</td>
</tr>
<tr>
<td>147 to 175</td>
<td>Mice - tumour</td>
<td>n/a</td>
<td>Determined</td>
<td>Lassen et al. (1971)</td>
</tr>
<tr>
<td>130</td>
<td>Rat - pituitary cells</td>
<td>50</td>
<td>Calibration on lysed cells, using extracellular readings</td>
<td>Hallam and Tashjian Jr (1987)</td>
</tr>
<tr>
<td>140</td>
<td>Rat – carotid bodies</td>
<td>n/a</td>
<td>Not stated</td>
<td>Buckler and Vaughan-Jones (1990)</td>
</tr>
<tr>
<td>120</td>
<td>Rabbit - gland</td>
<td>10-50</td>
<td>Approximation</td>
<td>Negulescu and Machen (1990)</td>
</tr>
<tr>
<td>122.8</td>
<td>Rabbit – kidney tubules</td>
<td>20</td>
<td>Not stated</td>
<td>Dubbin et al. (1993)</td>
</tr>
<tr>
<td>110</td>
<td>Rat - skeletal muscle</td>
<td>20</td>
<td>Not stated</td>
<td>Cody et al. (1993)</td>
</tr>
<tr>
<td>140</td>
<td>Rat - cardiac myocytes</td>
<td>10</td>
<td>Not stated</td>
<td>Wu et al. (1994)</td>
</tr>
<tr>
<td>140</td>
<td>Rat – carotid bodies</td>
<td>10</td>
<td>Not stated</td>
<td>Richmond and Vaughan-Jones (1997)</td>
</tr>
<tr>
<td>135</td>
<td>Rat - lymphocytes</td>
<td>3</td>
<td>BCECF null-point with nigericin</td>
<td>Balkay et al. (1997)</td>
</tr>
<tr>
<td>100**</td>
<td><em>Emiliania huxleyi</em></td>
<td>10 µmol kg⁻¹</td>
<td>Used relative pH values in this study: Values for [K⁺] have been reported over a wide range from 100 to 260 mmol l⁻¹</td>
<td>Suffrian et al. (2011)</td>
</tr>
<tr>
<td>60</td>
<td>Common carp sperm</td>
<td>3</td>
<td>BCECF null-point with nigericin</td>
<td>Balkay et al. (1997)</td>
</tr>
<tr>
<td>63</td>
<td>Carp – sperm quiescent</td>
<td>n/a</td>
<td>Nigericin pH determination (however, false assumption of no alteration of [K⁺])</td>
<td>Krasznai et al. (2003)</td>
</tr>
<tr>
<td>37</td>
<td>Carp – sperm - 20 s after activation</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Carp – sperm - 60 s after activation</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Carp – sperm - 300 s after activation</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3-3: Source of internal potassium values used for a range of tissues and cells in new study organisms. Where possible, source identifies original study organism and citation. Concentrations listed in descending order for different taxa.

<table>
<thead>
<tr>
<th>mM K⁺</th>
<th>New study organism</th>
<th>Original study organism &amp; source</th>
<th>New study reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not stated</td>
<td>Coral</td>
<td>Not explicitly stated but implied from previous study (Venn <em>et al.</em>, 2009)</td>
<td>Gibbin <em>et al.</em> (2014)</td>
</tr>
<tr>
<td>200 to 240</td>
<td>Sea urchin - eggs</td>
<td>Various studies</td>
<td>Tupper (1973) &amp; Robinson (1976) In Shen and Sui (1989)</td>
</tr>
<tr>
<td>140</td>
<td>Human – NS0</td>
<td>From mouse, in Thomas <em>et al.</em> (1979)</td>
<td>Bond and Varley (2005)</td>
</tr>
</tbody>
</table>
3.3.4 Summary and final calibration buffer formulation

Within the assumptions and limitations, SNARF-1 provides a tool to produce calibrated, fine-resolution pH\textsubscript{i} measurements. This chapter has outlined the steps and some considerations necessary for optimised use of SNARF-1 in *E. chloroticus* sperm cells.

In summary:

1. Optimal SNARF-1 incubations were obtained by a standard 4 µL aliquot of dry sperm being added to 75.4 µL of FSW and 0.64 µL of stock SNARF-1 in a brown 1.5 mL micro-centrifuge tube to give a final dye concentration of 10 µM for a 1.5 hr incubation. Incubations were left on ice in the dark.

2. For the calibration of pH\textsubscript{i} using the nigericin null-point method, a 1 µM final concentration and a 1 minute incubation were used.

3. The formulation of calibration buffers for use with SNARF-1 stained *E. chloroticus* sperm (20 mM HEPES-KOH, 446 mM KCl, 120 mM CoCl\textsubscript{2}) were modified from Nakajima *et al.* (2005), for asteroid sperm, and adjusted to pH values (6.8, 7.00, 7.25, 7.5, 7.75, 8.00 and 8.1) with 466 mM KCl in 3 M HCl. To ensure equal osmolality between buffers, an aliquot of 3.466 M CoCl\textsubscript{2} was also added to standardise the combined volume added to each buffer. Calibration buffers were stored in the fridge prior to use.
Chapter 4. Swimming performance and internal pH of sperm

4.1 Introduction

Ocean acidification (OA) is having a pervasive effect across biological systems from cellular processes through to ecosystem function (Helmuth, 2009; Hale et al., 2011; Andersson et al., 2015; Yates et al., 2015). Broadcast spawning marine invertebrates release gametes into the surrounding water where changes in levels of fertilisation success are attributed to OA induced changes in gamete performance (Havenhand et al., 2008; Byrne, 2011; Schlegel et al., 2012). Both negative and positive effects of OA on sperm motility and swimming speed are evident across a range of species including bivalves (Havenhand & Schlegel, 2009; Vihtakari et al., 2013), cnidarians (Morita et al., 2010; Nakamura & Morita, 2012), polychaetes (Lewis et al., 2013; Schlegel et al., 2014), asteroids (Uthicke et al., 2013a), holothurians (Morita et al., 2010), as well as echinoids (Havenhand et al., 2008; Caldwell et al., 2011). The most common mechanism proposed to underpin the change in echinoid sperm performance under OA conditions is a change in sperm internal pH (pHi); although decreased motility has recently been linked to lower mitochondrial membrane potential (Schlegel et al., 2015).

Equipped with only the cellular machinery provisioned during spermiogenesis, sperm cells are described as being limited in their capacity to defend an optimal homeostasis and to tolerate environmental change (Melzner et al., 2009). Both activation and sperm motility are under tight control of pH, with pathways of both energy supply and energy utilisation being pH dependent (Cummins, 2009; Nishigaki et al., 2014). As an integrator of multiple cellular processes and mechanisms that affect or are effected by pH, there is an intricate balance in the H⁺ loading and H⁺ extrusion from the cell towards maintaining an optimal steady state pH (Busa & Nuccitelli, 1984; Putnam, 2001; reviewed in: Bevensee & Boron, 2008). The dilution of echinoid sperm in seawater results in the alkalisation of pHi through the release of CO₂ and H⁺, both at high levels in the gonad (Johnson et al., 1983; Trimmer & Vacquier, 1986). When pH is above 7.2-7.3 there is an increase in both motility and respiration, with the maximum rate of increase at about pH 7.5 (Christen et al., 1982; Neill & Vacquier, 2004). The increased pCO₂ and decreased pH of OA are suggested to have a narcotic effect on sea urchin sperm through an impaired
ability to regulate pH, (Havenhand et al., 2008; Byrne, 2011; Reuter et al., 2011); although oxygen also plays a role in activation (Chia & Bickell, 1983).

A diminished capacity of sea urchin sperm to attain an optimal pH may have consequences for overall sperm performance due to a suite of pH dependent processes that are involved with activation and motility of sea urchin sperm. These include activity of the dynein ATPase flagellar motors that drive swimming (Gibbons & Fronk, 1972; Christen et al., 1986; Neill & Vacquier, 2004; Nishigaki et al., 2014), the regulation of the phosphocreatine shuttle that supplies energy to the motors (Golding et al., 1995; Ellington, 2001), and the pH sensitive lipases that provide substrate for mitochondrial ATP generation (Cardin & Meara, 1953; Mohri & Yasumasu, 1963). At the same time pH also indirectly impacts a number of regulatory enzymes and secondary messengers (Brokaw, 1987; Nomura et al., 2005; Vacquier et al., 2014). Collectively, we might expect that a decrease in pH of activation is potentially going to result in decreased motility and swimming speed.

Sperm quality and performance are of particular interest for agriculture, aquaculture as well as for human infertility, resulting in the development of techniques for quantifying key quality and performance measures (Cabrita et al., 2008). In this study we apply one of these techniques, computer assisted sperm analysis (CASA) (Partyka et al., 2012), to assess sperm motility in sperm of a broadcast spawning marine invertebrate in response to OA. Corresponding pH analysis using fluorescent dyes and flow cytometry (Gillan et al., 2005; Hossain et al., 2011; Robles & Martínez-Pastor, 2013) allowed the testing of the hypothesis that under atmospheric CO2 conditions projected for 100 to 300 years in the future, under ‘business as usual’ emission scenarios, sea urchin sperm performance will be compromised due to impaired ability to defend an optimal pH.

4.2 Methods

4.2.1 Seawater manipulation and chemistry

Protocols for the manipulation and analysis of elevated pCO2 experimental seawaters were based on recommendations from Dickson et al. (2007) and Riebesell et al. (2010). In brief, seawaters were bubbled with target CO2 concentrations to give 1) Control/Low for present day at 380 ppm (parts per million); 2) Medium for near-future
concentrations based on IPCC scenarios of RCP 8.5 for the year 2100 (1000 ppm); and 3) High for far-future concentrations based on projections for the year 2300 (1800 ppm). Seawater for all experiments was obtained pre-filtered from Kelly Tarlton’s Undersea World and re-filtered prior to \( pCO_2 \) manipulation through two nested, 1 micron filter bags (FSW). Air was initially dried and stripped of CO\(_2\) before being mixed with instrument grade CO\(_2\) (99.98%) using dial-a-gas mass flow controlled (Smart Trak2, Sierra Instruments) mixing setup based on the experimental system outlined by Fangue et al. (2010), and calibrated with an infrared CO\(_2\) analyser (Qubit S151). To ensure equilibrium of gas partial pressures between target gas mixtures and experimental seawaters, self-contained 20 L containers of FSW were bubbled for a minimum of 16 hours using a temperature controlled (20±1 °C) circulation system where gas mixtures were added through a venturi injector (Mazzei, MK-384) to optimise gas exchange efficiency.

Sets of treatment waters (Control, Medium and High) for experiments (OA seawaters) were handled as per SOP1 (Dickson et al., 2007). In brief, OA seawaters were taken from the bubbling buckets with water samples siphoned through a silicone hose over-filling 250 mL boro-silicate sample bottles by at least 50% for experiments and seawater analysis. A separate set of OA waters were prepared for each motility and pH\(_i\) experiment, and were placed in a 20 °C waterbath (Grant W28) at least two hours prior to experiments to allow temperature equalisation. To minimise temperature and CO\(_2\) changes during the motility and pH\(_i\) experiments, each set of bottles was held in a custom Styrofoam insulator from where aliquots were taken at least mid-water column with rapid replacement of the lid of both bottle and insulator. Water temperature (Thermoworks Thermapalm; certified calibrated accuracy 0.05 °C), and salinity (YSI 30) were measured prior to experiments.

Sets of OA seawaters were analysed for pH\(_{\text{Total}}\) and TA for further carbon chemistry calculations. All pH\(_{\text{Total}}\) analyses were performed within two hours of sampling and TA samples (250 mL) were preserved with a 50 µL aliquot of saturated mercuric chloride and stored at 4 °C in the fridge for later analyses. Spectrophotometric pH\(_{\text{Total}}\) determination used \( m\)-cresol purple (Sigma) to measure changes in absorbance at 434 nm and 578 nm wavelengths based on SOP6b (Dickson et al., 2007) and Fangue et al. (2010), with calibration performed using TRIS standard (SOP3a) (Dickson et al., 2007) with accuracy confirmed against certified reference standards to be 8.104 pH (Dickson et al., 2007). Open cell potentiometric total alkalinity (TA) measurements were run using a
Mettler-Toledo T50 automated titrator based on SOP3b (Dickson et al., 2007) and Fangue et al. (2010) with accuracy of TA values confirmed using Scripps certified reference seawater (batch 108 at TA = 2218 µmol/kg). Titrations over equivalence points initially used 0.1 mL aliquots of a 0.6 M NaCl 0.1 M HCl acid titrant to a pH of 3.6, a 360 second stabilisation period, followed by aliquots of 0.05 mL of titrant to pH 3.0. Both TA and pH_total values were calculated using formulae from SOPs 6b and 3b, based on MSExcel calculation spreadsheets modified from Gretchen Hofmann’s lab (University of California, Santa Barbara).

Carbonate chemistry for experimental waters was recalculated from measured parameters (salinity, temperature, pH_total, TA) to experimental temperature points using the seacarb (v. 3.0.8) package in R (v. 3.1.2); this package was selected based on comparisons of ten ocean carbonate chemistry packages by Orr et al. (2015) using CO2 constants as outlined in “Guide for best practices in ocean acidification research” (Dickson et al., 2007). Seacarb uses the recommended formulations for first and second dissociation constants K_1 and K_2 (Lueker et al., 2000), K_f (Perez & Fraga, 1987) and K_s (Dickson, 1990). Additionally the recommended (Dickson et al., 2007) boron/chlorinity ratio from Uppström (1974), and default values for atmospheric and hydrostatic pressures, silicate and phosphorous content were used. All our experiments were within the specified salinity (19-43 ppt) and temperature (2-35 °C) limitations for constants K_1 and K_2 (Orr et al., 2015).

4.2.2 Sea urchin collection and spawning

During the austral summer of 2014-2015 Evechinus chloroticus were collected, held, fed and spawned as outlined in Chapter 3, Section 3.2.1.
4.2.3 Sample preparation

Over a two week period sperm from thirteen different males were used in paired sperm motility and internal pH experiments (Figure 4-1). Sperm quality was visually assessed for high levels of motility using a Sedgewick rafter slide at 100x magnification. Sperm concentration for all experiments was standarised through haemocytometer counts from a standard dilution of 10 µL dry sperm mixed with 30 mL FSW. From these counts the volume of dry sperm to give a target sperm concentration of 1 million cells/mL for experiments was calculated for each male. Multiple males were run on a single day and the order of OA seawaters was randomly selected for each male, the order being repeated for the paired motility and internal pH experiments.

Figure 4-1: Infographic of sperm motility and internal pH experiments for each male (n = 13) including levels of replication. Coloured circles represent: OA seawaters (blue = Control; yellow = Medium; red = High); and calibration series (green). Further flow cytometry controls not illustrated here included: control and treatment seawater with no sample; control seawater with non-SNARF-1 incubated sperm; and viability test on non-SNARF-1 incubated sperm using PI.
4.2.4 Motility experiment

Optimised video capture for sperm motility analysis used an inverted microscope (Nikon Eclipse Ti at 5x magnification) adjusted for differential interference contrast (DIC) imagery. The importance of using a consistent within-experiment equipment setup and video capture frame rate are discussed in Boryshpolets et al. (2013) and Castellini et al. (2011). Motion was captured in a 250 image TIF-stack with an ANDOR iXon camera using proprietary IQ2 software with 2x2 binning to give a capture rate of 34 frames per second. A set of custom PVC, deep and relatively large-volume imaging chambers was designed to minimise boundary (or wall) effect from glass surfaces (Gee & Zimmer-Faust, 1997) and to maximise thermal inertia of the sample. The 3 mm thick PVC chambers with attached coverslip on the base had a volume of approximately 339 µL (Figure 4-2).

Figure 4-2: Custom sperm motility chambers made from 3 mm thick PVC plastic. Two chambers per plate of 12 mm diameter with coverslip glued to base (green dotted line) and coverslip on top (blue line) closes the chamber.

Motility recordings were performed as rapidly as logistically possible, with all video recordings started ~10 seconds after initiating each sperm dilution. The pre-calculated volume of dry sperm was added to a 14 mL aliquot of OA seawater in a 15 mL Falcon tube and agitated gently for 4 seconds to make a relatively homogenous sperm suspension of ~1 million cells/ mL. To minimise air bubbles when the chamber was closed with the top coverslip, a 350 µL aliquot of the sperm suspension was gently added to over-fill the imaging chamber and the top coverslip lowered in place to close. The chamber was immediately put on the inverted microscope stage with the focus pre-set for midway between the two coverslips and the 250 image TIF-stack acquisition started. Sperm dilutions were mixed in rapid succession producing back to back recordings with technical replication of 10 at each CO2 treatment (control, mid and high) (Figure 4-1). Preliminary analyses showed a high degree of technical replication was required to characterise variance within males.
4.2.5 Internal pH experiment

Internal pH (pHi) of sperm was examined using a flow cytometer and the pH sensitive dye carboxy-SNARF-1 (seminaphthorhodafluor), acetoxymethyl ester, acetate (Invitrogen, Molecular Probes C1272, Lot # 1151593) (referred to as SNARF-1 in this thesis). Within the assumptions and limitations, SNARF-1 provides a tool to produce calibrated, fine-resolution pH measurements in *E. chloroticus* sperm cells using the optimum dye incubation protocols developed for SNARF-1 and *E. chloroticus* sperm (Chapter 3, Section 3.3.4). In summary, to minimise activation and metabolism of sperm prior to experimental incubations the dry sperm were minimally diluted and kept on ice. To ensure optimum dye performance, a new 1.25 mM SNARF-1 stock was prepared in anhydrous dimethyl sulfoxide (DMSO, from Sigma-Aldrich) every second day and stored in the dark at -18 °C between use. A standard 4 µL aliquot of dry sperm was added to 75.4 µL of FSW and 0.64 µL of stock SNARF-1 to give a final dye concentration of 10 µM in a brown 1.5 mL Eppendorf tube. A parallel incubation with the SNARF-1 replaced with FSW was used as a control (Figure 4-1). Incubations were mixed well by hand (holding the tube upright and gently flicking the tip of the tube until suspension appeared evenly mixed) and left in ice-water for 90 minutes in the dark.

4.2.5.1 Fluorescence acquisition

After the 90 minute incubation a predetermined volume of sperm (calculated for each male from sperm counts) was diluted in 3 mL of OA seawater to give a target sperm concentration of 1 million cells/mL. Sample analysis was performed as outlined in Chapter 3, Section 3.2.4. In brief, immediately after mixing, samples were analysed in a flow cytometer (BD Biosciences FACS Calibur) optimised for *E. chloroticus* sperm cells with a combination of forward and side scatter (FSC & SSC respectively) used to identify the sperm cells. The SNARF-1 emission collected through detectors FL2 and FL3 (585/42 nm and 670 nm long-pass respectively) with voltages adjusted for autofluorescence and position on the axes, allowed identification of the stained and unstained sperm. An event rate of 350 to 150 events per second resulted in a single consolidated population of sperm cells identifiable by FSC & SSC, and data from approximately 5000 events was collected for each sample. As with the analysis of sperm motility, consecutive replicate dilutions (n = 10) were mixed for each OA seawater from the SNARF incubation mix (Figure 4-1) and run in immediate succession followed by controls for: 1) seawater alone, 2) sperm without SNARF-1 and 3) sperm viability confirmed with 1 mM propidium iodide (PI). The
SNARF-1 fluorescence ratio (R) calculated from FL3/FL2 is used in further calculations of pH\(_i\) (see Section 4.2.7.3 for more detail).

### 4.2.5.2 Calibration

Calibration of SNARF-1 fluorescence allows quantification of pH\(_i\) using a series of pH buffers across the expected pH\(_i\) range and the ionophore nigericin to equalise hydrogen ion concentrations [H\(^+\)] on either side of the cellular membrane (Thomas et al., 1979). Correct buffer formulation is important for effectively limiting any nigericin induced shifts in SNARF-1 fluorescence ratio to changes in [H\(^+\)]\(_i\). Calibration buffers and internal potassium concentration are discussed in Chapter 3 with a target buffer [K\(^+\)] of 466 mM. A final buffer formulation giving [K\(^+\)] of 472.5 mM (well within the variability of [K\(^+\)]\(_i\) among analytical techniques) consisted of 22.5 mM HEPES-KOH, 450 mM KCl, and 120 mM CoCl\(_2\) (modified from: Nakajima et al., 2005). The buffer series was prepared to target pH values (6.81, 7.00, 7.26, 7.51, 7.75, 8.00 and 8.11) with 472.5 mM KCl in 3 M HCl and 3.472 M CoCl\(_2\) to standardise the combined volume added to each buffer. Calibration buffers were stored in the fridge and two hours prior to experiments were put in the same 20 °C waterbath as OA seawaters. A calibration was performed for each male at the end of the pH\(_i\) experiment (Figure 4-1).

### 4.2.6 Data analysis

#### 4.2.6.1 CASA with ImageJ

The image analysis software ImageJ (v. 1.50a running on FIJI “Fiji Is Just ImageJ”; National Institutes of Health, Bethesda, MD, USA) was used to process videos and perform particle tracking with computer assisted sperm analysis (CASA); ImageJ produces outputs similar to commercially available systems (Boryshpolets et al., 2013). A customised batch-processing macro (inspired by: Purchase & Earle, 2012) incorporating the CASA plugin (Wilson-Leedy & Ingermann, 2007) was used to analyse a two second sub-stack (images 18 to 85) of the original TIF-stacks. Despite a pre-set microscope setup (Section 4.2.4), each sub-stack had to be independently adjusted for threshold by eye for comparable analyses. This was achieved by using a standard procedure to highlight a general low level background scatter by eye, the threshold was then increased by 5 points to leave a very fine background noise - a scatter of pixels that was removed through the CASA parameterisation. The CASA plugin was parameterised for identification of *E. chloroticus* sperm cells and definition of motion characteristics for motile and non-motile
sperm (Appendix 2). For each sample, output data on sperm swimming performance include measures of motility and swimming speeds of motile sperm as described in Table 4-1.

Table 4-1: ImageJ CASA macro output parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent motility</td>
<td>Percent of sperm moving in a manner fitting motility determination parameters</td>
<td>%</td>
</tr>
<tr>
<td>Velocity curvilinear (VCL)</td>
<td>Average velocity over entire track (total distance travelled) per second</td>
<td>µm.s⁻¹</td>
</tr>
<tr>
<td>Velocity average path (VAP)</td>
<td>Average path velocity based on a roaming average of 5 ²/₃ frames (¹/₆th of the frame rate).</td>
<td>µm.s⁻¹</td>
</tr>
<tr>
<td>Velocity straight line (VSL)</td>
<td>Straight line velocity between the first and last points of the average path</td>
<td>µm.s⁻¹</td>
</tr>
<tr>
<td>Linearity (LIN)</td>
<td>Linearity given by VSL/VAP, describing path curvature</td>
<td>-</td>
</tr>
<tr>
<td>Wobble (WOB)</td>
<td>VAP/VCL, describes side to side movement of the sperm head</td>
<td>-</td>
</tr>
<tr>
<td>Progression (PROG)</td>
<td>The average distance of the sperm from its origin on the average path during all frames analysed</td>
<td>µm</td>
</tr>
</tbody>
</table>

4.2.6.2 Flow cytometry analysis

The flow cytometer output .fcs files were processed in FlowJo (version 10.0.8) by gating the single population of sperm cells using FSC & SSC. The SNARF-1 stained cells of this gated population were isolated by setting additional active gates on both FL2 and FL3 histograms based on the unstained control. A small proportion (average for FL2 and FL3 for each sample < 5%) of the samples were determined to be unstained and excluded from further analysis. Using the mean fluorescence intensities of the gated populations, the fluorescence ratio (R) was calculated from FL3/FL2 for all males across control and OA seawaters, and also for the calibration solutions. The PI fluorescence of non-SNARF-1 stained sample was checked for high levels of viability after the incubation period.

4.2.7 Statistical approaches

Statistical analyses were performed using the software RStudio (version 0.99.486) and ‘R’ (RCoreTeam, 2015), unless specified otherwise, with basic plotting done using the ggplot2 package. Data from seawater pH_Total and pH_i calibrations were separately analysed with a one-way ANOVA. Assumptions of normality were checked with Q-Q plots and
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Shapiro-Wilk for levels of factor. Heteroscedasticity was checked with Levene’s test and post hoc test for comparisons between means using Tukey-Kramer’s ($\alpha = 0.05$).

### 4.2.7.1 LnRR

For both motility and pH$_i$ measures, the magnitude of effect of elevated CO$_2$ seawater on sperm was calculated on non-standardised data using the logarithmic response ratio (LnRR) and combined with an estimate of precision using confidence intervals (Nakagawa & Cuthill, 2007; Schlegel et al., 2012). This approach is generally considered to provide a more effective assessment of the biological importance of the data than using $p$-values alone (Nakagawa & Cuthill, 2007). The LnRR is defined by the natural-log proportional change in the means of the treatment groups and control group (LnRR = ln[$\bar{x}_{\text{treatment}}$/\$\bar{x}_{\text{control}}$]). Technical replicates were averaged to the level of the individual and the LnRR was calculated for all sperm performance measures (CASA outputs from Table 4-1 and both pH$_i$ and the SNARF-1 fluorescence ratio (R)). Each parameter’s LnRR for all 13 individuals were then bootstrapped in the statistical software ‘R’ running boot package and the resulting mean and 95% normal confidence intervals returned from 100,000 iterations. Bootstrapping provides an estimate of precision with the large number of iterations reducing the effect of random sampling errors. This approach involves sampling with replacement under the assumption that the sample data provides a good estimate of the population sampled, with the distribution of bootstrap-sampled means used to infer the estimate of precision (Quinn & Keough, 2002; Kulesa et al., 2015). The confidence intervals provide a measure of the variance between individuals and results are interpreted as significant where CIs do not overlap with zero (Schlegel et al., 2015).

### 4.2.7.2 Multivariate analysis

Multivariate techniques allow the relationships between multiple variables to be examined at the same time, in response to the experimental treatments (different levels of $p$CO$_2$) (Anderson et al., 2008). The permutational multivariate analysis of variance (PERMANOVA) simultaneously evaluates differences in both location and spread to test the null hypothesis that there is no difference in the attributes of the different groups in multivariate space. Compared to multpile analysis of variance (MANOVA), the PERMANOVA allows use of any distance measures and no assumption of normality applies as any distribution is removed through the use of permutations (Anderson et al., 2008).
For multivariate analysis, all data were standardised to a mean of zero and a variance of one to prevent distortion of analysis from 3 orders of magnitude difference between values for different motility parameters (Quinn & Keough, 2002). To remove the possibly redundant information of correlated variables and thereby reduce associated multi-collinearity expected from the CASA outputs (Fitzpatrick et al., 2012; Dormann et al., 2013; Kabacoff, 2015) the seven CASA motility parameters (Table 4-1) were condensed into principal components (PC) using principal component analysis (PRIMER v. 6, Plymouth Marine Laboratory). This procedure was performed using all technical replicates (n = 10 for the majority of analyses across all treatments, n = 9 where a replicate returned no result) for each treatment level and all 13 individuals. PCs with eigenvalues greater > 1 were selected; indicating that the PC explained more of the variation than the original variables.

All technical replicates were included in PCA for the generation of PCs; however, as motility and SNARF-1 analyses were on separate sub-samples from the same individual, and to avoid pseudo-replication, technical replicates for PCs and pH\textsubscript{i} were averaged for further calculation of the resemblance matrix using Euclidean distance. Multi-dimensional scaling (MDS) and principal coordinate ordination (PCO) plots were used to assess the distributions based on resemblance matrix ranks and distances respectively. Based on the resemblance matrix an initial (PERMANOVA) with two factors (male and OA seawater treatment) running 9999 permutations of residuals under a reduced model was used to assess the variability and differences between treatments. A lack of replication at the lowest level (i.e. due to the averaging of technical replicates to a single value) resulted in the automatic exclusion of the highest level interaction term due to being confounded by the variance of the residuals (Anderson et al., 2008). Multivariate dispersion using PERMDISP tested for homogeneity in the cluster dispersion around the centroids for the three OA seawater treatments. A final analysis of the resemblance matrix used PERMANOVA with pairwise comparisons of the factor ‘OA seawater’.

### 4.2.7.3 pH\textsubscript{i} calibration

A 3\textsuperscript{rd} order polynomial regression was fitted to calibration data as suggested by Wieder et al. (1993) and used by Gibbin et al. (2014) for BCECF, based on data from all individuals using a linear model in the statistical software ‘R’. Estimations of pH\textsubscript{i} were calculated from the 3\textsuperscript{rd} order polynomial expression for the calibration curve as used by Bond and Varley (2005).
4.3 Results

4.3.1 Seawater carbonate system

The OA seawater parameters measured at the time of experiments gave a clear and consistent separation between $pCO_2$ levels. Low $pCO_2$ (Control) treatment with a pH$_{Total}$ of 8.09, had a 0.32 pH unit drop to 7.77 pH$_{Total}$ for the Medium treatment, and the high treatment had a 0.58 pH unit drop to 7.51 pH$_{Total}$. These differences in pH$_{Total}$ were highly significant (ANOVA: $F_{(2,9)} = 727.4, p < 0.0001$).

Table 4-2: Treatment seawater parameters: Low (Control), Medium, and High. Measured values of pH$_{Total}$ and total alkalinity (TA) used with salinity and experimental temperature (T) to calculate (using seacarb R-package) remaining carbonate parameters of partial pressure of CO$_2$ and saturation states for calcite ($\Omega_{Ca}$) and aragonite ($\Omega_{Ar}$).

<table>
<thead>
<tr>
<th>OA seawater</th>
<th>pH$_{Total}$</th>
<th>T(°C)</th>
<th>Salinity (μequiv kg$^{-1}$)</th>
<th>TA (μatm)</th>
<th>pCO$_2$ (μatm)</th>
<th>$\Omega_{Ca}$</th>
<th>$\Omega_{Ar}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low/ Control</td>
<td>8.09 (±0.01)</td>
<td>20</td>
<td>34.4</td>
<td>2308</td>
<td>354.4</td>
<td>4.79</td>
<td>3.11</td>
</tr>
<tr>
<td>Medium</td>
<td>7.77 (±0.03)</td>
<td>20</td>
<td>34.5</td>
<td>2314</td>
<td>846.4</td>
<td>2.57</td>
<td>1.67</td>
</tr>
<tr>
<td>High</td>
<td>7.51 (±0.02)</td>
<td>20</td>
<td>34.4</td>
<td>2309</td>
<td>1623.7</td>
<td>1.48</td>
<td>0.95</td>
</tr>
</tbody>
</table>

4.3.1 Sperm performance

The parameters of sperm swimming and pH$_i$ were significantly altered under OA conditions, and among males some traits were appreciably more variable than others (Table 4-3, Figure 4-3). The size of effect of $pCO_2$ on the quality and performance of sperm showed a general pattern that sperm performance (swimming and pH$_i$) was significantly negatively impacted by elevated $pCO_2$ seawaters, with the exception of VCL and VAP (Figure 4-3). Although not significant, on average VCL was slightly elevated, and VAP very slightly reduced. All sperm parameters negatively impacted by OA exhibited a ‘stepwise’ decrease relative to the control, with no significant differences evident between the effect of Medium and High OA seawaters. The size of the 95% confidence intervals is indicative of the variability in response to elevated $pCO_2$ among different males.
Table 4-3: Sperm performance characteristics in different OA seawaters (Low [Control], Medium and High; seawater pH_{Total} values given in brackets). % motility = percentage of sperm defined as motile; VCL = velocity curvi-linear (µm.s^{-1}); VAP = velocity average path (µm.s^{-1}); VSL = velocity straight line (µm.s^{-1}); LIN = linearity; WOB = head wobble; PROG = progression; pH_i = internal pH. (n = 13 with technical replication of 9 or 10 for each male at each treatment level; ± standard error).

<table>
<thead>
<tr>
<th></th>
<th>Low (8.09)</th>
<th>Medium (7.77)</th>
<th>High (7.51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% motility</td>
<td>83 ± 3</td>
<td>74 ± 4</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>VCL</td>
<td>125.6 ± 3.48</td>
<td>128.78 ± 1.63</td>
<td>128.43 ± 1.38</td>
</tr>
<tr>
<td>VAP</td>
<td>100.8 ± 1.78</td>
<td>100.33 ± 0.97</td>
<td>99.19 ± 1.79</td>
</tr>
<tr>
<td>VSL</td>
<td>162.7 ± 8.80</td>
<td>141.60 ± 6.79</td>
<td>133.64 ± 9.64</td>
</tr>
<tr>
<td>LIN</td>
<td>1.61 ± 0.08</td>
<td>1.41 ± 0.06</td>
<td>1.35 ± 0.09</td>
</tr>
<tr>
<td>WOB</td>
<td>0.81 ± 0.01</td>
<td>0.78 ± 0.01</td>
<td>0.77 ± 0.01</td>
</tr>
<tr>
<td>PROG</td>
<td>317.4 ± 16.83</td>
<td>262.25 ± 11.35</td>
<td>271.35 ± 15.27</td>
</tr>
<tr>
<td>pH_i</td>
<td>7.52 ± 0.02</td>
<td>7.35 ± 0.04</td>
<td>7.31 ± 0.02</td>
</tr>
</tbody>
</table>
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Figure 4-3: Average effect size of elevated $pCO_2$ seawaters on sperm performance characteristics using natural-log response ratio (LnRR). Bootstrap means (100,000 times) with 95% confidence intervals (error bars); blue dashed line represents zero effect of $pCO_2$ seawater. Yellow data points are effect size of Medium $pCO_2$ seawater (or -0.32 pH units from Control); red are High $pCO_2$ seawater (-0.58 pH units from Control). VCL = velocity curvi-linear ($\mu$m.s$^{-1}$); VAP = velocity average path ($\mu$m.s$^{-1}$); VSL = velocity straight line ($\mu$m.s$^{-1}$); LIN = linearity; PROG = progression; R = SNARF-1 fluorescence ratio; pHi = internal pH. (n = 13 with technical replication of 9 or 10 for each male at each treatment level).

4.3.1.1 Motility

Motility analysis showed samples in all treatments swam with a high degree of directionality, with a lack of circular paths, suggesting the edge effect on swimming behaviour was avoided (Elgeti et al., 2010).

Decreasing pH of seawaters had a significant negative effect on levels of motility with average reductions of 16.5% with a -0.32 pH change and 27.4% with a -0.58 pH change. Although the sperm swimming parameter VAP showed no noticeable effect of OA seawaters, the velocity measure of VCL indicates that, although not statistically different from the Control, sperm swam slightly faster in Medium and High OA seawaters (+3.17, +2.82 $\mu$m.s$^{-1}$ respectively) (Figure 4-3 and Table 4-3). As suggested by a significantly lower LIN and VSL (-21.16, -29.12 $\mu$m.s$^{-1}$ respectively for Medium and High
OA seawaters) the actual path taken was significantly more curved and there was an overall significant decrease in PROG (Figure 4-3 and Table 4-3).

4.3.1.2 pHᵢ

Procedural controls (Fig. 4-1) showed no contamination of the OA seawaters (no, or very low events recorded) and that non-stained sperm showed high levels of sperm cell viability (minimal PI signal with intact cellular membranes), (see Chapter 3, Section 3.2.5). The pHᵢ of sperm was significantly impacted by OA conditions with all sperm exhibiting a similar effect size (LnRR) with relatively limited among-male variation (narrow CIs) (Figure 4-3; Table 4-3). The stepwise decrease of -0.18 and -0.21 pH units was significantly different from pHᵢ measured in Control seawater (for Medium and High OA-seawaters respectively); however, the two OA treatments were not statistically different (Figure 4-3; Table 4-3).

4.3.2 Overall quality and performance characteristics

Overall differences in sperm performance between OA seawaters were assessed by multivariate analysis including PERMANOVA. In the PCA analysis of CASA output variables, a high degree of correlation occurred between some variables (Table 4-4). Consolidation of these variables gave three PCs with eigenvalues > 1; combined these explained more than 75% of the variation. Minimal pattern of variables contributing to the different PCs was evident beyond PC1. PC1 was primarily influenced by directionality of sperm (VSL, LIN and PROG), PC2 by negative contribution of non-linear measurements (VCL and VAP) and PC3 by the head movement and actual path taken (WOB and VCL respectively); while % motility contributed almost evenly to PC1 and PC3 (Table 4-4).
Table 4-4: Sperm motility primary principal components (Eigen values > 1) from principal component analysis of standardised (\( \bar{x}, \sigma \)) CASA (computer assisted sperm analysis) output variables from *E. chloroticus* sperm. VCL = velocity curvi-linear (\( \mu \text{m.s}^{-1} \)); VAP = velocity average path (\( \mu \text{m.s}^{-1} \)); VSL = velocity straight line (\( \mu \text{m.s}^{-1} \)); LIN = linearity; WOB = head wobble; PROG = progression. (\( n = 13 \) with technical replication of 9 or 10 for each male at each treatment level).

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>% motility</td>
<td>0.284</td>
<td>-0.05</td>
<td>0.276</td>
</tr>
<tr>
<td>VCL</td>
<td>0.001</td>
<td>-0.62</td>
<td>-0.540</td>
</tr>
<tr>
<td>VAP</td>
<td>0.325</td>
<td>-0.625</td>
<td>0.068</td>
</tr>
<tr>
<td>VSL</td>
<td>0.561</td>
<td>0.22</td>
<td>-0.329</td>
</tr>
<tr>
<td>LIN</td>
<td>0.504</td>
<td>0.367</td>
<td>-0.357</td>
</tr>
<tr>
<td>WOB</td>
<td>0.391</td>
<td>-0.101</td>
<td>0.622</td>
</tr>
<tr>
<td>PROG</td>
<td>0.304</td>
<td>-0.171</td>
<td>0.071</td>
</tr>
</tbody>
</table>

| Eigenvalue | 2.36 | 1.58 | 1.15 |
| % Variation Explained | 34.9 | 23.3 | 17.0 |
| Cumulative % Variation | 34.9 | 58.2 | 75.2 |

The test for multivariate dispersion showed no significant difference in the deviations from centroid for all three OA treatment seawaters (PERMDISP: \( F_{(2,36)} = 0.001647; p(perm) = 0.9843 \); High, \( \bar{x} = 0.866, \ SE = 0.0878 \); Medium, \( \bar{x} = 0.857, \ SE = 0.0724 \); Low, \( \bar{x} = 0.876, \ SE = 0.0684 \)); pairwise comparisons confirmed homogeneity of multivariate dispersion between all pairs (Table 4-5). As a result, variance between treatment groups in the PERMANOVA can be attributed to data from motility (PCs) and SNARF-1 (pHi).

Table 4-5: PERMDISP Pairwise comparisons for homogeneity of multivariate dispersion of sperm swimming performance between different \( p\text{CO}_2 \) seawaters: \( l = \) Low (Control); \( m = \) Medium; \( h = \) High.

<table>
<thead>
<tr>
<th>Groups</th>
<th>( t )</th>
<th>( P(perm) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( (l,m) )</td>
<td>0.19736</td>
<td>0.8403</td>
</tr>
<tr>
<td>( (l,h) )</td>
<td>0.095748</td>
<td>0.9255</td>
</tr>
<tr>
<td>( (m,h) )</td>
<td>0.079059</td>
<td>0.9382</td>
</tr>
</tbody>
</table>

PCO analysis revealed that most of the variation in sperm performance between OA treatment groups could be explained by PCO1 (63.4%) and PCO2 (21.3%) (Figure
There were highly significant differences in sperm swimming and pH, depending on the three OA treatment seawaters, but no difference between males (PERMANOVA: pseudo $F_{(2,24)} = 15.329, p = 0.0001$; pseudo $F_{(12,24)} = 0.0031, p = 1$). Compared to the Control, PERMANOVA pairwise comparison showed differences in sperm performance at both elevated CO₂ treatment seawaters were highly significant, but not between Medium and High OA seawaters (Table 4-6). This pattern is also seen with greatest distance between both High and Medium OA seawaters and the Control (Table 4-7).

Figure 4-4: Principal coordinate ordination (PCO) of sperm performance parameters in 13 males at three treatment (treat) $pCO_2$ seawaters: $l$ = Low (Control); $m$ = Medium; $h$ = High.
Table 4-6: PERMANOVA pairwise comparisons of sperm performance at different $p$CO$_2$ seawaters: $l =$ Low (Control); $m =$ Medium; $h =$ High.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$t$</th>
<th>$P($perm$)$</th>
<th>Unique perms</th>
</tr>
</thead>
<tbody>
<tr>
<td>$l, m$</td>
<td>4.0463</td>
<td>0.0001</td>
<td>9945</td>
</tr>
<tr>
<td>$l, h$</td>
<td>5.2462</td>
<td>0.0001</td>
<td>9953</td>
</tr>
<tr>
<td>$m, h$</td>
<td>1.3186</td>
<td>0.1651</td>
<td>9943</td>
</tr>
</tbody>
</table>

Table 4-7: PERMANOVA average distance between and within pairwise comparisons of sperm swimming performance between different $p$CO$_2$ seawaters: $l =$ Low (Control); $m =$ Medium; $h =$ High.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$l$</th>
<th>$m$</th>
<th>$h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$l$</td>
<td>1.2675</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$m$</td>
<td>2.6797</td>
<td>1.3454</td>
<td></td>
</tr>
<tr>
<td>$h$</td>
<td>3.3865</td>
<td>1.4733</td>
<td>1.3345</td>
</tr>
</tbody>
</table>

4.3.2.1 Individual variation

Within the overall differences in sperm performance between OA seawaters, the relationship between pH$_i$ and the proportion of sperm attaining activation (% motility) was tested. Underlying the highly correlated relationship between overall % motility and pH$_i$ ($R^2 = 0.8668$), was a highly significant among-individual variation in response to the three OA seawater levels (PERMANOVA: pseudo $F_{(12,24)} = 6.0362$, $p = 0.0001$) (Figure 4-5).

The test for multivariate dispersion showed no significant difference in the deviations from centroid between different males (PERMDISP: $F_{(12,26)} = 1.4875$; $p($perm$) = 0.704$) and pairwise comparisons indicated a range of differences among male pairs; although most differences were not significant (PERMMANOVA: $P($perm$) = 0.0151$ to 0.7434 across the 78 pairs). This is reflected in the range of relatively small but different distances among individuals (Table 4-8). Although on average males exhibited a positive relationship between % motility and sperm pH$_i$ the different slopes show within-individual variation in response to OA (Figure 4-5). Responses ranged from positive to negative, with some individuals exhibiting a higher sensitivity to $p$CO$_2$ than others.
Figure 4-5: Relationship between internal pH (pHi) and % motility of *E. chloroticus* sperm cells in response to exposure to three different OA conditions. Solid regression line = average response (R² = 0.8668); dotted regression lines = individual male responses.

Table 4-8: PERMANOVA average distance between and within pairwise comparisons of sperm % motility and pHᵢ between different males (1 – 13).  

<table>
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<th>Males</th>
<th>1</th>
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<th>4</th>
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<td>0.31</td>
<td>0.13</td>
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</table>
4.3.3 pH; calibration

Across the calibration buffer series the expected curve of the SNARF-1 fluorescence ratio (R) showed a high level of agreement across males with highly significant differences between R values for each buffer (Figure 4-6; ANOVA: $F_{(6,98)} = 656.8, p < 0.0001$). The ANOVA was performed despite the calibration data being non-normally distributed, as the one-way ANOVA is considered to be robust to violations of normality (Quinn & Keough, 2002).

![Calibration curve for internal pH of sperm cells using SNARF-1 and nigericin.](image)

Figure 4-6: Calibration curve for internal pH of sperm cells using SNARF-1 and nigericin. (n = 14, error bars = SEM). Fluorescence ratio (R) calculated from FL3/FL2. Red line = linear regression 3rd order polynomial.
4.4 Discussion

OA seawaters with lower pH and higher $pCO_2$ had significant negative impacts on most, but not all, motility parameters and pHᵢ of *Evechinus chloroticus* sperm. Sperm were unable to attain the same pHᵢ of activation in Control seawater; in both the near- and far-future OA conditions there was a stepwise decrease in pHᵢ. Although higher OA conditions resulted in an increasing proportion of immotile sperm, those attaining activation (% motility) swam through the water faster (VCL), but due to a more curved path had a reduced straight line speed (VSL). The suggested relationship between increasing OA and the impact on sperm activation and motility through pH dependent cellular processes is not straightforward; while an increasing proportion of sperm do not attain activation, the remaining sperm swim slightly faster.

Although the alkalinisation of quiescent sperm was not explicitly examined here, under present day $pCO_2$ the activation of *E. chloroticus* sperm resulted in a pHᵢ of 7.52, a figure consistent with the observed alkalinisation found in other urchin species using a range of experimental techniques. These include fluorescent amine probes used to determine the pHᵢ increase of activation to be from a pH of about 6.9 or 7.0 to about pHᵢ 7.4 in buffered artificial seawater (Christen *et al.*, 1982; Lee *et al.*, 1983). Nuclear magnetic resonance (NMR) gave a slightly wider alkalinisation range from about pHᵢ 7.0 to between 7.4 and 7.6 in artificial seawaters (Christen *et al.*, 1983; Johnson *et al.*, 1983). Fluorescent dyes have been used to show fine scale changes in sperm pHᵢ, the two most popular dyes being BCECF (Guerrero & Darszon, 1989; Seifert *et al.*, 2015) and SNARF-1 (Sase *et al.*, 1995; Nakajima *et al.*, 2005). The use of SNARF-1 in this study provided fine resolution pHᵢ values in agreement with the range described in the literature, with a high degree of precision enabling comparison and changes across treatments to be characterised.

The overall decreased motility and overall swimming performance of *E. chloroticus* sperm occurs despite a quicker through-the-water speed (VCL); however, due to a more curved swimming path, the speed between two points is effectively slower (reflected in lower VSL and PROG). A similar increase in velocity over a curved path (VCL) was reported in *Paracentrotus lividus* (Graham *et al.*, 2015) and in *Psammechinus miliaris* (Caldwell *et al.*, 2011) with the suggestion in the latter that current day $pCO_2$ levels were sub-optimal for sperm swimming. Despite an increase in VCL in *E. chloroticus*, the accompanying decrease in directionality and lower straight line speed (PROG and VSL), combined with lower percentage of motile sperm, will have modelled...
negative impacts on fertilisation success (Vogel et al., 1982; Levitan et al., 1991; Lewis et al., 2002). In an OA context, the direct impacts of lower sperm motility and/or speed are seen in lower fertilisation rates observed for the urchins *Heliocidaris erythrogramma* (Havenhand et al., 2008), and *Paracentrotus lividus* (Graham et al., 2015), or indirectly through the effect of sperm concentration on fertilisation rates in *Mesocentrotus franciscanus* (Reuter et al., 2011), *Heliocidaris erythrogramma* (Schlegel et al., 2012), or *Stereochinus neumayeri* (Ho et al., 2013; Sewell et al., 2014). The changing parameters of sperm performance may well have further implications for concepts such as sperm limitation, polyspermy and plasticity of egg size (Millar & Anderson, 2003; Luttikhuiizen et al., 2011; Reuter et al., 2011). For example, the faster VCL observed for sperm under OA conditions will be a disadvantage in conditions of sperm limitation, as sperm longevity will be limited by faster depletion of finite endogenous energy stores (noted in: Chapter 2) (Fitzpatrick et al., 2012).

Internal pH is suggested to play a fundamental role in the control of sea urchin sperm activation and continued swimming. The involvement of CO₂ and pHᵢ is well described (e.g.: Christen et al., 1982; Johnson et al., 1983) and has led to the suggestion that sea urchin sperm will be unable to compensate for the changes in seawater carbon-chemistry of OA, with consequences for activation and motility (Havenhand et al., 2008; Kurihara, 2008; Graham et al., 2015). The effect of OA on pHᵢ of sea urchin gametes has recently been demonstrated in the unfertilised eggs of *Strongylocentrotus droebachiensis* with a stepwise decrease in pHᵢ; as shown through changes in un-calibrated fluorescence ratio (R) from the pH sensitive dye BCECF (Bögner et al., 2014). Under similar conditions in this study, a calibrated stepwise decrease in the average pHᵢ of activated *E. chloroticus* sperm confirms the suggested significant negative impact of OA. Why a stepwise decrease and not a linear decline with external pH decrease is not clear, possible explanations include experimental limitations or changes in physiological processes. Although the calibration curve indicates a precise measure of pHᵢ, SNARF-1 has previously been used to describe the fine resolution changes in pHᵢ across cellular compartments, such as between the mitochondria and the nucleus in boar sperm (Kamp et al., 2003) and between mitochondria and cytoplasm in rat myocytes (Ramshesh & Lemasters, 2012). If under increasing OA any differential shifts in fluorescence occurred between compartments of the sperm, such as from the mitochondria, these would have been averaged across the cell (Chapter 3; Kamp et al., 2003; Ramshesh & Lemasters,
Alternatively, an OA induced disruption of H\(^+\) extrusion from the cell may occur through the lower activity of the voltage and sAC dependent Na\(^+\)/H\(^+\) exchanger (Lee, 1985; Neill & Vacquier, 2004) leading to an increased reliance on passive diffusion of H\(^+\) across the cellular membrane (Mohri & Yasumasu, 1963; Hamamah & Gatti, 1998; Missner & Pohl, 2009; Boron et al., 2011). The effects of OA on the maintenance of optimal pH\(_i\) may have knock-on impacts to other cellular processes, including possible involvement in the proposed on/off state of the sperm mitochondrion (Schlegel et al., 2015).

The suggested narcosis of sperm in an elevated pCO\(_2\) world, through the pH\(_i\) inhibition of dynein-ATPase activity and bioenergetic pathways, is not supported in this study on *E. chloroticus* sperm. Although the pH sensitivity of dynein-ATPase has been clearly described (Gibbons & Fronk, 1972; Christen et al., 1986; Neill & Vacquier, 2004), this is not the primary point of regulation under OA conditions. This can be seen in the slightly elevated swimming speeds (VCL) of *E. chloroticus* sperm indicating that the overall operation of the dynein motors is permitted despite the stepwise decrease in pH\(_i\). The same applies for other pH dependent processes including the kinetics of the regulatory enzyme sAC (Nomura et al., 2005; Vacquier et al., 2014). The apparent lack of pH\(_i\) specific decreases in VCL performance suggest other factors are involved with efficient sperm swimming. These include the possibility that echinoid sperm are ‘running-on-idle’ under current day seawater pH and pCO\(_2\) conditions (with capacity to compensate for a lower pH\(_i\)); or, more fundamentally, that pH\(_i\) is not the primary regulator of sperm motility under OA conditions. The latter is supported by the long established understanding that oxygen tension may play a greater role in sperm activation than currently acknowledged in many OA studies (Cohn, 1918; Mohri & Yasumasu, 1963; Webster & Giese, 1975; Chia & Bickell, 1983; Byrne, 2011), or more recently the proposed role of decreased mitochondrial membrane potential in lower swimming speed (Schlegel et al., 2015).

The relationship between OA, pH\(_i\) and the activation and swimming of sea urchin sperm does not appear to be straightforward with apparent differences in among-male responses. As discussed above, although increasing OA conditions resulted in a slight increase in through-the-water velocity (VCL) of the activated sperm, an increasing proportion of immotile sperm also occurred; whether this latter portion were exhibiting narcosis was not possible to determine here. Within male variation in sperm traits are not uncommon with sperm size and swimming speed in mammals, birds, and sea urchins shown to be highly correlated within individuals but not among (Fitzpatrick et al., 2010;
Simpson et al., 2014), and may also apply to the differences in within-male sensitivity (% motility) to OA. The high degree of within-treatment variability in the motility parameters of *E. chloroticus* also indicates among-individual differences in capacity to compensate for lower environmental pH. A similar high degree of among-individual variability is seen in the sea urchin *Heliocidaris erythrogramma* with differences in effect of OA on sperm % motility as well as between +14% to -44% change in level of fertilisation success (Schlegel et al., 2012).

Although other studies have looked at sperm motility parameters in a range of marine invertebrates, the very different implications of the different speed parameters (i.e. VCL, VAP or VSL) meant direct comparisons were only able to be drawn with those reporting parameters. Future studies would benefit from a level of consistency in parameter reporting to enable comparisons and advance the understanding of how sperm motility is impacted by OA over a range of different organisms.

This study provides the first quantitative analysis of the impacts of OA on urchin sperm and the suggested link between pHᵢ and motility parameters when measured on the same individuals. The findings do not support the simple hypothesis that sea urchin sperm performance will be compromised under OA conditions due to impaired ability to defend an optimal pHᵢ. Instead sperm response to OA is non-linear with evidence of two alternate states. First, the increased swimming speed (VCL) and corresponding stepwise decrease in pHᵢ show that OA is not negatively impacting pH dependent processes (such as dynein-ATPase) that regulate sperm swimming. Second, the increasing proportion of immotile sperm is potentially due to narcosis. Variation in sperm response suggests that the impacts of OA are subject to within-male differences in sensitivity. Despite the faster through-the-water speed (VCL), the more curved path results in a slower straight line speed and progress that will have consequences for fertilisation kinetics. These results provide a significant contribution to the understanding of how OA will affect the physiology and some processes involved with activation and swimming in sea urchin sperm. Overall, the lower number of slower swimmers under future OA conditions is likely to have negative consequences for fertilisation in *E. chloroticus*, with implications for reproduction and long term maintenance of populations.
Chapter 5. Fertilisation success

5.1 Introduction

Rising atmospheric CO\textsubscript{2} and the resulting ocean acidification (OA) are identified as contributing to severe and pervasive negative impacts across natural systems (IPCC, 2014). OA has important implications for marine organisms as their surrounding environment experiences concurrent decreases in carbonate availability and increases in hydrogen ion concentration; the latter resulting in a decrease in pH. With [H\textsuperscript{+}] already 30\% higher than pre-industrial levels, further [H\textsuperscript{+}] increases of up to 150\% are projected by the year 2300 under the Representative Concentration Pathway (RCP) 8.5, with examples of biological effects at these levels described in previous studies (Doney \textit{et al.}, 2009; Dupont \textit{et al.}, 2010\textsuperscript{b}; Hofmann & Todgham, 2010; Riahi \textit{et al.}, 2011). The potential disruption of optimal cellular conditions under large scale changes in pH can lead to the impairment of normal cellular processes and activity (Kaniewska \textit{et al.}, 2012; Sokolova, 2013; Stillman & Paganini, 2015). Due to their small size and limited capacity for regulating cellular homeostasis, gametes of marine broadcast spawners are suggested to be some of the most susceptible to OA; any impacts to cellular processes (Melzner \textit{et al.}, 2009) will likely have implications for fertilisation success (Bögner, 2016).

The effect of OA on fertilisation success has been examined in a variety of broadcast spawning marine invertebrates – most studies show a no- to negative-response that is species-specific, with some species being more resilient to OA than others (Kurihara \textit{et al.}, 2004; Kurihara, 2008; Byrne \textit{et al.}, 2010\textsuperscript{b}; Ross \textit{et al.}, 2011; Byrne, 2012). In sea urchins, although FS may appear to be generally robust to OA (Dupont \textit{et al.}, 2010\textsuperscript{b}; Byrne, 2011), exceptions do exist with both elevated sensitivities (Martin \textit{et al.}, 2011; Sung \textit{et al.}, 2014) and tolerance to pH values of 7.0 or lower (Kurihara & Shirayama, 2004; Ericson \textit{et al.}, 2010).

The experimental approach adopted can contribute to some of the general differences in observed response of OA on fertilisation (Byrne, 2012). Typically, studies examine the differences in fertilisation success (FS) at a few set-point pCO\textsubscript{2} levels (e.g. ambient with near- or far-future conditions yr 2100 to 2300 respectively) to inform the broadscale effects of OA. Alternatively, studies using a finer scale pCO\textsubscript{2} gradient provide a more detailed description of changes in FS including the characterisation of any rapid
Chapter 5. Fertilisation success

decline (tipping point) of FS (Martin et al., 2011; Moulin et al., 2011; Dorey et al., 2013; Bögner et al., 2014; Frieder, 2014).

A typical early approach to FS studies involved experiments using pooled gametes to give an indication of the population level response. However, underlying a population level response to increasing OA, FS at the individual male:female cross level can reveal a high degree of within species variability (Schlegel et al., 2012; Kelly et al., 2013; Foo et al., 2014; Hofmann et al., 2014; Sewell et al., 2014). This individual variability in response to OA is potentially masked in experiments using pooled gametes from multiple individuals (Evans & Marshall, 2005); as seen in the contrasting conclusions about the response of Heliocidaris erythrogramma (Havenhand et al., 2008; Byrne et al., 2009) or Sterechinus neumayeri (Ericson et al., 2011; Sewell et al., 2014) to elevated levels of $p$CO$_2$.

In this study the effect of OA on FS and early cleavage was evaluated over a 10-step $p$CO$_2$ gradient using individual male:female crosses of the endemic New Zealand sea urchin, Evechinus chloroticus. The fine scale 10-step gradient between ambient and projected far-future OA levels (1800 µatm $p$CO$_2$) aimed to test the generalised robustness of echinoid FS, and characterise any tipping point. With urchins collected from the same location, individual male:female crosses indicate the level of standing variation in FS response to OA attributable to adaptive potential (e.g.: Foo et al., 2012; Kelly et al., 2013). Using a non-saturated sperm concentration allowed for sensitivity to detect both a degree of positive as well as negative responses in FS (Schlegel et al., 2012); > 75% being considered a non-impaired FS level (Byrne, 2011). This study also aimed to examine the potential for gender to contribute to differences in FS response to OA using nested single male:female crosses.

5.2 Methods

5.2.1 Seawater manipulation and chemistry

Treatment seawaters (OA seawaters) were prepared to give a 10 step gradient from control (target ~390 µatm) to high (target ~1800 µatm) $p$CO$_2$ concentrations. Final seawater carbonate chemistry was determined from analysis of total alkalinity (TA) and pH on the total scale ($p$H$_{Total}$) using the software RStudio (version 0.99.486) and ‘R’ (version
3.1.2; RCoreTeam, 2015) and the seacarb package (Gattuso et al., 2015). Further details of preparation and analysis procedures and protocols are provided in Chapter 4, Section 4.2.1.

The gradient of $p$CO$_2$ seawaters for experiments in this chapter were prepared using a standard stepwise dilution across 10 Schott bottles (500 mL, with actual volume of 625 mL) between control and high OA seawaters. To achieve this, all bottles were rinsed three times with control OA seawater siphoned from the bubbling buckets, completely emptied, and the calculated volume of control seawater added using a graduated measuring cylinder (Table 5-1). Bottles were then topped up with free flowing High $p$CO$_2$ seawater from a silicone siphon tube leaving a small headspace (about 1 mL), immediately capped, and inverted three times to maximise mixing within each bottle.

### Table 5-1: Seawater gradient mixing volumes for 500 mL Schott bottles, with an actual volume of 625 mL. Target $p$CO$_2$ for control 380 µatm and High 1800 µatm. Step between control (step 1) and step 2 is 125 mL, all other steps are 62.5 mL increments.

<table>
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<th>Control pCO$_2$</th>
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<td>500</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>562.5</td>
<td>65.5</td>
<td>562.5</td>
</tr>
</tbody>
</table>

Prepared OA seawaters were carefully added by siphoning through a silicone tube, as per SOP1 (Dickson et al., 2007), to 24 mL glass scintillation vials and tightly sealed. Lids with PTFE inners were only uncapped briefly to allow addition of gametes, minimising exposure to ambient air. A constant temperature (20 °C) was achieved for all experiments using two identical insulated aluminium temperature blocks (315x445 mm) with a 6x10 grid of chambers. The chambers within the block contained de-ionised water to optimise heat transfer to the scintillation vials, with temperature controlled using a pair...
of re-circulating waterbaths for each block (Figure 5-1)(Sewell & Young, 1999). Once stabilised, block and water bath temperatures remained steady (± 0.1 °C) over extended periods.

![Figure 5-1: Aluminium temperature control block for fertilisation experiments encased in polystyrene insulation to optimise temperature stability. Temperature controlled water (20 °C) was circulated through each end of the block (blue arrows). 24 mL scintillation vials (SV) were inserted into chambers (30 mm ø holes) of the block containing de-ionised water to optimise heat transfer.](image)

**5.2.2 *Evechinus chloroticus* collection, spawning and gamete preparation**

*Evechinus chloroticus* were collected from the shallow subtidal (1-3 m depth) at Mathesons Bay (36°18’6.58” S; 174°48’0.71″ E) within the Hauraki Gulf, New Zealand, during the austral summer breeding season. Within 2 hours of collection urchins were transported in sealed buckets containing icepacks to the seawater facilities at the University of Auckland where they were held in environmentally controlled conditions (18 °C, 12:12 dark:light cycle). Aerated aquaria (100 L) were stocked to a maximum of 50 urchins per tank with 50-75 % water changes every 2-3 days to minimise faecal matter and debris build up. Urchins were fed to satiation on seaweed (*Ecklonia radiata* and *Carpophyllum* spp.) and spawned within two weeks of collection.
Spawning was induced using a standard coelomic injection of up to 5 mL of 0.55 M KCl (potassium chloride) through the peristomal membrane. Injected urchins were gently agitated to mix coelomic fluids before being inverted over a 100 mL beaker with 1 μm bag filtered sea water (FSW) covering the gonopores. On initiation of sperm release, males were placed oral side down and sperm collected ‘dry’ using a Pasteur pipette while females were left to spawn their eggs into the beakers of FSW. Egg batches and sperm collections were individually labelled.

*E. chloroticus* eggs are approximately 110 μm in diameter allowing each egg batch to be gently filtered through a 220 μm mesh to remove unwanted material (e.g. faeces, spines), with the mesh kept immersed in FSW to minimise egg damage. After being filtered the eggs were rinsed three times with FSW followed by visual assessment under a dissection microscope (10x magnification) for absence of clumping (a characteristic of abnormal jelly layers), a smooth regular shape, and lack of germinal vesicle indicating completed meiosis (Foltz *et al.*, 2004). A constant egg number of 2500 eggs per scintillation vial, or approximately 104 eggs mL\(^{-1}\), was used in all fertilisation experiments; based on concentrations used by Sewell & Young (1999). The concentration of each egg batch was determined by averaging egg counts from three 100 μL aliquots of an even egg suspension on a Sedgewick Rafter slide at 25x magnification, and adjusted to ensure that the final volumes for ~2500 eggs was between 100-200 μL. In all experiments eggs were added to scintillation vials containing OA seawaters for a 30-45 minute acclimation period prior to the addition of sperm.

Sperm were assessed to ensure a standard concentration of high quality sperm were used in all experiments. Initially an activated sperm (AS) solution was prepared using a standard dilution of 10 μL of dry sperm mixed with 30 mL of FSW, followed by aspiration with a wide bore transfer pipette, to minimise damage to the flagella (Brokaw, 1986), and achieve an even suspension. AS were assessed under a compound microscope at 100x magnification for vigorous swimming activity of the majority of cells. The concentration of dry sperm was calculated from the same AS using haemocytometer counts (La Fontaine with a depth of 0.1 mm and a grid square size of 0.0025 mm\(^2\)), and used to prepare a premix sperm solution (PS) with target of 24 million cells mL\(^{-1}\). A 50 uL aliquot of PS was added to each scintillation vial for a final target sperm concentration of 50,000 mL\(^{-1}\) so that all experiments achieved a target of ~80 % fertilisation success, combined with low rates of polyspermy (Franke, 2005). The target of 80 % aimed to allow both negative and
positive effects of treatment OA seawaters to be detected by using non-saturating sperm concentrations (Marshall, 2006). In 2010, when these experiments were designed, a target of 80% was thought appropriate to allow for both positive and negative responses to be detected. Subsequent research has suggested that a lower target FS (50%) might be more appropriate to ensure a positive response can be detected (Schlegel et al., 2012).

To ensure high quality gametes were used, and to minimise degradation, all fertilisation experiments were started within 2 hours of spawning, with eggs kept cool at around 16 °C and ‘dry’ sperm held on ice prior to use (Vacquier, 1986; Adams et al., 2003). Gamete compatibility and quality were checked for fertilisation success under sperm saturated conditions by mixing two drops (~40 µL) of AS with a small sample of approximately 1000 eggs on a Sedgewick Rafter slide. Individual male:female crosses were checked for presence of a fertilisation membrane (FM) in 100 eggs after 10 minutes and all crosses exhibited > 95 % fertilisation.

### 5.2.3 Scoring fertilisation success

Fertilisation success and early stages of embryogenesis in *E. chloroticus* were scored after a consistent 2 hour incubation period to allow accumulation of fertilisations (Meidel & Yund, 2001) and to capture early cell divisions. Eggs and embryos were collected with a single 1 mL sample from the bottom of each scintillation vial, and added to a 1.5 mL microcentrifuge tube containing borate-buffered formalin (pH 8.1) to give a final concentration of 4%. A single 100 µL aliquot of preserved eggs from each vial was viewed under a microscope (at 100x magnification) with the first 100 eggs on transects across the Sedgewick Rafter cell scored for normal fertilisation and cell division stages as described in Table 5-2.

Successful fertilisation and early cell divisions were categorised as Normal-fertilisation success (N-FS) and comprised of eggs with a symmetrical raised fertilisation membrane as well as the symmetrical first and second cell divisions (Table 5-2). Unsuccessful fertilisation and early cell divisions comprised of two categories: abnormal fertilisation, defined by uneven cell divisions (number and/or size of blastomeres, both indicating occurrence of polyspermy); and Non-raised fertilisation membrane, defined by a lack of evidence of normal slow polyspermy block, however the hyaline layer may be evident (Table 5-2) (Bögner et al., 2014; Sewell et al., 2014).
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Table 5-2: Scoring categories for fertilisation and cell division success.

<table>
<thead>
<tr>
<th>Scoring category</th>
<th>Description</th>
<th>Contributes to normal fertilisation success (N-FS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg</td>
<td>Unfertilised egg</td>
<td>No</td>
</tr>
<tr>
<td>Normal fertilisation success</td>
<td>Clear and symmetrical raised fertilisation membrane</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-raised fertilisation membrane</td>
<td>Incomplete raised fertilisation membrane – irregular or non-raised</td>
<td>No</td>
</tr>
<tr>
<td>Abnormal fertilisation</td>
<td>Evidence of polyspermy through irregular cell divisions – e.g. 3 or 5 cell</td>
<td>No</td>
</tr>
<tr>
<td>2 cell</td>
<td>Normal 2 cell embryo</td>
<td>Yes</td>
</tr>
<tr>
<td>4 cell</td>
<td>Normal 4 cell embryo</td>
<td>Yes</td>
</tr>
</tbody>
</table>

5.2.4 Experimental design and statistical analysis

Three different balanced experimental designs were used to assess areas of sensitivity of fertilisation in *E. chloroticus*, and single male:female crosses in all experiments provided a measure of among-individual variation in sensitivity across a gradient of $pCO_2$ (Table 5-3): First, a single male:female cross to determine different sensitivities between individual pairs (Individual Cross or IC) with technical replication ($n = 3$) at each CO$_2$ level. Two further experiments looked to assess males and females for differing sensitivity to OA as an indication of the presence of genetic variation required for broad-sense heritability of tolerance. This was done using a nested sibling design (Figure 5-2) combining both full and half-sibling crosses to distinguish components of variation (Lynch & Walsh, 1998; Conner & Hartl, 2004). These experiments comprised a group of single male:female crosses where a single female was crossed with three separate males (Male Variability or MV) or a single male with three separate females (Female Variability or FV), with technical replication ($n = 2$) at each CO$_2$ level. The limited number of positions on the temperature control block (Figure 5-1) required technical replication to be decreased to two. The nested genders (i.e. males in MV; females in FV) were levels within the nesting gender (i.e. female in MV; male in FV) (Table 5-3). As the interest here was to define any changes in FS across a fine scale gradient of $pCO_2$, logistical limitations meant a full factorial blocked design (Sunday *et al.*, 2014) was not feasible across the 10 levels of OA seawater used in this study. This was due to the experimental design including technical replication ($n = 2$) and the limited number of positions on the temperature control
block (Figure 5-1). A full factorial blocked experiment across 10 $p$CO$_2$ levels would require an increase in total number of vials from 60 for the experimental design used here (e.g. for 1 male x 3 females) to 180 vials in a full factorial blocked experiment (3 females x 3 males).

Table 5-3: Summary of fertilisation success experimental designs for IC, MV and FV experiments. Given male and female numbers are per-cross or per-experimental-run, $n$ in brackets indicates total of each gender per experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Males</th>
<th>Nested in</th>
<th>Females</th>
<th>Technical Replication per cross</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC (Individual Cross)</td>
<td>1 (n = 9)</td>
<td>-</td>
<td>1 (n = 9)</td>
<td>3</td>
</tr>
<tr>
<td>MV (Male Variability)</td>
<td>3 (n = 24)</td>
<td></td>
<td>1 (n = 8)</td>
<td>2</td>
</tr>
<tr>
<td>FV (Female Variability)</td>
<td>1 (n = 5)</td>
<td></td>
<td>3 (n = 15)</td>
<td>2</td>
</tr>
</tbody>
</table>

All N-FS scores were converted to proportions to account for uneven total counts before being arcsin transformed ($x = \sin^{-1}\sqrt{y}$) for statistical analyses; this transformation has more effect on the upper and lower bounds of proportional data, thereby tending to normalise the distribution (Quinn & Keough, 2002). Where noted, technical replication was averaged and thereby increased the precision of reported values.
Figure 5-2: Example of nested sibling design for Male Variability (MV) experiment; repeated for 8 females in MV experiments (total of 24 nested males) and 5 males in Female Variability (FV) experiments (total of 15 nested females). In the results section the ‘Nesting gender’ is always represented with a capital letter; ‘nested gender’ with lower case. Modified from: Lynch and Walsh (1998).

Statistical analyses were performed using the software RStudio (version 0.99.486) and ‘R’ (version 3.1.2; RCoreTeam, 2015), unless specified otherwise, with basic plotting completed using the ggplot2 or Lattice packages. Data from seawater pH\textsubscript{Total} was analysed with a one-way ANOVA. Analysis differed between the two experimental designs: IC data were analysed using two-way ANOVA with interaction (factors: cross [random], \( p\text{CO}_2 \)); FV and MV data were analysed using mixed-effects two-level nested ANOVA using CO\(_2\) and the nesting gender as a fixed factor with nested gender as a random factor. As categories of the nested factor only occur within a single category of the nesting factor, main effects are only performed on the nesting gender (i.e. male in FV; female in MV).

The model (aov) for nested-analysis produced multiple ANOVAs, one for each of: the main effect; nested factor; and remaining fixed factor, the latter two each with separate residuals.

Assumptions of normality were checked with Q-Q plots and Shapiro-Wilk for levels of factor. Homogeneity of variance was checked with Levene’s test and post hoc test for pairwise comparisons between means, using Tukey-Kramer’s test (\( \alpha = 0.05 \)). Where the assumption of homogeneity of variance was violated, the suggested use of Welch’s robust one-way ANOVA was not possible due to the requirement to use the two-factor nested ANOVA. Differences in variance was likely due to the very different responses to increasing \( p\text{CO}_2 \) between and within the nesting genders of MV and FV experiments. When the Levene’s test shows variances to be significantly different, caution is required on interpreting marginally significant differences, and \( F \)-test Type I error rates can be altered (Quinn & Keough, 2002). Analysis proceeded by acknowledging these limitations and that the sensitivity of the ANOVA is considered to be minimised through a combination of meeting assumptions of normality, when samples sizes are the same, and the ANOVA \( F \)-statistic is highly significant (Quinn & Keough, 2002).

**5.2.4.1 LnRR of fertilisation and development stages**

The magnitude of effect of increasing \( p\text{CO}_2 \) on all six categories of fertilisation and embryogenesis (Table 5-2) was examined on IC data using the logarithmic response ratio
(LnRR) (Chapter 4, Section 4.2.7.1). In brief, technical replicates were averaged to the level of the individual male:female cross for all fertilisation categories (Table 5-2). To avoid the potential artificial semblance of developmental delay driven by the increase in proportion of unfertilised eggs, a two-step calculation was performed: 1) effect size was calculated for unfertilised eggs as a proportion of the six scoring categories; then, 2) unfertilised eggs were excluded and proportions recalculated for the remaining five categories, followed by calculations of effect size. The latter step was taken to limit how the proportion of each of the division stages (within those classified as fertilised) change with increasing OA. By limiting the effect size calculations to the five fertilised and division categories, the analysis avoids the negative bias that an increasing proportion of unfertilised eggs has on the decreasing overall proportion of these five categories, thereby allowing a direct comparison between, for example, changes in 2 cell stage compared with 4 cell stages. Data were adjusted for zero proportions by adding 1 to all parameters prior to calculating LnRR (LnRR = ln(x treatment + 1)/x control + 1)). A trial was done as in Molloy et al. (2008) using data adjustments of 0.001, 0.01, 0.1 and 1 for categories without zero proportions; data adjustment of 1 was found to have least effect on the LnRR values. Each parameter’s LnRR for all 9 individuals were then bootstrapped in the statistical software ‘R’ running boot package and the resulting mean and 95% normal confidence intervals returned from 100,000 iterations. The confidence intervals provide a measure of the variance between individuals and results are interpreted as significant when CIs do not overlap with zero (α = 0.05).

5.3 Results

5.3.1 Seawater and carbonate system

Seawater carbonate chemistry for all experiments showed a clear expected separation in pH values between an average low $p$CO$_2$ of 382 µatm to high of 1728 µatm (Table 5-4). Analysis of the carbon chemistry across the 10 step gradient between the low and high $p$CO$_2$ treatments indicated that there was a consistent non-linear gradient (Figure 5-3). Oxygen levels remained above 80% for the ~2 hour duration of the fertilisation trials (Microx TX3 oxygen optrode).

Table 5-4: Seawater carbonate parameters for OA seawater used for each end of the 10 step OA gradient, CO$_2$ levels: Low (Control) and High. Measured values of pH$_{Total}$ and
total alkalinity (TA) used seacarb with salinity and experimental temperature (T) to calculate remaining carbonate parameters of partial pressure of CO₂ ($pCO₂$) and saturation states for calcite ($Ω_{Ca}$) and aragonite ($Ω_{Ar}$). $pH_{Total}$ brackets denote error = ±SE; n = 9.

<table>
<thead>
<tr>
<th>OA Seawater</th>
<th>$pH_{Total}$</th>
<th>T(°C)</th>
<th>Salinity</th>
<th>TA (μequiv kg⁻¹)</th>
<th>$pCO₂$ (µatm)</th>
<th>$Ω_{Ca}$</th>
<th>$Ω_{Ar}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low/ Control</td>
<td>8.13 (0.02)</td>
<td>20</td>
<td>34.0</td>
<td>2241</td>
<td>382</td>
<td>4.93</td>
<td>3.20</td>
</tr>
<tr>
<td>High</td>
<td>7.52 (0.02)</td>
<td>20</td>
<td>34.0</td>
<td>2241</td>
<td>1728</td>
<td>1.48</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Figure 5-3: Summary of seawater $pCO₂$ (µatm) across experimental 10-step gradient mixed between high and control $pCO₂$ OA seawaters. Mixing steps described in Table 5-1. Fitted red line = 3rd order polynomial regression. Error bars = ±SE, n = 9.

5.3.2 Fertilisation experiments

5.3.2.1 Individual Cross (IC)

Fertilisation and early embryogenesis in the Mathesons Bay population of *Evechinus chloroticus* was fairly robust to acute near-future OA, with incremental decreases in N-FS across the $pCO₂$ gradient from a maximum N-FS of 74.6% at 382 µatm
to a minimum of 46.4% at 1728 µatm \( p\text{CO}_2 \) (-13.5 and -28.2% respectively; Figure 5-4). At the population level (technical replication averaged within individual cross, average of 9 crosses), average N-FS remained above 60% until near-future levels with further decreases to far-future \( p\text{CO}_2 \), with significant differences evident between low (637), and high \( p\text{CO}_2 \) treatments (1234 or more) (Tukey HSD groups; Figure 5-4). Importantly, underpinning this population level response was a high degree of variation among different individual male:female crosses (Figure 5-4 and Figure 5-5); as shown by the highly significant interaction term (ANOVA: \( F_{(72,180)} = 4.055, p < 0.0001 \) (Table 5-5). Some individuals were clearly more sensitive to increasing \( p\text{CO}_2 \) than others, with cross A and G exhibiting a rapid decline compared to D, J and H that maintained high levels of N-FS across the \( p\text{CO}_2 \) gradient (Figure 5-5). Similar high levels of variation among individual crosses was evident in both MV and FV experiments (Figure 5-6 and Figure 5-7).
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Figure 5-4: Mathesons Bay population level normal-fertilisation success (N-FS) in *Evechinus chloroticus* (n = 9 pairs) across a gradient of elevated $p$CO$_2$ seawaters (382 to 1728 µatm). Individual variation indicated by error bars = ±SE. Technical replication n = 3 averaged within individual; fitted red line = 3$^{rd}$ order polynomial regression. Letters denote Tukey HSD groups ($\alpha = 0.05$) calculated on arcsin transformed data.
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Figure 5-5: Individual male:female cross level normal-fertilisation success (N-FS) in *Evechinus chloroticus* across a gradient of elevated $p$CO$_2$ seawaters (380 to 1800 µatm). Data points include all technical replicates ($n = 3$) for each $p$CO$_2$ level; blue fitted line = Loess locally weighted smoothing line.

Table 5-5: Two-way ANOVA results for Individual Cross ($n = 9$) comparing changes in normal-fertilisation success across a gradient of elevated $p$CO$_2$ seawaters, using arcsin transformed proportion scores, and all replicates within each cross ($n = 3$). Significant results are shown in bold ($P < 0.0001$).

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross</td>
<td>8</td>
<td>17.112</td>
<td>2.1390</td>
<td>149.987</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$p$CO$_2$</td>
<td>9</td>
<td>7.256</td>
<td>0.8062</td>
<td>56535</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cross*$p$CO$_2$</td>
<td>72</td>
<td>4.164</td>
<td>0.0578</td>
<td>4.055</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residuals</td>
<td>180</td>
<td>2.567</td>
<td>0.0143</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


5.3.2.2 Nested sibling design

As with the IC experiment, fertilisation and early embryogenesis of *E. chloroticus* in both MV and FV experiments were fairly robust to acute near-future OA, with N-FS decreasing with further $pCO_2$ increase towards far-future levels (Figure 5-6; Figure 5-7). The nested sibling design and analysis with the two-level nested ANOVA showed $pCO_2$ to have a highly significant negative impact on N-FS that differed between individuals of the nesting gender in both FV and MV experiments. Although the effect of $pCO_2$ on N-FS is not the same for all nesting individuals (ANOVA-MV, $[pCO_2 \times Female]$: $F_{(65,333)} = 2.111$, $P < 0.0001$; Table 5-6; and ANOVA-FV, $[pCO_2 \times Male]$: $F_{(37,240)} = 9.173$, $P < 0.0001$; Table 5-8), within each nesting individual there was no significant difference in N-FS among the three nested individuals (ANOVA-MV, [Male(Female)]: $F_{(6,8)} = 2.116$, $P = 0.161$; Table 5-6; and ANOVA-FV, [Female(Male)]: $F_{(6,4)} = 5.038$, $P = 0.0697$; Table 5-8). Individual identity appears to play a greater role in variability in N-FS than gender does, with both eggs and sperm contributing to the highly significant differences in average N-FS among the nesting gender (Table 5-6; Table 5-8; Post-hoc pairwise comparisons: Table 5-7; Table 5-9).
Figure 5-6: Normal-fertilisation success (N-FS) in Male Variability nested half-sibling design. Each panel represents 3 different males (different colours, total = 24) nested in each female (panels A-H, total = 8; capital letter represents nesting gender) across a gradient of elevated $p$CO$_2$ seawaters (380 to 1800 µatm). Coloured fitted lines = Loess locally weighted smoothing line for each male calculated from mean of technical replicates ($n = 2$).
Table 5-6: Nested ANOVA results for Male Variability nested sibling design (individual sets of crosses with three males nested within a single female; n = 8) comparing changes in fertilisation and early cell divisions across a gradient of elevated pCO2 seawaters, using arcsin transformed proportion scores, and all replicates within each cross (n = 2). Residuals and pCO2 appear twice due to separate allocation to each ANOVA within the nested ANOVA calculation. The Nested ANOVA is testing two hypotheses: 1. That the means of pCO2 treatment within nesting gender are the same, 2. That the means of pCO2 treatment between nested gender within nesting gender are the same. The output of the nested random effect (df = 6, from [2 technical replicates] x [3 nested individuals]) is presented separately from the fixed effect, with df values attributed accordingly. Significant results are shown in bold (P < 0.0001).

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main effect:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>15.63</td>
<td>2.232</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nested random effect:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male(Female)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCO2</td>
<td>6</td>
<td>2.854</td>
<td>0.4757</td>
<td>2.116</td>
<td>0.161</td>
</tr>
<tr>
<td>Residuals</td>
<td>8</td>
<td>1.798</td>
<td>0.2248</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fixed effects: Within</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCO2</td>
<td>10</td>
<td>6.338</td>
<td>0.6338</td>
<td>34.472</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Female*pCO2</td>
<td>65</td>
<td>2.523</td>
<td>0.0388</td>
<td>2.111</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Residuals</td>
<td>333</td>
<td>6.123</td>
<td>0.0184</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5-7: Female pairwise comparisons for Male Variability nested sibling design (three males nested within single female; n = 8; nesting gender presented in capital letters) comparing changes in fertilisation and early cell divisions across a gradient of elevated pCO₂ seawaters, using arcsin transformed proportions, and all replicates within each cross (n = 2). Significant results are shown in bold (α = 0.05).

<table>
<thead>
<tr>
<th>Females</th>
<th>difference</th>
<th>lower</th>
<th>upper</th>
<th>p.adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-A</td>
<td>-0.0918</td>
<td>-0.2087</td>
<td>0.0250</td>
<td>0.2472</td>
</tr>
<tr>
<td>C-A</td>
<td>-0.1639</td>
<td>-0.2790</td>
<td>-0.0489</td>
<td>0.0005</td>
</tr>
<tr>
<td>D-A</td>
<td>0.2368</td>
<td>0.1189</td>
<td>0.3547</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>E-A</td>
<td>0.0770</td>
<td>-0.0414</td>
<td>0.1954</td>
<td>0.4953</td>
</tr>
<tr>
<td>F-A</td>
<td>-0.4159</td>
<td>-0.5412</td>
<td>-0.2905</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G-A</td>
<td>-0.0050</td>
<td>-0.1374</td>
<td>0.1274</td>
<td>1.0000</td>
</tr>
<tr>
<td>H-A</td>
<td>-0.3236</td>
<td>-0.4582</td>
<td>-0.1891</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C-B</td>
<td>-0.0721</td>
<td>-0.1890</td>
<td>0.0448</td>
<td>0.5661</td>
</tr>
<tr>
<td>D-B</td>
<td>0.3287</td>
<td>0.2090</td>
<td>0.4483</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>E-B</td>
<td>0.1689</td>
<td>0.0487</td>
<td>0.2891</td>
<td>0.0006</td>
</tr>
<tr>
<td>F-B</td>
<td>-0.3241</td>
<td>-0.4511</td>
<td>-0.1970</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G-B</td>
<td>0.0868</td>
<td>-0.0472</td>
<td>0.2208</td>
<td>0.5013</td>
</tr>
<tr>
<td>H-B</td>
<td>-0.2318</td>
<td>-0.3679</td>
<td>-0.0956</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>D-C</td>
<td>0.4008</td>
<td>0.2829</td>
<td>0.5186</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>E-C</td>
<td>0.2410</td>
<td>0.1226</td>
<td>0.3594</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>F-C</td>
<td>-0.2520</td>
<td>-0.3773</td>
<td>-0.1266</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G-C</td>
<td>0.1589</td>
<td>0.0265</td>
<td>0.2913</td>
<td>0.0069</td>
</tr>
<tr>
<td>H-C</td>
<td>-0.1597</td>
<td>-0.2943</td>
<td>-0.0251</td>
<td>0.0080</td>
</tr>
<tr>
<td>E-D</td>
<td>-0.1598</td>
<td>-0.2809</td>
<td>-0.0386</td>
<td>0.0018</td>
</tr>
<tr>
<td>F-D</td>
<td>-0.6527</td>
<td>-0.7807</td>
<td>-0.5247</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G-D</td>
<td>-0.2418</td>
<td>-0.3767</td>
<td>-0.1069</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>H-D</td>
<td>-0.5604</td>
<td>-0.6974</td>
<td>-0.4234</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>F-E</td>
<td>-0.4929</td>
<td>-0.6214</td>
<td>-0.3645</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G-E</td>
<td>-0.0821</td>
<td>-0.2174</td>
<td>0.0533</td>
<td>0.5885</td>
</tr>
<tr>
<td>H-E</td>
<td>-0.4007</td>
<td>-0.5381</td>
<td>-0.2632</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G-F</td>
<td>0.4109</td>
<td>0.2694</td>
<td>0.5524</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>H-F</td>
<td>0.0923</td>
<td>-0.0512</td>
<td>0.2358</td>
<td>0.5113</td>
</tr>
<tr>
<td>H-G</td>
<td>-0.3186</td>
<td>-0.4683</td>
<td>-0.1689</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
Figure 5-7: Normal-fertilisation success (N-FS) in Female Variability nested half-sibling design. Each panel represents 3 different females (different colours, total = 15) nested in each male (panels A-E, total = 5; capital letter represents nesting gender) across a gradient of elevated \(pCO_2\) seawaters (380 to 1800 µatm). Coloured fitted lines = Loess locally weighted smoothing line for each male calculated from mean of technical replicates (n = 2).
Table 5-8: Nested ANOVA results for Female Variability nested sibling design (individual sets of crosses with three females nested within single male; n = 5) comparing changes in fertilisation and early cell divisions across a gradient of elevated $p$CO$_2$ seawaters, using arcsin transformed proportions, and all replicates within each cross (n = 2). The Nested ANOVA is testing two hypotheses: 1. That the means of $p$CO$_2$ treatment within nesting gender are the same, 2. That the means of $p$CO$_2$ treatment between nested gender within nesting gender are the same. The output of the nested random effect (df = 6, from [2 technical replicates] x [3 nested individuals]) is presented separately from the fixed effect, with df values attributed accordingly. Significant results are shown in bold ($P < 0.0001$).

<table>
<thead>
<tr>
<th>Df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>4</td>
<td>11.99</td>
<td>2.997</td>
<td></td>
</tr>
<tr>
<td>Nested random effect: Female(Male)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$CO$_2$</td>
<td>6</td>
<td>2.2034</td>
<td>0.3672</td>
<td>5.038</td>
</tr>
<tr>
<td>Residuals</td>
<td>4</td>
<td>0.2916</td>
<td>0.0729</td>
<td></td>
</tr>
<tr>
<td>Fixed effects: Within</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$CO$_2$</td>
<td>10</td>
<td>3.445</td>
<td>0.3445</td>
<td>36.787</td>
</tr>
<tr>
<td>Male*$p$CO$_2$</td>
<td>37</td>
<td>3.178</td>
<td>0.0859</td>
<td>9.173</td>
</tr>
<tr>
<td>Residuals</td>
<td>240</td>
<td>2.247</td>
<td>0.0094</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-9: Male pairwise comparisons for Female Variability nested sibling design (three females nested within single male; n = 5; nesting gender presented in capital letters) comparing changes in fertilisation and early cell divisions across a gradient of elevated $p$CO$_2$ seawaters, using arcsin transformed proportions, and all replicates within each cross (n = 2). Significant results are shown in bold ($\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Males</th>
<th>difference</th>
<th>lower</th>
<th>upper</th>
<th>p.adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-A</td>
<td>-0.3866</td>
<td>-0.4816</td>
<td>-0.2917</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C-A</td>
<td>0.0873</td>
<td>-0.0089</td>
<td>0.1835</td>
<td>0.0952</td>
</tr>
<tr>
<td>D-A</td>
<td>0.1469</td>
<td>0.0498</td>
<td>0.2440</td>
<td>0.0004</td>
</tr>
<tr>
<td>E-A</td>
<td>0.1354</td>
<td>0.0387</td>
<td>0.2320</td>
<td>0.0014</td>
</tr>
<tr>
<td>C-B</td>
<td>0.4740</td>
<td>0.3763</td>
<td>0.5716</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>D-B</td>
<td>0.5335</td>
<td>0.4350</td>
<td>0.6320</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>E-B</td>
<td>0.5220</td>
<td>0.4239</td>
<td>0.6201</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>D-C</td>
<td>0.0596</td>
<td>-0.0401</td>
<td>0.1593</td>
<td>0.4733</td>
</tr>
<tr>
<td>E-C</td>
<td>0.0480</td>
<td>-0.0512</td>
<td>0.1473</td>
<td>0.6739</td>
</tr>
<tr>
<td>E-D</td>
<td>-0.0115</td>
<td>-0.1117</td>
<td>0.0886</td>
<td>0.9978</td>
</tr>
</tbody>
</table>
5.3.3 Effect of pCO$_2$ on fertilisation and development parameters

The magnitude of effect of increasing pCO$_2$ on fertilisation and early cleavage differed between the measured parameters, with some significant differences, and evidence of delayed cell divisions (Figure 5-8). In agreement with overall decrease in FS reported from IC experiments (Figure 5-4) the numbers of unfertilised eggs increased significantly across the gradient of increasing OA (Figure 5-8). FM and non-raised FM were relatively unchanged, and although the occurrence of polyspermy (Abnormal FS) appeared to decrease, the effect of increasing OA was variable with no significant trend. Of those eggs showing a fertilisation membrane or cell divisions, a delay in development is evident with decreasing proportion of 4 cell stage embryos, with an increasing significantly negative impact mostly occurring at $\geq 465$ µatm increase in pCO$_2$, and a small, non-significant increase in number of 2 cell embryos (Figure 5-8).
Figure 5-8: Average effect size of elevated $pCO_2$ seawaters on fertilisation and development using natural-log response ratio relative to control conditions ($LnRR = \ln[\bar{x} \text{ treatment} + 1]/\ln[\bar{x} \text{ control} + 1]$). Bootstrap means (100,000 times) with 95% confidence intervals (error bars); blue dashed line represents zero effect of $pCO_2$ seawater. Values for Egg were calculated from proportions of total counts; values for other categories excluded unfertilised eggs from proportion calculations. Data points are effect size across a gradient of increasing levels of $pCO_2$ seawater relative to the control (382 µatm $pCO_2$). Egg = no evidence of a fertilisation membrane; FM = normal raised fertilisation membrane; Non-raised FM = incomplete raised fertilisation membrane – irregular or non-raised; Abnormal FS = evidence of polyspermy; 2 cell = normal 2 cell embryo; 4 cell = normal 4 cell embryo. $n = 9$ with averaged technical replication ($n = 3$) for each male:female cross at each treatment level.
Chapter 5. Fertilisation success

5.4 Discussion

Fertilisation in *Evechinus chloroticus* is fairly robust to acute near-future OA, N-FS levels decreasing by ~15% as $pCO_2$ concentrations increase from present day levels to those predicted for the end of this century, and a relatively linear decrease in N-FS from current day to far-future levels of $pCO_2$. At the level of individual male:female crosses, most crosses exhibited a decrease in N-FS with increasing $pCO_2$ with both male and female identity contributing to differences in response. The importance of looking beyond the population to individual level responses has been clearly shown in previous studies of sea urchin fertilisation and early development (Schlegel *et al.*, 2012; Kelly *et al.*, 2013; Foo *et al.*, 2014; Hofmann *et al.*, 2014; Sewell *et al.*, 2014). Here with *E. chloroticus*, a large degree of variability in response to elevated $pCO_2$ can be seen underlying an otherwise fairly tolerant population, with some crosses being more tolerant to elevated $pCO_2$ than others. This individual variation and indications of resilience to acute exposure to elevated $pCO_2$ suggest that the genetic basis for continued successful fertilisation and early development in *E. chloroticus* is already present within the population, and suggesting the possibility for adaptation to future OA.

Population level estimates of FS have previously been used to indicate the broadscale average response to increasing levels of OA and allow generalised patterns and comparisons of species tolerances. The resilience of FS in *E. chloroticus* reflects the generalised suggestions of sea urchins being tolerant to increasing $pCO_2$ and the effects of OA (Byrne, 2011). In previous studies the highest tolerance levels are generally seen in population level studies that used pooled gametes (Appendix 3), such as seen for *Hemicentrotus pulcherrimus* (Kurihara & Shirayama, 2004) and *Echinometra mathaei* (Uthicke *et al.*, 2013b). However, evidence of extreme tolerance in species such as *Paracentrotus lividus* with no change in FS up to $pCO_2$ levels of about 6600 µatm (Martin *et al.*, 2011) contrasts with rapid decline in FS in *Strongylocentrotus nudus* by only ~750 µatm (Sung *et al.*, 2014). The alternative approach of studies using individual male:female crosses generally show an impact to FS response at lower $pCO_2$ concentrations, as seen in *Pseudoboletia indiana* (Foo *et al.*, 2014) and in *Mesocentrotus franciscanus* (Reuter *et al.*, 2011). Consistent with these examples, the individual crosses in *E. chloroticus* also showed that underlying the broadscale population level tolerance to OA, there was a high
level of variation in FS response to increasing $pCO_2$ between pairs. Sensitivity to $pCO_2$ clearly differs among individuals.

The fine scale $pCO_2$ gradient used in this study provided a high resolution picture of how FS responses among individual male:female crosses vary; some individuals maintaining high FS levels across the gradient, and others experiencing tipping points at or just beyond near-future OA levels. However, the averaged results mask the range of sensitivities observed, with the relatively steady decrease in *E. chloroticus* N-FS of 13.5% and 28.2% across OA levels projected for near- and far-future respectively, providing no evidence of a population level tipping point. Biological tipping points have been identified as potentially occurring under global climate change and threaten to limit species survival and impact community structure as tolerance levels are exceeded (Lenton *et al.*, 2008; McNeil & Matear, 2008; Monaco & Helmuth, 2011). At the early life stage, tipping points have been identified at fairly high $pCO_2$ levels for larval survival (Dorey *et al.*, 2013), while rapid declines in FS occur at very different $pCO_2$ levels for different species including 750 µatm in *Strongylocentrotus nudus* (Sung *et al.*, 2014), 1797 µatm in *Strongylocentrobus purpuratus* (Frieder, 2014); or above ~5000 µatm in *Hemicentrotus pulcherrimus* (Kurihara & Shirayama, 2004). The limited evidence of a tipping point in FS for most *E. chloroticus* individual crosses suggests a general tolerance beyond the $pCO_2$ levels of far-future OA; however, FS was also shown to be a variable trait with some crosses reaching the limits of tolerance much closer to near-future OA than others.

As traits can include both phenotypic and genetic components of variation (Gienapp *et al.*, 2008), in this study phenotypic variation was minimised not only through rigorous experimental control of gamete handling and environmental conditions, but also by collecting all adults from the same location to minimise differences in local acclimation and adaptation. By minimising phenotypic differences in gamete and FS response to OA, the resulting variation in cross-pair FS was assumed to be indicative of a level of broad-sense heritability of adaptation potential and ongoing tolerance of FS to increasing OA.

In general, compared to species from areas with relatively stable environmental conditions, those species from coastal margins with a naturally variable pH have been suggested to be pre-adapted and therefore more tolerant to near-future OA (Feely *et al.*, 2008; Wootton *et al.*, 2008; Byrne *et al.*, 2009; Dorey *et al.*, 2013). The overall tolerance of *E. chloroticus* FS is therefore not unexpected for a species that inhabits the
heterogeneous shallow sub-tidal coastal margin over a wide latitudinal distribution (Zaslavskaya et al., 2012). Within this distribution a level of population heterogeneity is evident at different spatial scales, including a significant phylogeographic barrier at Cook Strait, between the North and South Islands of New Zealand (Nagel et al., 2015). Cautious extrapolation of the significant variability in N-FS among all single male:female crosses from all experiments (IC, FV & MV) in this study raises the possibility that the *E. chloroticus* population north of the Cook Strait phylogeographic barrier possess the necessary variation to adapt to near- and far-future OA.

If there is a genetic basis for the variability in gamete and FS traits, it is possible that selective pressure of OA and transgenerational effects will lead to longer term adaptation (Agrawal et al., 1999; Kelly et al., 2013; Pespeni et al., 2013b; Thor & Dupont, 2014). Adaptation to OA can be seen to have occurred in breeding programmes (Parker et al., 2012), *in-vitro* multi-generation studies (Lohbeck et al., 2014; Schaum & Collins, 2014) including modelling (Fitzer et al., 2013), as well as in natural populations (Calosi et al., 2013). However, if the rate of environmental change is too fast for a sustained population level adaptation, the risk of a genetic bottleneck may present an additional complication through the loss of genetic variance (Blows & Hoffmann, 2005).

Local adaptation can contribute to within-species variability in FS response through the exposure of adults to seawaters with different carbonate chemistry (Kelly et al., 2013; Pespeni et al., 2013a). This is also seen in the FS response reported by Moulin et al. (2011) where individuals from the same species and same coastal area had significantly different responses depending on whether the adults were collected from the open coast or tidepools. As tidepools can experience large shifts in pH over the diurnal tidal cycle and the oscillation in day and night photosynthetic and respiratory CO2 loading, the adults from tide pools are regularly subjected to high pH (Truchot & Duhamel-Jouve, 1980; Feely et al., 2008; Melzner et al., 2009). Under OA conditions, higher levels of FS from the urchins inhabiting the tide pools suggest that there is plasticity in the underlying mechanisms for tolerance. Experimentally, the level of tolerance can also have a temporal component with longer duration of adult exposure to OA conditions resulting in increased acclimation and corresponding increase in reproductive success (Dupont et al., 2013; Uthicke et al., 2013b; Suckling et al., 2014; Suckling et al., 2015). Although a species’ FS response to OA conditions can be influenced by the environmental conditions adults are exposed to over a range of different timescales (diurnal through to evolutionary), the
apparent benefits appear to be species specific; also requiring an experimental design with appropriate sensitivity to detect change (Byrne, 2012; Schlegel et al., 2012; Sewell et al., 2014; Vihtakari et al., 2016).

Gamete traits are well understood to influence egg and sperm interactions and therefore play an important role in changes in FS (Levitan et al., 1991; Levitan, 1996; Evans et al., 2007). In general, environmental stress can impact aspects of reproduction through both paternal and maternal effects (Foo et al., 2012; Kelly et al., 2013; Jensen et al., 2014; Guillaume et al., 2016); in the OA context this includes changes in FS that may be underpinned by changes in both sperm and egg effects respectively. For example sperm effects can result in fertilisation curves that require higher sperm concentrations to attain similar levels of FS, or egg effects that result in decreased maximal levels of FS despite higher sperm concentrations (Reuter et al., 2011; Frieder, 2014; Sewell et al., 2014). Additionally, the fertilisation kinetics models describe a positive relationship between both sperm swimming speed and % motile with FS levels (Vogel et al., 1982; Levitan, 2000), traits that are all lower under increased pCO2 (Havenhand et al., 2008). Egg effects can result in elevated incidence of polyspermy and abnormal FS, through OA impacts to polyspermy blocks (Reuter et al., 2011; Sewell et al., 2014); however, an increased occurrence of abnormal-FS was not evident in E. chloroticus, suggesting that the fast-block to polyspermy was not noticeably impaired by OA. The significant role of both males and females in the variability of N-FS in E. chloroticus suggests that overall, both eggs and sperm contributed to differences in response to OA.

Important steps towards successful fertilisation and early development come from maintaining gamete performance under the high CO2 and low pH of OA seawaters. The earliest developmental stages of marine invertebrates are thought to have the narrowest tolerance to environmental stress, with later stages attaining the ability to actively respond through increasing cellular defence mechanisms (Hamdoun & Epel, 2007; Melzner et al., 2009; Tadros & Lipshitz, 2009). Gamete activation involves multiple processes that are dependent on factors such as pHi, O2 and CO2 (reviewed in: Nakazawa et al., 1970; Chia & Bickell, 1983; Epel, 1990). The effects of OA are suggested to disrupt cellular processes including the pH-dependent cell-membrane bound ion balance machinery, protein and enzyme functions, and energetic pathways through direct impacts or indirectly through signal transduction pathways (reviewed in: Bevensee & Boron, 2008; Place & Smith, 2012; Chapter 4). The OA induced declines in FS have been directly associated with
decreased sperm performance and correlated to an impaired ability to maintain optimal physiological homeostasis; as measured through changes in mitochondrial membrane potential (Schlegel et al., 2015) and pH\(_i\) (Chapter 4).

Less well studied are the effects of OA on the sea urchin egg and the first cell divisions; unlike plutei larvae they are unlikely to possess the capability to compensate for OA (Hamdoun & Epel, 2007; Tadros & Lipshitz, 2009; Stumpp et al., 2012). OA has been shown to impact cleavage success (Kurihara, 2008; Moulin et al., 2011; Foo et al., 2012; Place & Smith, 2012) with some of the variation in response of cleavage success linked to female identity (Foo et al., 2016); a result also found to be the case here in *E. chloroticus*. Gamete physiology is likely to underpin aspects of these changes in response, with the first mitotic divisions being dependent on a plethora of cellular processes that are regulated by changes in membrane potential, Ca\(^{2+}\) and pH\(_i\) that occur post fertilisation. For example, pH\(_i\) is involved in the regulation of the architectural reorganisation of the cell, growth of microvilli, rearrangement and syngamy of the maternal and paternal pronuclei, increased protein and DNA synthesis as well as regulating cell-to-cell signalling through gap junctions after first division (Spray et al., 1981; Peracchia, 1987; Schatten & Schatten, 1989; Epel, 1990; Chandler, 1991; Miller & Epel, 1999; Place & Smith, 2012; Ciapa & Philippe, 2013). Any increased energetic cost of processes involved with attaining and maintaining an optimal cellular homeostasis, such as H\(^+\) efflux through the Na\(^+\)/H\(^+\) exchanger, may have negative implications for all other energy consuming processes (Place & Smith, 2012; Sokolova, 2013). Although not all species have been shown to exhibit a delay in developmental progression at these early stages (Yu et al., 2013), the resulting impact on cellular processes or bioenergetics of the activated egg may well contribute to the increasing delay seen in the early cell divisions as pCO\(_2\) levels increase (this study; Kurihara, 2008; Cohen-Rengifo et al., 2013).

In this study on *E. chloroticus*, the fine scale pCO\(_2\) gradient enabled a detailed investigation into the individual response of FS to future predicted levels of OA, and highlighted a high degree of variability between individuals. Clearly some individuals are more sensitive to increasing OA than others. The overall averaged tolerance of FS across a wide range of pCO\(_2\) concentrations, along with indications of a possible genetic basis for adaptation, suggest the possibility for the long term maintenance of the natural population in the North Island of New Zealand as OA increases. Through the FS response, this study contributes to a more informed basis for predictions of long term impacts of increasing
\( p\text{CO}_2 \) on *E. chloroticus* populations, including the potential implications for community and ecosystem resilience (Harvey *et al.*, 2016).
Chapter 6. General discussion

Ocean acidification is considered to be a global scale environmental issue that will have far reaching implications for many levels of biological systems, from cellular physiology to species survival and ecosystem resilience. The projected extent and scale of possible impacts puts OA together with seawater temperature increase as the two major climate change stressors in marine systems. The chemical changes associated with OA are projected to continue to increase with cumulative global emissions during the 21st century and possibly into the far-future (IPCC, 2014). Despite increasing research examining the biological impacts of OA, there remains a high level of uncertainty about some of these impacts including the physiological mechanisms underlying how marine species will respond to this continued environmental change. An environmental challenge that is beyond the limits of a species physiological tolerance can impact on growth, reproduction, species interactions or species survival, and determine whether species might be winners or losers in a high $p$CO$_2$ world. Building on previous research on the impact of OA on fertilisation and early development, this thesis makes a significant contribution to the understanding of sea urchin sperm physiology under OA conditions, and the impacts of OA on the earliest life stages of a broadcast spawner. In particular, this study set out to answer the following questions:

1. Is *E. chloroticus* sperm swimming performance impacted by OA and is sperm narcosis, lower % motility and lower swimming speed, driven by changes in pH$_i$?

2. How will OA impact fertilisation success in the ecologically important *E. chloroticus*?

3. Are there intraspecific differences in fertilisation success that suggest possible adaptation of tolerance to OA?

6.1 Summary and synthesis

The empirical research addressing the three main research questions has been presented in the four data chapters: Chapter 2. Comparative ultrastructure of spermatozoa from two regular and two irregular New Zealand echinoids; Chapter 3. Methods for the
analysis of internal pH (pHi) in sea urchin sperm; Chapter 4. Swimming performance and internal pH of sperm, and; Chapter 5. Fertilisation success. With consideration of the three main thesis questions, this section draws on findings of these empirical chapters to provide an overview of the main findings and the theoretical implications of the study.

6.1.1 Sperm morphology and performance

The comparative ultrastructure of four echinoids from NZ populations (the clypasteroid sand dollar *Fellaster zelandiae*, spatangoid heart urchin *Echinocardium cordatum*, the endemic camarodont *E. chloroticus*, and the recently arrived (Pecorino et al., 2012) diadematoid black urchin *Centrostephanus rodgersii*) confirmed that these species possessed sperm with a morphology similar to other echinoids: with an acrosome-tipped conical-nucleus, and a posteriorly located uni-flagellum powered by a single annulus mitochondrion. Differences in parameters within the general echinoid sperm morphology were observed between the species, with sperm from *E. chloroticus* showing the greatest level of within male variability in sperm morphology. Particular sets of differences grouped the irregular urchins as having shorter and wider sperm heads than the regular urchins; a finding that differs from previous descriptions of irregular species (Summers & Hylander, 1974; Drozdov et al., 2012a).

The calculation of cell volume was a specific goal of Chapter 2 as this information was required for the development of analytical techniques used for detailed sperm cell analysis, including the calculation of an appropriate [K⁺]i in Chapter 3, and the quantitative analysis of pHi in Chapter 4. Cell volume was used in the analysis of the focus species, *E. chloroticus*, as well as during protocol development using *F. zelandiae*, providing key information for the use and calibration of pHi sensitive dyes.

Sperm quality characteristics, such as morphology and physiological tolerance, may also be optimised to specific sets of environmental conditions under the selective pressures of sperm-limitation and sperm-competition (Snook, 2005; Crean & Marshall, 2008). For example, *Fellaster zelandiae* – which inhabits only shallow subtidal soft sediments – shows a much lower variability in sperm morphology traits than *E. chloroticus* – which inhabits a much great range of environments. As sperm performance (e.g. velocity) has previously been correlated with morphological traits, the variability in *E. chloroticus* sperm morphology may also contribute to the variability seen in *E. chloroticus* sperm performance (e.g. swimming speed; Chapter 4) (Fitzpatrick et al., 2010).
Considering multiple sperm characteristics in combination, such as morphology, swimming speed and % motility, has been shown to be integral in determining overall reproductive success (Fitzpatrick et al., 2012); with swimming speed being a key component of variation in FS (Levitan, 1996; Havenhand et al., 2008; Graham et al., 2015).

### 6.1.2 SNARF-1 and internal potassium

The advantages of the physiologically relevant fluorescent dye SNARF-1 combined with the nigericin calibration resulted in a reliable and repeatable methodology ideal for measuring pHi in marine invertebrate sperm. Suitable SNARF-1 incubation parameters for *E. chloroticus* sperm were rigorously determined. In addition, an accurate value for [K+]i was a crucial consideration to avoid K+ induced shifts in fluorescence readings from incorrect formulation of buffers for the nigericin calibration. As an appropriate [K+]i value for *E. chloroticus* sperm could not be determined from the literature, a more appropriate value was determined experimentally.

Cellular [K+]i in *E. chloroticus* sperm is approximately 3-4 times higher than that reported in mammals, and at least double that previously determined in sea urchins (Lee et al., 1983; Guerrero & Darszon, 1989; Darszon et al., 2004). A range of different methods have previously been used in determining the [K+]i of other marine invertebrate cells and tissues; however, only cnidarian tentacle tissues have values close to the 466 µM targeted for use in SNARF-1 calibration buffers for *E. chloroticus* sperm (Herrera et al., 1989). As K+ is acknowledged to play an important and efficient role as an osmotically active cation (Measures, 1975; Bowlus & Somero, 1979; Robertson, 1980; Yancey et al., 1982; Kirschner, 1991; Oren, 2011), this comparatively high level of [K+]i was perhaps not surprising. However, this does raise the importance of using appropriate ion concentrations in any analyses of cells, including sperm, and provides a validated value of [K+]i to be considered in future studies of other marine invertebrate sperm.

### 6.1.3 OA impacts on the earliest life stages of an echinoid

The hypothesis that sea urchin sperm may exhibit a form of narcosis due to the elevated pCO2 and low pH of the surrounding seawater was first proposed by Kurihara (2008). Here this hypothesis was tested in Chapter 4 through the novel development and application of paired analyses of sperm pHi, using a flow cytometer, and sperm motility, using CASA analysis, under OA conditions predicted for the years 2100 and 2300. The
effect of OA seawaters on *E. chloroticus* sperm performance differed between individual males and was not a simple case of pH$_i$ dependent narcosis, and certainly not in a linear fashion. With the seawater pH decrease, pH$_i$ of *E. chloroticus* sperm exhibited a stepwise decrease that corresponded to a decreasing number of active sperm that swam slightly faster (as seen in % motility and VCL). Sperm with the same pH$_i$ appeared to exhibit two alternate states, one group appeared to experience a potential narcosis that lead to a lower % motility, while another group that were able to attain activation swim faster. These results suggest that additional factors may play a larger role in activation and swimming performance of echinoid sperm than are currently addressed in the literature; these might include factors such as oxygen concentration, or mitochondrial performance.

As the endpoint assessment of how gamete performance and interactions are affected by OA, fertilisation success and early cell divisions were assessed across a relatively fine scale pCO$_2$ gradient in Chapter 5. The ten step gradient allowed the high resolution description of *E. chloroticus* FS response. Underlying the steady population decline in FS across present day to far-future OA conditions, individual male:female cross responses varied from no change in FS to a rapid decline with increasing pCO$_2$. The sperm concentration used in this study for *E. chloroticus* was previously determined to minimise polyspermy (Franke *et al.*, 2002). However, this was at ambient pCO$_2$ and therefore the single sperm concentration chosen may have played a role in differences in response among individual *E. chloroticus* male:female crosses.

For broadcast spawning echinoids, a change in seawater chemistry as a result of OA will have a direct effect on gametes and FS through exposure to their surrounding environment. As a fundamental regulator of many cellular processes, the maintenance of pH$_i$ within optimal parameters is essential for normal cellular function (Busa & Nuccitelli, 1984; Bevensee & Boron, 2008). The stepwise decrease of pH$_i$ in *E. chloroticus* sperm evident under both near- and far-future OA is also seen in echinoid eggs (Bögner *et al.*, 2014). In this study, the significantly lower pH$_i$ in sperm occurred concurrently with changes in sperm swimming performance including lower % motility and changes in sperm swimming speed.

The potential sperm narcosis experienced by a proportion of *E. chloroticus* sperm might offer a plausible explanation for some of the decrease in FS; however, the broader applicability of the extent of any narcosis is unclear. For example, some other sea urchin
studies have shown a similar OA induced increase in VCL swimming speed (Caldwell et al., 2011; Graham et al., 2015), but without a negative effect on % motility. Instead Caldwell et al. (2011) reported a higher proportion of active sperm, while Graham et al. (2015) found no change. Other sea urchin studies also indicate a link between FS and sperm swimming speeds; however the speed parameter type is not explicitly stated (Havenhand et al., 2008; Schlegel et al., 2012).

In an ecological context, although less sperm swam faster (VCL), under increasing OA a significant decrease in straight line speed (VSL) was found. As both % motility and straight line speed are correlated with fertilisation success (Moore & Akhondi, 1996; Havenhand et al., 2008; Graham et al., 2015) and are key parameters in fertilisation kinetics models, the decrease in these parameters have both modelled and practical implications for FS (Vogel et al., 1982; Levitan et al., 1991; Lewis et al., 2002; Havenhand et al., 2008); preliminary evidence indicates that this is also the case in *E. chloroticus* (Appendix 4). At the same time, as higher through-the-water swimming speeds are expected to impact sperm longevity through an increased energy requirement, this will lead to more rapid depletion of finite endogenous energy reserves; primarily an issue in situations of sperm limitation (Levitan, 2000; Caldwell et al., 2011; Reuter et al., 2011).

This study provides the first direct experiments on the impact of OA on pH$_i$ of sperm using SNARF-1. The paired analysis using CASA and flow cytometry allowed direct correlations to be drawn between the impacts of OA on changes in pH$_i$ and swimming performance of sperm from individual males. In addition, some preliminary work indicates impaired sperm performance (% motility) correlates with a decrease in FS.

### 6.1.4 Adaptation of FS response to OA

Some of the variability in sea urchin FS can also be attributed to the contribution of a range of male and female effects (Evans & Marshall, 2005; Evans et al., 2007). The relatively few sea urchin studies to date show mixed responses to OA, including males affecting success of early cleavage (Foo et al., 2012; Foo et al., 2014) contrasting with different species where male effects are not evident (Foo et al., 2016). Here we build on these studies to show that in *E. chloroticus* both male and female effects contributed to the difference in variability in FS and early cell divisions.
Chapter 6. General discussion

The variation in FS and early cell divisions among individual *E. chloroticus* crosses showed that urchins exposed to the same set of local environmental conditions in Mathesons Bay produced gametes with very different tolerances to OA – a finding supported in Chapter 4 with the high degree of variability in swimming performance and % motility among individual males. High levels of variability in individual sperm performance has previously been found to be linked to FS (Levitan, 2000) including under OA conditions (Schlegel et al., 2012; Schlegel et al., 2014). Resilience can include both short term acclimation from phenotypic plasticity as well as steps towards longer term adaptation through selection for beneficial traits (Blows & Hoffmann, 2005; Chevin et al., 2010; Peck et al., 2015; Stillman & Paganini, 2015). Whether the variability in tolerance seen in *E. chloroticus* under the acute exposure of gametes to OA can lead to a population level adaptation is still to be determined. If there is a genetic basis for the variability in resilience traits, it is possible that selective pressure of OA and transgenerational effects will lead to longer term adaptation (Agrawal et al., 1999; Kelly et al., 2013; Thor & Dupont, 2014).

6.2 Future directions

This work provides valuable information using novel approaches towards better understanding the impacts of OA on the early life stages of gametes, fertilisation success and the first mitotic divisions in *E. chloroticus*. Important questions have been raised that were out of the original scope of this study, but form a basis for future research directions. Logistical requirements of the simultaneous measurement of pHi and sperm swimming performance along with seawater chemistry involved two teams; the additional requirements for FS assays were not available. However, this section looks beyond these issues to present some key future directions.

6.2.1 Further applications for analysis of pHi

Using *E. chloroticus* sperm, the methodological tools developed in this study provided the first calibrated changes in pHi for marine invertebrate sperm in response to OA conditions. Extending this work in different ways, initially to include other species is essential to develop a better understanding of how OA will impact sperm across a range of organisms. Spatial changes in pHi due to compartmentalisation (Kamp et al., 2003; Ramshesh & Lemasters, 2012) can be described using confocal microscopy or imaging.
flow cytometers (Dubbin et al., 1993; Basiji et al., 2007; Buckman et al., 2009). Finally, a more integrated understanding of the impacts of OA on sperm cell physiology can be achieved through combining SNARF-1 with measurements of other key sperm parameters such as membrane potential using TMRM (Tetramethylrhodamine, methyl ester), or Ca²⁺ using Fluo-4 (Gatti & Christen, 1985; Shapiro et al., 1990; Darszon et al., 1994).

### 6.2.2 Role of oxygen

Historically oxygen has been identified as one of a number of factors essential for activation of sea urchin sperm (reviewed in: Mohri & Yasumasu, 1963). Sperm physiological studies from almost 100 years ago concluded that pH, CO₂ and oxygen all play an essential part in determining the “fertilising power” of sperm (Cohn, 1918). Further studies looking at measures of sperm activation and motility are required to test the suggestion that oxygen tension may play a greater role in sperm activation than currently acknowledged in many OA studies. Currently, from limited studies looking at these two stressors in combination, hypoxia has been shown to, in general, have a higher impact when studied in combination with OA, for example in larval scallops (Gobler et al., 2014) and larval mussels, although the effect was species-specific in the latter (Frieder et al., 2014).

### 6.2.3 Synergistic impacts of more than one stressor

The focus of this study was to establish the effects of OA on FS and sperm performance in isolation. A key aim was to not only allow the impacts on FS to be described in detail, but to understand the role of pH; and sperm narcosis as a possible mechanism underlying changes in FS. Applying the knowledge and methodology developed in this study to include examining the effect of multiple stressors, such as hypoxia or temperature, will provide valuable information towards understanding the impacts of anthropogenic climate change. Protocols can also be further developed to look beyond sea urchins to include sperm from other marine organisms.
7.2 Appendix 2. Motility experiment and CASA parameterisation

The CASA plugin for Image-J and FIJI (available from [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) was parameterised by performing repeated video analyses while adjusting individual or sets of parameters to achieve an arbitrary correlation between motility levels in the original video and that of the analysis output (Appendix 2). Average motility statistics as well as images containing both motile and immotile sperm tracks were used in this assessment. As the helical swimming motion of the sperm resulted in the head repeatedly moving in and out of the field of view, parameterisation focussed on minimising the identification of multiple tracks from a single sperm. The filters 2, 3, and 4 (Appendix 2) were designed to maximise detection of slow moving sperm against any bulk background flow (Wilson-Leedy & Ingermann, 2007). Once set, the input parameters were kept the same for all analyses across males.


<table>
<thead>
<tr>
<th>Input code</th>
<th>Input description</th>
<th>Value</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Minimum sperm size (pixels)</td>
<td>5</td>
<td>Set to ensure background noise was not identified as sperm cells</td>
</tr>
<tr>
<td>b</td>
<td>Maximum sperm size (pixels)</td>
<td>300</td>
<td>Set to ensure that large distorted sperm are captured as a single sperm trail, not divided into separate sperm trails. But to exclude refractive rings.</td>
</tr>
<tr>
<td>c</td>
<td>Minimum track length (frames)</td>
<td>10</td>
<td>Set to minimise inclusion of sperm cells swimming vertically through the field of view, but sufficient to capture drifting immotile sperm</td>
</tr>
<tr>
<td>d</td>
<td>Maximum sperm velocity between frames (pixels)</td>
<td>20</td>
<td>Optimised for realistic, smooth sperm tracks</td>
</tr>
<tr>
<td>e</td>
<td>Minimum VSL for motile (µm.s⁻¹)</td>
<td>6</td>
<td>Below this value sperm are categorised as immotile. This is set to allow for bulk fluid flow and immotile sperm drift</td>
</tr>
<tr>
<td>f</td>
<td>Minimum VAP for motile (µm.s⁻¹)</td>
<td>8</td>
<td>Set to ensure non-directional swimming sperm are included in % motility calculations</td>
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<tr>
<td>g</td>
<td>Minimum VCL for motile (µm.s⁻¹)</td>
<td>15</td>
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</table>
### Appendices

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<thead>
<tr>
<th>Filter 1</th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
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<tbody>
<tr>
<td>h</td>
<td>Low VAP speed ($\mu$m.s$^{-1}$)</td>
<td>10</td>
<td>Less than this value (but above “f”) defines “low” VAP</td>
</tr>
<tr>
<td>i</td>
<td>Maximum % of path with zero VAP</td>
<td>1</td>
<td>Sperm classed as motile if greater than 1% of sperm track is above “f”</td>
</tr>
<tr>
<td>j</td>
<td>Maximum % of path with low VAP</td>
<td>50</td>
<td>Sperm classed as motile if greater than 50% of sperm track is above “h”</td>
</tr>
</tbody>
</table>

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<th>Parameter</th>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>K</td>
<td>Low VAP speed 2 ($\mu$m.s$^{-1}$)</td>
<td>30</td>
<td>Above this set of conditions identifies sperm moving faster than is characteristic of bulk flow regardless of their motion characteristics$^1$</td>
</tr>
<tr>
<td>L</td>
<td>Low VCL speed ($\mu$m.s$^{-1}$)</td>
<td>35</td>
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<th>Parameter</th>
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<th>Description</th>
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<td>M</td>
<td>High WOB (% VAP/VCL)</td>
<td>80</td>
<td>This set of conditions is intended to find sperm that are in motion for most of the time analysed and that do not move in a perfectly straight line; as would be expected of sperm moving due to bulk flow$^1$</td>
</tr>
<tr>
<td>n</td>
<td>High LIN (% VSL/VAP)</td>
<td>80</td>
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</tbody>
</table>

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<th>Filter 4</th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
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</thead>
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<tr>
<td>o</td>
<td>High WOB two (% VAP/VCL)</td>
<td>80</td>
<td>This set of conditions identifies slow moving sperm with a high degree of path curvature, as such curvature is not characteristic of sperm moving due to bulk flow$^1$</td>
</tr>
<tr>
<td>p</td>
<td>High LIN two (% VSL/VAP)</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>q</td>
<td>Frame rate (frames per sec)</td>
<td>34</td>
<td>Frame rate of video acquisition</td>
</tr>
<tr>
<td>r</td>
<td>Microns per 1000 pixels</td>
<td>730</td>
<td>Calibrated in ImageJ using a micrometer to give an average (n = 10) 137.018 (n = 10, SE = 0.23) pixels per 0.1 mm</td>
</tr>
<tr>
<td>s</td>
<td>Print xy coordinates for all tracked sperm?</td>
<td>0</td>
<td>Not used</td>
</tr>
<tr>
<td>t</td>
<td>Print motion characteristics for all sperm?</td>
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<td>Not used</td>
</tr>
<tr>
<td>u</td>
<td>Print median values for motion characteristics?</td>
<td>0</td>
<td>Not used</td>
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</tbody>
</table>
### 7.3 Appendix 3. Summary of select echinoid fertilisation studies

Appendix 3: Summary of select studies examining fertilisation success and early cleavage in sea urchins from Order Camerodonta indicating closest taxonomic level to *Evechinus chloroticus*. Pictorial FS response to OA indicated in “Response” column by ‘=’ representing no change and ‘−’ a negative response. Abbreviations: *CO₂ or HCl column*: C = calculated, T = target; pHT = pH on Total scale, otherwise unknown; FS = fertilisation success. *pCO₂* levels presented as ppm unless stated as µatm. (Modified from: Albright *et al.*, 2012).

<table>
<thead>
<tr>
<th>Closest taxonomic group to <em>E. chloroticus</em></th>
<th>Species</th>
<th>CO₂ or HCl (ppm)</th>
<th>pH</th>
<th>Temp °C</th>
<th>Climate zone</th>
<th>Game Pre-exposure time</th>
<th>Development time</th>
<th>Individual or pooled crosses</th>
<th>Effect on fertilisation and cleavage</th>
<th>Response</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Evechinus chloroticus</em></td>
<td>382, 401, 471, 541, 637, 748, 847, 1044, 1234, 1489, 1728 (C)</td>
<td>7.47, 7.53, 7.6, 7.67, 7.75, 7.80, 7.86, 7.93, 7.98, 8.05 (pHT)</td>
<td>20</td>
<td>Temperate</td>
<td>20 min</td>
<td>2 h</td>
<td>Individually</td>
<td>Slow decrease in FS &amp; cleavage from 74% to 42% at 1728 µatm. Significant variability among individual females and males</td>
<td>== --</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>Family Echinometridae</td>
<td><em>Echinometra mathaei</em></td>
<td>HCl and CO₂ (365–10,360)</td>
<td>6.8–8.1</td>
<td>Tropical</td>
<td>15 min</td>
<td>15 min</td>
<td>Pooled</td>
<td>pH &lt; 7.8, FS starts to decrease. Sig decreased FS by pH 7.1</td>
<td>==</td>
<td>(Kurihara &amp; Shirayama, 2004)</td>
<td></td>
</tr>
<tr>
<td>Family Echinometridae</td>
<td>Echinometra mathaei</td>
<td>487, 785, 1261, 1768 C</td>
<td>7.60, 7.76, 7.94, 8.12</td>
<td>26.2 - 27.7</td>
<td>Tropical</td>
<td>15 min</td>
<td>2 h</td>
<td>Pooled</td>
<td>Cleavage in all treatments was &gt; 80%</td>
<td>===</td>
<td>(Uthicke et al., 2013b)</td>
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<tr>
<td>Family Echinometridae</td>
<td>Heliocidaris erythrogramma</td>
<td>CO₂ (ambt., 1000)</td>
<td>7.7, 8.1</td>
<td>20.5</td>
<td>Temperate</td>
<td>3 h</td>
<td>Individua l</td>
<td>Decreased sperm motility, swimming speed, FS, cleavage, and swimming larvae</td>
<td>===</td>
<td>(Havenhand et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>Family Echinometridae</td>
<td>Heliocidaris erythrogramma</td>
<td>7.6, 7.8, 7.9, 8.2 (pH)</td>
<td>20–26</td>
<td>Temperate</td>
<td>20 min</td>
<td>2 h</td>
<td>Pooled</td>
<td>No effect of CO₂ on FS or development; Temp effect on development, not FS; no Temp * CO₂ interaction</td>
<td>===</td>
<td>(Byrne et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Family Echinometridae</td>
<td>Heliocidaris erythrogramma</td>
<td>CO₂ (330–1828)</td>
<td>7.6–8.25</td>
<td>18–26</td>
<td>Temperate</td>
<td>15 min</td>
<td>Pooled</td>
<td>No effect of Temp or CO₂ on FS at the sperm concentration used</td>
<td>===</td>
<td>(Byrne et al., 2010a)</td>
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<tr>
<td>Family Echinometridae</td>
<td>Heliocidaris tuberculata</td>
<td>CO₂ (330–1828)</td>
<td>7.6–8.25</td>
<td>18–26</td>
<td>Temperate</td>
<td>15 min</td>
<td>Pooled</td>
<td>No effect of Temp or CO₂ on FS at the sperm concentration used</td>
<td>===</td>
<td>(Byrne et al., 2010a)</td>
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</tr>
<tr>
<td>Superfamily Odontophora</td>
<td>Hemicentrotus pulcherrimus</td>
<td>HCl and CO₂ (365–10,360)</td>
<td>6.8–8.0</td>
<td>Temperate</td>
<td>15 min</td>
<td>15 min (FS), 105 or 210 min (cleavage)</td>
<td>Pooled</td>
<td>pH &lt; 7.8, FS starts to decrease Sig decreased FS by pH 6.8 Decreased cleavage</td>
<td>===</td>
<td>(Kurihara &amp; Shirayama, 2004)</td>
<td></td>
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<tr>
<td>Superfamily Odontophora</td>
<td>Hemicentrotus pulcherrimus</td>
<td>380–1500 (T)</td>
<td>7.59–7.99</td>
<td>20</td>
<td>Temperate</td>
<td>20 min</td>
<td>20 min</td>
<td>Pooled</td>
<td>Pretreated sperm = stable until decline to ~50% by 1000 µatm</td>
<td>===</td>
<td>(Sung et al., 2014)</td>
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<tr>
<td>Superfamily Odontophora</td>
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<td><strong>Pseudoboletia indiana</strong></td>
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<tr>
<td>Temperature: 348, 617, 924 C (22°C) 319, 706, 1071 C (25°C)</td>
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<tr>
<td>pH: 7.71, 7.88, 8.08 (22°C) 7.66, 7.82, 8.11 (25°C)</td>
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<tr>
<td>Decreased FS with increasing CO₂ at 22°C. 25°C increased FS levels in several crosses. High degree of intraspecific variation</td>
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<tr>
<td><strong>Strongylocentrotus droebachiensis</strong></td>
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<tr>
<td>Location: 180, 380, 980, 1400, 3000 (T)</td>
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<td>pH: 7.6, 7.8, 8.0</td>
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<tr>
<td>Pooled</td>
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<tr>
<td>Decrease in FS &amp; cleavage at higher CO₂, no difference at 180 µatm Egg pH decreases &gt; 980 µatm</td>
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<td><strong>Mesocentrotus franciscanus</strong></td>
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<tr>
<td>Time: 10 Polar</td>
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<tr>
<td>High levels of FS unchanged, but requiring higher sperm concentration at increased pCO₂; general increase in time to polyspermy block at 1580 ppm</td>
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<td>pH: 8.00-7.26</td>
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<td>Time: 15 Temperate</td>
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<td>Sperm:eggs ratio specific decreases Linear decrease between pH 8.0 and 7.36</td>
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<td><strong>Strongylocentrotus nudus</strong></td>
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<td>pH: 7.96-7.20 8.01-7.55</td>
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<td>Time: 20 Temperate</td>
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<td>Pretreated eggs = high FS to &gt; 3000 µatm Pretreated sperm = steep decline to 5% by 750 µatm</td>
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<tr>
<td>Odontophora</td>
<td>Camarodonta</td>
<td>Strongylocentrotus</td>
<td>purpuratus</td>
<td>501-2801°C</td>
<td>15 Temperate</td>
<td>10 min</td>
<td>20 min</td>
<td>Pooled</td>
<td>Slight decline in FS between 7.6 and 7.44, tipping point at 7.44</td>
<td>(Frieder, 2014)</td>
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<tr>
<td>Odontophora</td>
<td>Camarodonta</td>
<td>Tripneustes</td>
<td>gratilla</td>
<td>CO₂ (330–1828)</td>
<td>18–26 Temperate</td>
<td>15 min</td>
<td>Pooled</td>
<td>No effect of Temp or CO₂ on FS at the sperm concentration used</td>
<td>(Byrne et al., 2010a)</td>
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<tr>
<td>Camarodonta</td>
<td>Camarodonta</td>
<td>Paracentrotus</td>
<td>lividus</td>
<td>400, 700, 1100, 1900, 3600, 6600°C</td>
<td>20 Temperate</td>
<td>not stated</td>
<td>15 min</td>
<td>Pooled female, single male</td>
<td>No effect on FS</td>
<td>(Martin et al., 2011)</td>
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<tr>
<td>Camarodonta</td>
<td>Camarodonta</td>
<td>Paracentrotus</td>
<td>lividus</td>
<td>310-8335°C</td>
<td>14 Temperate</td>
<td>15 min</td>
<td>1 h</td>
<td>Pooled</td>
<td>FS differed between tidepools. Evidence of transgenerational tolerance from adults</td>
<td>(Moulin et al., 2011)</td>
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<tr>
<td>Camarodonta</td>
<td>Camarodonta</td>
<td>Sterechinus</td>
<td>neumayeri</td>
<td>392, 928, 1405°C</td>
<td>0 polar</td>
<td>not stated</td>
<td>not stated</td>
<td>Pooled</td>
<td>No difference in FS despite acclimation over 6 and 17 months. Higher FS in elevated temp treatment</td>
<td>(Suckling et al., 2014)</td>
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<tr>
<td>Camarodonta</td>
<td>Camarodonta</td>
<td>Sterechinus</td>
<td>neumayeri</td>
<td>384, 473, 666°C</td>
<td>-0.3, -0.5</td>
<td>0 polar</td>
<td>20 min</td>
<td>18-24 h</td>
<td>Individual</td>
<td>Variable response among pairs to increased pCO₂: general increase in time to polyspermy block; decrease in FS</td>
<td>(Sewell et al., 2014)</td>
</tr>
<tr>
<td>Order</td>
<td>Species</td>
<td>pH</td>
<td>Temperature</td>
<td>Hours</td>
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<td>Treatment Notes</td>
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| Camarodonta  | Sterechinus neumayeri    | 7.5, 7.7, 8.0 | 0-3 polar   | 15 min | 4 h | Pooled
No effect of CO$_2$ at ambient temperature, significant decrease in FS at elevated temperature; non-significant decrease in cleavage at same temperatures | (Ericson et al., 2011)   |
| Camarodonta  | Sterechinus neumayeri    | 433, 927, 1417 T | 7.6, 7.8, 8.0 | 1, 3, 5 polar | 15 min | 20 h | Pooled
No effect of pH on FS - even at low sperm:egg ratio. Temp and sperm:egg ratio effect FS. | (Ho et al., 2013)         |
| Camarodonta  | Sterechinus neumayeri    | 410, 510, 730 C | -1.5 polar  | not stated | not stated | Pooled
(20 female, 1 male)
No developmental delay, no difference in 2 cell stage between treatments | (Yu et al., 2011)         |
| Camarodonta  | Sterechinus neumayeri    | 442, 1010, 1444 C (-1°C)
528, 1065, 1533 C (2°C) | 7.50, 7.66, 7.97 (-1°C)
7.49, 7.65, 7.93 (2°C) (pH) | -1, 2 polar | na | 24 h | Individually
Single fertilisation event in ambient SW, split to pH and temp treatments. Lower cleavage with synergistic effect of increasing pCO$_2$ and temp, with differences among individual pairs | (Foo et al., 2016)        |
7.4 Appendix 4. Relationship between sperm % motility and fertilisation success

As the $pCO_2$ of OA seawaters increased (354, 846 and 1623 µatm $pCO_2$; levels reported in Chapter 4) both average sperm % motility ($n = 13$; data from Chapter 4) and N-FS ($n = 9$; using different males) decreased; with a highly correlated linear relationship between the two ($r^2 = 0.984$) (Appendix 4).

Appendix 4: Relationship between sperm % motility and normal-fertilisation success (N-FS, data from Chapter 5) in *Evechinus chloroticus* (% motility data from Chapter 4).
List of references


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