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Santa Barbara

Transgenerational effects of environmental heterogeneity on marine invertebrate larvae

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution, and Marine Biology

by

Logan C. Kozal

Committee in charge:

Professor Gretchen Hofmann, Chair

Professor Erika Eliason

Professor Halley Froehlich

December 2022

The dissertation of Logan C. Kozal is approved.

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December 2022

Transgenerational effects of environmental heterogeneity on marine invertebrate larvae

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by

Logan C. Kozal

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Kozal, LC, Wong, JM, Leach, TS, & Hofmann, GE (2017). *Transgenerational Effects of Differential pCO_2 on Purple Sea Urchins (*S. purpuratus*).* Poster. International Larval Biology Symposium, Honolulu, HI.

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2019 **Scientific Diver for an NSF RAPID Grant** (PIs: Dr. Marie Strader, Auburn University & Dr. Gretchen Hofmann, UCSB) studying coral bleaching of *Acropora hyacinthus* and recovery in Moorea French Polynesia

2018-present **Cawthron Institute** in Nelson, NZ studying the effects of marine heatwaves on the greenshell mussel, *Perna canaliculus*

2017-present **Santa Barbara Coastal Long Term Ecological Research Program** studying long term pH, dissolved oxygen, and temperature dynamics in the Santa Barbara Channel and physiological effects on purple urchins, *Strongylocentrotus purpuratus*

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ABSTRACT

Transgenerational effects of environmental heterogeneity on marine invertebrate larvae

by

Logan C. Kozal

Marine species are under mounting threat from stressors associated with climate change, such as ocean acidification and warming. Extreme events such as marine heatwaves (MHWs) are also forecasted to increase in frequency, intensity, and duration. The environment varies greatly with regard to the factors associated with these climatic stressors. Parameters of the physical environment are heterogeneous spatially across gradients associated with marine macrophytes such as kelp forests, and variable across time associated with regular cycles such as seasons, and events such as upwelling or MHWs. Therefore, in this dissertation, I investigated whether this physicochemical variability associated with kelp forest ecosystems or MHWs can drive transgenerational effects in marine invertebrate larvae exposed to stressors associated with climate change (temperature and pH). Early life history stages are believed to be particularly vulnerable to the stressors associated with global change, therefore I focused my investigations on the early larval stages of two marine invertebrate species of ecological and commercial importance: the purple urchin, *Strongylocentrotus purpuratus* and the green-lipped mussel, *Perna canaliculus*.

First, I monitored the conditions inside and outside a kelp forest environment in the Santa Barbara Channel (SBC) and investigated the maternal effects of conditioning *S. purpuratus* inside and outside the kelp forest for 6 months spanning gametogenesis. pH and

temperature were slightly elevated and more predictably variable inside the kelp forest as compared to outside. After acclimation, the urchins were spawned and their larvae were raised under high and low pCO_2 conditions in lab to assess their physiological response to the maternal and developmental environments. Larvae from outside conditioned mothers were more resilient to an additional stressor in the form of an acute thermal tolerance trial, and tended to have more skeletal development than those from inside conditioned mothers. Two experiments were also conducted to explore the paternal effects of simulated MHWs, on *P. canaliculus* aquaculture in the Marlborough Sound region of New Zealand, which has suffered devastating MHW events in recent years. In the first experiment, I exposed male *P. canaliculus* to an acute 1-week exposure to MHW or ambient temperatures and then raised their larvae under warm of ambient temperatures to the veliger stage. Paternal and developmental heat exposure had a negative impact on successful development of the larvae, but veliger larvae from heat exposed fathers had the highest thermal tolerance in a lethal tolerance assay. In the final experiment, I conditioned *P. canaliculus* males from wildtype lineages and lineages selectively bred for heat tolerance to a chronic 1-month exposure to a slightly lower MHW temperature in order to investigate the interaction between selective breeding and paternal effects. Elevated paternal and larval temperatures had negative impacts on successful larval development and size for larvae from both selectively bred and wildtype lineages. Overall, the results of this dissertation indicate that heterogeneity in abiotic factors can have transgenerational effects through the maternal and paternal line with consequences for how key marine invertebrates respond to stressors associated with global change. It will be critical to consider the impact of natural gradients on plasticity and how transgenerational effects can be used to inform aquaculture practices to mitigate the effects of MHWs.

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Introduction

The objective

The core objective of my thesis is to investigate how environmental heterogeneity influences organismal performance and plasticity across generations in the context of global change. This heterogeneity may exist across space as in the case of physicochemical gradients created by marine macrophyte habitats such as kelp forests (Nielsen et al. 2018). It may also exist across time in the case of events such as episodic upwelling or marine heatwaves (MHWs) (Oliver et al. 2021a) and the regular oscillations of diurnal and seasonal cycles (Delille et al. 2009). My investigations span two study systems, kelp forests in coastal California, and mussel farms in New Zealand. Each system is characterized by its own set of environmental factors which define the acclimation environment for the species of interest and the impetus for transgenerational effects including kelp associated gradients in water properties and temperatures correlated with MHW events of different phenologies. These factors and the target species on which their transgenerational impacts are being investigated, the purple sea urchin (*Strongylocentrotus purpuratus*) and the green-lipped mussel (*Perna canaliculus*), are introduced below. Early life stages are believed to be highly vulnerable periods during the life history of most marine organisms and may be of particular concern with respect to environmental stressors (Kurihara 2008, Gibson et al. 2011). Therefore, in this dissertation the transgenerational effects of the environmental stressors of interest were investigated by assessing the phenotype of the early larval stages of the focal species.

The global context

Anthropogenic emissions of greenhouse gases, such as carbon dioxide (CO_2) are driving climactic changes in our oceans such as sea-level rise, warming, and acidification (OA) (Pörtner et al. 2022). Trends associated with human induced climate change are coupled with a rise in extreme events including storms and heatwaves (Coumou & Rahmstorf 2012). Pairings between gradual changes in environmental stressors and "pulses" of extreme events are having devastating consequences for terrestrial and marine ecosystems (Scheffer et al. 2001, Harris et al. 2018). Extreme events can push marine ecosystems over thresholds leading to shifts in community assemblage and whole state shifts (Sanz-Lázaro 2016, Wernberg et al. 2016, Michaud et al. 2022). In addition to driving more extreme climactic events such as heatwaves, global climate change is also shifting patterns in episodic processes such as upwelling (Diffenbaugh et al. 2004, Feely et al. 2008). When events impact foundation species they can alter not only the community composition of an ecosystem (Hoegh-Guldberg et al. 2007, Rogers-Bennett & Catton 2019, Seuront et al. 2019, Raymond et al. 2022) but even the abiotic parameters of the habitat itself as the organisms which form a foundation can also drive the local conditions through their biological processes (Helmuth et al. 2002, Hofmann et al. 2011, Kleypas et al. 2011, Frieder et al. 2012).

Environmental heterogeneity in the context of rapid global climate change and ocean acidification (OA) could have important consequences for species persistence and adaptation (Schindler et al. 2015, Morelli et al. 2016, Webster et al. 2017). As average pCO_2 concentrations and global temperatures rise, stochastic variability in pH and temperature is also influenced by climate change. Not only do marine sea surface temperatures (SST) continue to climb, but marine heatwaves (MHWs) are increasing in frequency and duration

(Frölicher et al. 2018, Oliver et al. 2021a, Barkhordarian et al. 2022, Jacox et al. 2022). In this dissertation I sought to investigate the impacts of variation in environmental stressors including temperature, dissolved oxygen (DO), and pH associated with MHWs and kelp forest habitat on transgenerational plasticity in two important species: the purple sea urchin (*Strongylocentrotus purpuratus*) and the green-lipped mussel (*Perna canaliculus*).

Transgenerational plasticity

Parental experience, especially during gametogenesis can influence offspring performance when exposed to the same suite of environmental conditions. Transgenerational plasticity (TGP), where parental environment modifies the phenotype of the offspring without affecting the genotype, can prime offspring for future stressful conditions, and could therefore be particularly important in conferring tolerance to environmental stressors in a global change context (Levis & Pfennig 2016, Donelson et al. 2018).

Transgenerational acclimation can be derived from maternal effects such as maternal RNAs, nutrient, hormone, protein and lipid provisioning to eggs or paternal effects mediated through sperm (Munday 2014). As males do not contribute energetic provisions to the offspring the primary area of investigation for paternal effects is epigenetic. Males and females could potentially pass down epigenetic markers such as DNA methylation, histone modifications, or non-coding RNA to their offspring driving phenotypic plasticity through heritable changes in gene expression (Gavery & Roberts 2010, Munday 2014, Hofmann 2017, Eirin-Lopez & Putnam 2019). Mechanistically, epigenetic modifications which can influence the phenotype without changing the genotype, have been flagged as a potentially important means for rapid adaptation to changing environmental conditions (Ho & Burggren

2010, Hofmann 2017, Eirin-Lopez & Putnam 2019). With regard to environmental stress, DNA methylation is a mechanism of epigenetic change that can drive phenotypic plasticity through heritable changes in gene expression (Gavery & Roberts 2010, Hofmann 2017). In addition to altering transcription, epigenetic changes can influence rates of DNA mutation, leading to potential evolutionary consequences (Holliday & Grigg 1993, Dixon et al. 2016).

Maternal effects are widely accepted to play an important role in offspring phenotype and performance under different environmental conditions (Mousseau & Fox 1998). However, whether or not a maternal effect appears adaptive can vary widely depending on the alignment of the maternal and offspring environment, and the balance between maternal and offspring fitness (J. Marshall & Uller 2007, Reed et al. 2010, Bonduriansky et al. 2012, Shama & Wegner 2014). Along the male line, transgenerational effects, in species without paternal care, are thought to be primarily epigenetic. Although they are less studied than maternal effects, research has shown that paternal effects, mediated through sperm, can significantly influence offspring performance both positively and negatively, and can sometimes have a larger impact than maternal effects (Crean et al. 2013, Jensen et al. 2014, Lane et al. 2015, Marshall 2015, Guillaume et al. 2016, Gasparini et al. 2018). In studies of transgenerational effects of pH and temperature different effects have been observed through the two parental lines across different phyla (Shama & Wegner 2014, Lane et al. 2015, Jonsson & Jonsson 2016, Venkataraman et al. 2019). Lastly, transgenerational effects through the two parental lines can interact, and paternal effects may be mediated by maternal effects such as resource allocation (Crean & Bonduriansky 2014, Lane et al. 2015)

Study systems

Kelp forests and purple urchins in the Santa Barbara Channel

The first chapter of this dissertation investigates whether the different physicochemical environments inside and outside a kelp forest in the Santa Barbara Channel (SBC) are associated with maternally driven transgenerational effects in larvae of the purple sea urchin, *Strongylocentrotus purpuratus* under different pCO_2 conditions. As a dominant herbivore, *S. purpuratus* has a major influence on the function of coastal kelp forest ecosystems in the California Current System (CCS) and beyond (Dayton et al. 1992, Yorke et al. 2019). As a calcifying species, urchins are vulnerable to future pH stress associated with ocean acidification (OA) and extreme low pH events, which can be further exacerbated by additional stressors such as temperature (Padilla-Gamiño et al. 2013).

With respect to OA, pH regimes can vary greatly over short distances and can fluctuate rapidly. Some of the most variable pH environments are found in temperate waters, eastern boundary current upwelling systems, and nearshore productive habitats such as macrophyte habitats and estuaries (Hofmann et al. 2011). In the CCS, episodic upwelling brings cold water, low in pH and dissolved oxygen (DO) but rich in nutrients, up from depth. The frequency, duration, and extent of upwelling is predicted to increase with climate change, extending the footprint of undersaturated waters with respect to aragonite (Diffenbaugh et al. 2004, Feely et al. 2008). In the SBC, in the southern CCS, pH and oxygen levels vary on diurnal and seasonal scales, as well as on "event" scales (Frieder et al. 2012, Kapsenberg & Hofmann 2016). The lowest pH events can be paired with warm temperatures driven by overnight respiration in the summer, or cold temperatures during times of strong upwelling (Kapsenberg & Hofmann 2016). The interaction between physical drivers such as

upwelling, and biological drivers, such as primary production, makes it critical to consider site specific differences in dynamic ecosystems such as the CCS.

Kelp forests can impact flow and modify local water chemistry through photosynthesis (Gaylord et al. 2007, Krause-Jensen et al. 2016) and have been shown to elevate mean pH and DO levels while also increasing diurnal variability due to photosynthesis and respiration (Delille et al. 2009, Frieder et al. 2012, Kapsenberg & Hofmann 2016, Hirsh et al. 2020). This process has piqued interest in whether macrophytes provide potential refuges from OA (Morelli et al. 2016, Nielsen et al. 2018, Woodson et al. 2019), and further, how differences in the magnitude and variability of pH and oxygen influence organismal performance (Mcleod et al. 2011, Morelli et al. 2016, Wahl et al. 2018). Since the change in mean pH around macrophyte habitats generally comes with a corresponding increase in variability, it has mixed consequences for processes such as calcification which can vary between taxa and ontogenetic timing with consequences for community assemblage (Cornwall et al. 2013, Cornwall et al. 2018, Kapsenberg et al. 2018, Wahl et al. 2018). The spatial heterogeneity and temporal variability of these habitats with respect to pH, oxygen and temperature creates a mosaic where conditions can range in hospitability across short distances (Krause-Jensen et al. 2015, Starko et al. 2022).

Given this existing abundance of small scale physicochemical variability, plasticity may play a key role for species persistence in a global change context. Phenotypic plasticity is critical to survival in variable environments. It may be sustained in a population by selection and could itself be the target of natural selection (Via 1993). It is necessary to better describe the abiotic conditions that might promote TGP and align investigations with ecologically relevant stressors and the duration of their exposure (Burgess & Marshall 2014,

Bautista & Crespel 2021). In variable environments, characterizing both the magnitude and predictability of variability will be critical for considering its implications for plasticity, the conditions which drive its evolution, and the resulting consequences for population vulnerability (Burgess & Marshall 2014, Fox et al. 2019, Bitter et al. 2021).

Environmental variability may align with particular life history stages. In the SBC, the period of strongest upwelling, and thus variation in pH, coincides with when larvae of *Strongylocentrotus purpuratus*, the purple sea urchin, are typically in the water column (Strathmann 2017). In addition, *S. purpuratus* begins gametogenesis in the summer going into the fall, the time period of highest temperatures in the SBC and when MHW events are most common worldwide (Sen Gupta et al. 2020). In addition to the variability in pH and DO associated with kelp forests, purple urchins and the kelp that supports them are experiencing increased thermal stress from MHWs which may impact biotic interactions and recruitment patterns (Muth et al. 2019, Okamoto et al. 2020, Starko et al. 2022). This system was selected due to the ecological importance of *S. purpuratus* in shaping kelp forest ecosystems and the alignment with key periods of their reproductive life cycle with key environmental stressors.

Marine heatwaves and mussel farms in the Marlborough Sounds, New Zealand

The second and third chapters of this dissertation investigate whether paternal effects of exposure to marine heatwave (MHW) conditions can increase thermal tolerance of the larvae of *Perna canaliculus*, the green-lipped mussel. The green-lipped mussel is a major aquaculture species of incredible cultural and economic significance to the country of New Zealand and marine heatwaves are an increasing threat to the species and the aquaculture industry as a whole.

MHWs are increasing in frequency, intensity and duration with global climate change (Frölicher et al. 2018, Oliver et al. 2018, Oliver et al. 2021b). MHW events have doubled since 1982 and predictions indicate that MHWs may become annual events or permanent year round in some regions by the end of the century (IPCC 2021). Under "business as usual" emission scenarios (RCP 8.5), MHW events are predicted to increase by a factor of 50 in frequency and a factor of 10 in intensity relative to preindustrial levels by the year 2100 (IPCC 2019, IPCC 2021). These accelerating MHW events have devastating consequences for biodiversity and the corresponding essential ecosystem services such as fisheries (Caputi et al. 2016, Smale et al. 2019, Cheung & Frölicher 2020, Smith et al. 2021, Smith et al. 2023). As the global population continues to grow under these conditions, aquaculture is surpassing wild catch as a method of providing protein (Gentry et al. 2017, Martin 2017). However, like wild stocks, farmed species are susceptible to many of the same anthropogenic stressors that plague wild fisheries (Maulu et al. 2021).

New Zealand's aquaculture industry has been rapidly expanding to meet growing seafood demand sustainably (Alfaro et al. 2014, Stenton-Dozey et al. 2021). However the threat of environmental stressors such as MHWs necessitates innovation in order to meet this soaring demand. In recent years, New Zealand has needed to expand marine shellfish aquaculture into progressively more exposed waters to avoid stressors such as acidification and warming that are often most exacerbated closest to the coast (Heasman et al. 2020). Since 1981 the Tasman Sea Region has warmed at a linear rate of 0.2-0.3°C per decade (Sutton & Bowen 2019). In summer 2017-2018, Sea Surface Temperature (SST) anomalies in the Tasman Sea reached +2.5 to +3.7°C in the hottest eastern region, marking a more widespread and intense, though shorter, event than the MHW just two years earlier in 2015-

2016 which lasted for nearly a year (Perkins-Kirkpatrick et al. 2019, Salinger et al. 2019, Oliver et al. 2021b). In addition, current modelling efforts indicate that marine heatwaves will become more frequent and could even become permanent state by the end of the century (Behrens et al. 2022).

Over the past decade summer MHWs off the coast of New Zealand have been associated with large scale die-offs of *Perna canaliculus*, (Gmelin 1791), the green-lipped mussel (Thomsen et al. 2019, Li et al. 2020). The New Zealand green-lipped mussel (commercially 'Greenshell™') has immense cultural and economic significance as an endemic aquaculture species that makes up the majority of New Zealand's aquaculture harvest, accounting for 71% of the annual aquaculture exports and over $381 million NZD in revenue (Stenton-Dozey et al. 2021). Given the importance of this species, in this study we sought to explore the effects of MHW temperatures on *P. canaliculus* broodstock through the performance of their larvae under different developmental temperatures.

The threat of climate change and extreme heat events to *P. canaliculus* and many other valuable food species across the globe urges the need for research investigating the capacity of these organisms to cope with elevated temperatures and adapt to future change. Recent advances in MHW forecasting open up the possibility for managers and industries to intervene more proactively to protect critical ecosystem services (Caputi et al. 2016, Holbrook et al. 2020, Jacox et al. 2022). Aquaculture provides a unique opportunity to manipulate the biological or environmental features of a system that may confer greater resilience (Gavery & Roberts 2017). For instance, thermal priming can reduce susceptibility heat stress in macroalgae, cultivated in aquaculture (Jueterbock et al. 2021).

One tractable mechanism to explore adaptive capacity in aquaculture species is that of parental effects. Paternal transgenerational effects mediated by epigenetics could be particularly important in aquaculture where it is possible to control the parental experiences of the two sexes separately, breeding broodstock from different source sites, or condition sexes separately in a hatchery. However, paternal effects are largely understudied as compared with maternal effects. This raises the question whether, in aquaculture, it might be possible to induce a positive transgenerational effect solely by manipulating the paternal environment without metabolically taxing the maternal line and corresponding provisions to the eggs. This system was selected due to the importance of green-lipped mussels as an aquaculture species and the mounting threat that marine heatwaves pose to mussel farms. Through the investigations described in chapters 2 and 3 of this dissertation I sought to determine whether exposure to high temperatures could be leveraged to increase larvae thermal tolerance to through paternal effects in a hatchery setting or whether paternal exposure would only exacerbate negative outcomes of MHWs for this *P. canaliculus*.

I. Kelp associated variability in seawater chemistry connects to transgenerational effects in the purple urchin, *Strongylocentrotus purpuratus*

Abstract

Giant kelp (*Macrocystis pyrifera*) provides the foundation for immense biodiversity on California's coast. Kelp forests can change the retention time of seawater, altering water chemistry including pH and dissolved oxygen (DO), as well as the magnitude and predictability of variability in the same properties. Environmental heterogeneity across space and time could drive organismal performance and processes such as transgenerational plasticity (TGP), where parental experience modifies the offspring phenotype, potentially conferring tolerance to future environmental stress. We monitored environmental variability by deploying temperature, pH and DO sensors inside and outside a temperate kelp forest in the Santa Barbara Channel (SBC) throughout the gametogenesis period of a key herbivore, the purple urchin, *Strongylocentrotus purpuratus*. Over the 6-month period, pH and temperature were slightly elevated inside the kelp forest accompanied by more predictable, low frequency variability relative to outside. Adult *S. purpuratus* were conditioned inside and outside the kelp spanning gametogenesis. The urchins were spawned and their larvae were raised under high (1053 μatm) and low pCO_2 (435 μatm) at 15°C in lab to assess their physiological response to the maternal and developmental environments. Larvae raised under high pCO_2 were more susceptible to lethal thermal stress; however, within each larval treatment, progeny from outside mothers had higher thermal tolerance. Our results indicate that heterogeneity in abiotic factors associated with kelp can have transgenerational effects in

the field and interactions between multiple factors, including temperature and pH, will impact purple urchins as local variability associated with marine heatwaves and upwelling evolve with climate change.

1. Background & Overview

A recent study by Hoshijima and Hofmann, found that DO and pH variability was more predictable inside the kelp forest environment compared to outside (Hoshijima & Hofmann 2019). In this study, DO and pH levels were slightly higher outside of the kelp forest during the Spring upwelling season in the SBC. Adult urchins conditioned inside and outside of the kelp forest during gametogenesis revealed an increase in protein content of eggs produced by inside mothers, indicating elevated maternal provisioning. Previous lab-based studies have shown that conditioning adult purple urchins to different pH and temperature regimes during gametogenesis has transgenerational effects on size, lipid content, gene expression, and DNA methylation of their larvae raised under different pCO_2 conditions (Wong et al. 2018, Strader et al. 2019, Wong et al. 2019). However, aside from (Hoshijima & Hofmann 2019), little work has been done to look at this phenomenon in the field.

While most TGP studies are conducted in the lab, temporal variation in environmental conditions around reproduction has been shown to have transgenerational consequences in situ in Atlantic silverside, *Menidia menidia*, and European squid, *Loligo vulgaris* (Murray et al. 2014, Rosa et al. 2014) along with spatial temperature variation across the intertidal in California mussels, *Mytilus californianus* (Waite & Sorte 2022). In the field, high frequency fluctuations, regular cycles, and event scale shifts associated with

upwelling or MHWs all interact to define the acclimation environment. This temporal mosaic is further complicated across space as fluctuations are modified as water moves across habitat types and depths. It is difficult for lab studies to mimic these many axes of variation and their correlations accurately; therefore, it is critical to supplement lab studies of TGP with field studies in dynamic areas such as coastal kelp forests. Studies have explored ecological changes in recruitment dynamics and community assemblage across a kelp gradient (Duggins et al. 1990, Schroeter et al. 1996, Carrasco et al. 2017), but few have examined the physiological consequences of the abiotic gradient, as we do here mechanistically via TGP.

In this study, we investigate TGP driven by field acclimation using the natural heterogeneity within and outside a kelp forest. To accomplish this, sensors were deployed to monitor oxygen, temperature, and pH both inside and outside a giant kelp (*Macrocystis pyrifera*) forest in the SBC. Adult *S. purpuratus* were simultaneously acclimatized on the kelp forest benthos in close association with the sensor arrays. Caged adult sea urchins were conditioned in the field for 6 months during their gametogenesis period, then spawned in the laboratory in order to assess the physiological response of their larvae to pCO_2 stress (Figure 1). This field to lab experiment allowed us to examine whether and how maternal environmental experience inside or outside the kelp forest influences the phenotype and performance of their offspring when exposed to low pH/high pCO_2. Adult acclimation could result in a range of larval responses to pCO_2 stress as both positive and negative transgenerational effects have been observed in the literature and the suite of environmental factors inside and outside the kelp forest are complex (Figure 2).

2. Materials & Methods

2.1 Site instrumentation and adult conditioning

In early June 2018, a SeaFET™ pH and miniDOT™ DO sensor were deployed on a benthic mooring within the kelp forest (henceforth, "inside") at Arroyo Quemado reef, a Santa Barbara Coastal Long Term Ecological Research (SBC LTER) site (Rivest et al. 2016). The SeaFET™ recorded pH and temperature every 30 minutes and the miniDOT™ recorded DO and temperature every 10 minutes. A complementary pair of sensors were deployed at the base of the long term LTER mooring "outside" the kelp forest with the same sampling intervals (Figure 1). The sensors were replaced halfway through the experiment. There was a depth difference between the two moorings: inside ~9 m and outside ~14 m. The benthos inside was rocky reef while outside was sandy substrate. To calibrate the pH sensors, water samples were taken on scuba using Niskin Go-FLO sampling bottles (General Oceanics) and immediately poisoned on the boat using $HgCl_2$ at a final concentration of 0.02%. The pH of these water samples was measured in the lab using a UV spectrophotometer (Shimadzu UV-1800) and *m*-cresol purple dye and Total Alkalinity was measured by titration (Mettler-Toledo T50) following the procedures outlined by Dickson et al. (Dickson et al. 2007). Point calibrations were aligned with the time and temperature when a sample was taken in the field and pH was adjusted to the *in situ* levels using CO2Calc (Robbins et al. 2010). Calibration coefficients for the raw voltage data from the SeaFET™ were calculated using the seaCarb package in R (Gattuso et al. 2021).

Four plastic cages, reinforced with plastic garden fencing, were deployed at both locations close to the sensor arrays. Purple urchins, *Strongylocentrotus purpuratus*, were collected from the Arroyo Quemado kelp forest (California Department of Fish and Wildlife

Scientific Collecting permit #SC-9228) and randomly assigned to each of the 8 cages, ~12 in each cage (Figure 1). The urchins were held in these cages from early June through early December during their gametogenesis period and fed an excess of kelp every 2 weeks to control for the effects of food availability at each site. Every two weeks divers opened the cages, checked the health and number of urchins, removed any dead urchins and remaining kelp, and filled the cage with fresh kelp. A few urchins died in the first ~2 weeks presumably from the stress of collection. After this there was no mortality at either site.

2.2 Time series analysis

Temperature observations from the miniDOT™ oxygen sensors were used in all analysis. Power spectral density (PSD) estimates were conducted following the methods of (Hoshijima & Hofmann 2019). Time series analysis for DO and temperature was conducted on observations from June 10, 2018 to October 8, 2018 to avoid artifacts due to sensor conditioning at the beginning of the deployment and bio-fouling at the end. Due to a data gap between the first and second deployment, PSD estimates for pH were calculated separately for each half from June 8 to September 21 and September 28 to October 30 and a mean spectrum was computed by weighting each by their duration. A 24 hr moving average filter was applied to remove the diurnal signal and Beta statistics (β), to measure "environmental color", were calculated as the negative slope of the $\log_{10} - \log_{10}$ spectral density (Marshall & Burgess 2015, Hoshijima & Hofmann 2019). Using NOAA 1/4° Daily Optimum Interpolation Sea Surface Temperature (OISST) data (Huang et al. 2021) for the region between 34.25 and 34.5 degrees latitude and -120.5 and -119.5 degrees longitude, MHW

events were calculated using heatwaveR (v 0.4.5), as events where SST exceeded the 90th percentile of climatological observations for at least 5 days (Schlegel & Smit 2018).

2.3 Adult spawning and larval culturing

Adult urchins were recovered on December 4th after 6 months of field conditioning. Urchins from each cage were kept in separate bags and submerged in flowing ambient seawater until spawning 1 week later. Spawning, gamete collection, and quality screening was performed following (Wong et al. 2019). Three females with the highest quality eggs were selected from each of 6 cages (one cage was excluded from each site).Test diameters were measured for all females that contributed eggs. Eggs from each of the three females were pooled in equal numbers to create three egg pools per site, each representing a cage, and fertilized with the sperm from one inside conditioned male. All larvae were thus a mix of full and half siblings. Dilute activated sperm was added slowly to each pool of eggs until 95% fertilization was reached (Figure 1). After 95% fertilization was reached for all egg pools half of the embryos from each were added to a high (H: 1053.18 ± 14.24 µatm) and low (L: ~435 ± 6.23 µatm) pCO_2 culture vessel for a final concentration of 10 embryos mL^{-1}. This yielded three culture vessels for each of four treatment groups: embryos from mothers conditioned inside the kelp forest raised under high pCO_2 (IH), embryos from mothers conditioned inside the kelp forest raised under low pCO_2 (IL), embryos from mothers conditioned outside the kelp forest raised under high pCO_2 (OH), embryos from mothers conditioned outside the kelp forest raised under low pCO_2 (OL).

2.4 CO_2 mixing system and seawater chemistry

Larval cultures were held constant at 15°C, reflective of the collection site during the last weeks of adult conditioning. Temperature was maintained using a Delta Star heat pump with a Nema 4x digital temperature controller (AquaLogic). A flow through CO_2 system was modified from the design of Fangue et al. to create the desired carbonate chemistry parameters (Fangue et al. 2010). To generate filtered seawater (FSW), incoming seawater was UV- and 0.35 µm filter- sterilized. Desired pCO_2 treatments were created by mixing CO_2 gas with CO_2 scrubbed dry air in the appropriate ratios using Mass Flow Controllers (Sierra Instruments) and injecting the gas mixture into two reservoir tanks using venturi injectors. The two reservoir tanks then fed 6 culture vessels per treatment at a rate of 6 L hr^{-1} using irrigation drippers. Each culture vessel consisted of two nested 12 L buckets: the inner bucket had holes covered with 30 µm mesh allowing water to flow from the inner bucket to the outer and overflow without losing any larvae. Flow through and mixing was facilitated in each bucket by an acrylic paddle attached to a 12V motor. Temperature, pH, and salinity were measured daily for each bucket to confirm uniform conditions across each treatment. Temperature was measured using a thermocouple (Omega HH81A) and salinity was measured using a conductivity meter (YSI-3100). pH was measured following SOP 6b using a spectrophotometer and *m*-cresol purple dye (Dickson et al. 2007). Total Alkalinity (2236.89 ± 0.17 µmol kg^{-1}) was measured from incoming water samples poisoned with $HgCl_2$ following SOP3b (Dickson et al. 2007). CO2calc (Robbins et al. 2010) was used to calculate carbonate chemistry parameters using the equilibrium constants from (Mehrbach et al. 1973) refit by (Dickson & Millero 1987). Temperature was held constant throughout at 15.01 ± 0.52°C with a salinity of 33.2.

2.5 Egg and larval sampling

Prior to conducting fertilizations, eggs were sampled from all 18 females that would mother offspring (n = 9 females per treatment). From each female, 1000 eggs were placed in 6 replicate tubes for protein and lipid quantification. Eggs were centrifuged to remove excess seawater and flash frozen with liquid nitrogen. For morphometric analysis, 1000 eggs per female were preserved by addition of formalin (in 0.01 M phosphate buffered saline with borax) to the same volume of eggs in FSW for a final concentration of 2% formalin and stored at +4°C. The offspring from each bucket were also sampled as prism larvae, an early echinopluteus stage of pre-feeding larvae. The prism stage was defined by the archenteron merging to one side of the body and becoming tripartite, the first development of skeletal rods, and a pyramid like shape. 1000 prism larvae per bucket per stage were preserved in a final concentration of 2% formalin in FSW. Some larvae were also sampled at the prism stage and were immediately used for live assessment of thermal tolerance and whole animal respirometry.

2.6 Thermal tolerance

To assess the effect of maternal conditioning and larval pCO_2 treatment on thermal tolerance, larvae from each treatment were subjected to a range of acute heat shocks for one hour and then scored as alive or dead following a procedure adapted from (Hammond & Hofmann 2010). 3.5 mL of FSW in scintillation vials was brought to temperature across a gradient from 24.8°C to 33.4°C. At the prism stage, ~3333 larvae from each of the three larval cultures per treatment were combined into an aggregate pool. Larvae from each pool were gently mixed before 1000 larvae (0.5 mL) were pipetted into 10 treatment vials per

treatment across the temperature gradient were then incubated for 1 hour. Control larvae were held at 15°C. After 1 hour all vials were transferred to a 15°C cold room and scoring was performed immediately. 1 mL was loaded onto a rafter slide and the larvae were viewed through a compound microscope at the 4x objective. The first 100 larvae seen were scored as either alive (denoted by swimming and/or cilia movement) or dead. All scoring was blind to treatment and temperature. The survivorship curve for each treatment group was used to calculate the lethal temperature at which 50% of the individuals died, LT_{50}. Statistical analysis was performed in R (v 4.0.3) using the lme4 (v 1.1-26)(Bates et al. 2015), MASS (7.3-53) (Ripley 2011) and base packages.

2.7 Respirometry

To assess the effect of maternal conditioning and larval pCO_2 treatment on larval metabolism, oxygen consumption rates of prism stage larvae were measured using the method outlined by (Marsh & Manahan 1999) with minor modifications. A gradient of larvae (n = 100-600) from each culture bucket (n = 3 per treatment, n = 12 total) were loaded into 5 ground glass µBOD vials per culture bucket containing a known volume of high or low pCO_2 FSW with no headspace. One blank vial per culture bucket was also loaded with high or low pCO_2 FSW corresponding the larval treatment. End point oxygen concentrations for each vial were measured following a 5-7 hour incubation. ~315 ul of water from each µBOD vial was loaded into an optode cell using a gas tight syringe. Oxygen concentration was measured using a fiber optic oxygen probe inside the cell (Micro TX3; PreSens, Germany). The probe reading was calibrated using a two-point (0 and 100%) calibration using 0.01 g mL^{-1} $NASO_3$ solution and aerated 0.2 µm re-filtered FSW. Baseline respiration over the 5-7 hour

incubation, determined from blank vials, was subtracted from each μBOD vial measurement. Then, oxygen consumption rates per individual (in pmol O_2 hr^{-1} $larva^{-1}$) were calculated from a standard curve of oxygen consumption rate generated from the cumulative oxygen consumption over time at each larval concentration (n = 100-600). Oxygen consumption rates per individual were divided by larval volume

$$\left(\frac{Mean\ Body\ Length^3}{3}\right).$$

Average oxygen consumption rate per unit volume (pmol O_2 hr^{-1} mm^{-3}) for each bucket was used for analysis. Statistical analysis was performed in R (v 4.0.3) using the lme4 package (v 1.1-26) (Bates et al. 2007).

2.8 Morphometric analysis

Eggs and larvae preserved in 2% formalin were photographed on a compound microscope (Olympus BX50) with an attached digital camera (Motic 10MP) using the Motic Images Plus software. Images were calibrated using a stage micrometer at the 10x objective and analyzed using ImageJ (National Institutes of Health, USA). Average diameter was calculated for 35 eggs from each of the 18 females (n = 9 per treatment) using three roughly orthogonal diameters. Prism larvae were photographed from a lateral view where both the tip of the body rod and branching point of the postoral rod were in focus. Spicule length was measured as the length from the tip of the body rod to the branching point of the postoral rod and body length was measured as the top of the arch to the top of the pyramid parallel to the ventral plane. Spicule and body length for each individual were used to calculate the spicule:body length ratio. Statistical analysis was performed in R (v 4.0.3) using the lme4 (v 1.1-26) and lmer4 (Bates et al. 2007) and lmerTest (Kuznetsova et al. 2015) packages with

maternal and larval treatment as fixed effects and pool as a random effect. For egg morphometrics, maternal treatment was treated as a fixed effect while maternal identity was treated as a random effect. Post-hoc tests to compare individual treatments were conducted in the agricolae package (v 1.3-5) (de Mendiburu & de Mendiburu 2019) using the Tukey method to adjust for multiple comparisons.

2.9 Protein quantification

Total protein was extracted from frozen egg samples (n = 3 tubes per female) using the method described in (Wong et al. 2019), modified from (Byrne et al. 2008) and (Prowse et al. 2008). After extraction, the retained supernatant of total soluble protein was quantified at 562 nm on a microplate reader (Bio Rad) using a BCA protein assay kit following manufacturer's instructions (Catalog number 23225, Pierce Biotechnology).

2.10 Lipid quantification

Total lipid was extracted from frozen egg samples (n = 3 tubes per female) following the methods described by (Wong et al. 2019), based on (Sewell 2005), the only difference being that 250 µl of methanol was combined with 125 µl of chloroform in the first step as no internal standard was needed. Immediately prior to quantification, total lipid extracts were dried in glass vials using nitrogen gas. Total lipid was quantified using a spectrophotometric method (Marsh & Weinstein 1966). Briefly, 500 µl of sulphuric acid was added to each of the dried samples; the vials were covered with aluminum foil caps and heated in a furnace at 200°C for 15 minutes. The vials were allowed to cool for 15 minutes and then 2.5 mL of water was added to each, followed by an additional cooling period. The absorption of each

re-constituted lipid sample was read on a UV spectrophotometer (Shimadzu UV 1800) at 375 nm using disposable cuvettes. A standard curve of known mass of lipid ranging from 25 to 300 ug was prepared in the same way and measured alongside each batch of samples. The lipid profile of the standards was composed of the major lipid classes found in *S. purpuratus* eggs in the ratios reported in (Wong et al. 2019) (51% triacylglycerol, 38% phospholipid, 11% cholesterol). Principle component analysis was conducted on all egg metrics (size, protein, and lipid content) and maternal test diameter in R (v 4.0.3) using the ggfortify (v 0.4.11) (Tang et al. 2016) and base packages.

3. Results

3.1 in situ *water properties*

The average *in situ* water temperature varied between the inside and outside sites for the time period analyzed, with the shallower inside site warmer by approximately 1.5°C (17.65 ± 3.14°C) than the deeper outside site (16.10 ± 3.91°C) (Figure 3). The average DO concentration and saturation were also higher inside the kelp forest (7.28 ± 0.80 mg L^{-1}, 93.20 ± 11.78%) than outside (7.03 ± 0.67 mg L^{-1}, 87.49 ± 11.32%). With regard to pH, On average, seawater inside the kelp forest tended to be more alkaline than was measured outside the kelp; here, pH was 8.13 ± 0.13 inside the kelp forest, vs. 7.89 ± 0.07 outside (Figure 3). Lastly, there was a greater degree of high frequency variation in temperature, DO (concentration and saturation), and pH outside the kelp forest compared with inside. β values, a metric of environmental color, were higher inside the kelp forest relative to outside for all parameters, indicating a tendency to lower-frequency, more predictable, variation over time (Figure 3).

3.2 Thermal tolerance

Using acute thermal tolerance trials, we found an effect of both maternal conditioning environment and developmental pCO_2 treatment on larvae's capacity to deal with additional environmental stressor: temperature. Specifically, LT_{50}, the lethal temperature at which 50% of the larvae died from a 1-hour exposure, varied by up to 1.0°C across treatment groups (Figure 5). The effect of both maternal ($t = 3.051$, $p = 0.003$) and larval treatment ($t = 3.964$, $p = 0.001$) were significant. LT_{50} varied by ~ 0.6 °C between larvae raised under the low (L) versus High (H) larval pCO_2 treatment, within each maternal treatment (Figure 5, Table 1). Within each larval pCO_2 treatment, larval LT_{50} varied by 0.4°C across mothers with conditioned outside (O) versus inside (I) the kelp forest (Figure 5, Table 1). Within the same larval treatment (H or L) those from inside (I) mothers had lower thermal tolerance, while within a maternal group (O or I) those from exposed to high pCO_2 (H) had lower thermal tolerance (Figure 5). Larvae from mothers conditioned outside the kelp forest raised under low pCO_2 (OL) had the highest LT_{50} at 29.9 ± 0.1°C. Those from inside mothers who were raised under high pCO_2 conditions (IH) had the lowest LT_{50} at 28.9 ± 0.1°C. Larvae from outside mothers raised under high pCO_2 conditions (OH), and those from inside mothers raised under low pCO_2 (IL) had intermediate LT_{50}, 29.3 ± 0.1°C and 29.5 ± 0.1°C respectively. The same trends held for calculated LT_{10} and LT_{25} values, though overall variation was more extreme, with a difference of 1.4°C between the LT_{25} values and a difference of 2°C between the LT_{10} values of IH and OL larvae (Table 1). In pairwise contrasts between treatments, using a Tukey correction, all treatments were significantly different except for OH and IL which displayed intermediate LT50's, (IL - IH *z-ratio* = 4.134 $p = 0.0002$, IL – OL *z-ratio* = -3.954 $p = 0.0004$, IL - OH *z-ratio* = 1.095 $p =$

0.6926, IH – OL z-ratio = 7.910 $p < 0.0001$, IH – OH *z-ratio* = -3.065 $p = 0.0117$, OL – OH *z-ratio* = 5.035 $p < 0.0001$).

3.3 Metabolic rate

Using whole animal respirometry, we saw that larval pCO_2 treatment seemed to have a greater impact on offspring from mothers conditioned inside the kelp than those from mothers conditioned outside. Larvae from inside conditioned mothers raised under high pCO_2 conditions (IH) had the highest metabolic rate per unit size (17094.86 pmol O_2 hr^{-1} mm^{-3} ± 926.65 SE); the rate for IL larvae was lower (14812.77 pmol O_2 hr^{-1} mm^{-3} ± 744.60 SE) (Figure 6). The metabolic rates for both groups of larvae from outside mothers were similar (15415.00 pmol O_2 hr^{-1} mm^{-3} ± 1651.14 SE for OH larvae and 15250.96 pmol O_2 hr^{-1} mm^{-3} ± 717.69 SE for OL larvae) (Figure 6). Without size correction the pattern was similar (IH: 12.11 pmol O_2 hr^{-1} ± 0.66 SE, IL: 11.05 pmol O_2 hr^{-1} ± 0.56 SE, OH: 10.56 pmol O_2 hr^{-1} ± 1.13 SE, OL: 10.89 pmol O_2 hr^{-1} ± 0.51 SE). While larvae from inside conditioned mothers raised under high pCO_2 tended to have higher respiration rates than those raised under low pCO_2, the effect of maternal or larval treatment or their interaction were not statistically significant (Linear model: maternalO $t = 0.153$, $p = 0.882$, larvalH $t = 1.118$, $p = 0.296$, maternalO:larvalH $t = -0.094$, $p = 0.927$; *F-statistic*: 0.7452 on 3 and 8 *DF*, *p-value*: 0.5547).

3.4 Body size

Considering morphometrics, we found that initial skeletal development (spicule length) was mainly affected by larval pCO_2 treatment, though offspring from outside-

conditioned mothers tended to have longer spicules compared to those from inside conditioned mothers. At the prism stage there was not a significant effect of maternal or larval treatment on body size (Linear mixed effects model: maternalO $df = 3.57$, $t = -0.241$, $p = 0.823$, larvalL $df = 323.8288$, $t = 1.087$, $p = 0.278$). The average larval body length was tightly constrained across treatments: ranging from 126.25 μm for OH larvae to 129.50 μm for OL larvae (Figure 7B). There was a significant effect of larval but not maternal treatment on spicule length (Linear mixed effects model: maternalO $df = 3.886$, $t = 0.665$, $p = 0.543275$, larvalL $df = 409.676$, $t = 2.882$, $p = 0.005010$) and spicule to body ratio, (Linear mixed effects modelL: maternalO $df = 3.88274$, $t = 0.808$, $p = 0.46581$, larvalL $df = 407.03296$, $t = 2.886$, $p = 0.00437$). OL larvae had the longest spicules, 77.78 ± 14.62 μm, followed by IL, 69.08 ± 14.23 μm, and OH, 67.67 ± 15.59 μm, and IH larvae had the shortest spicules, 64.75 ± 12.58 μm (Figure 7A). The ratio of spicule length to body length was highest for OL larvae at 0.60 ± 0.10, followed by IL at 0.54 ± 0.10, and OH at 0.53 ± 0.11, while IH larvae had the lowest ratio of spicule length to body length at 0.51 ± 0.09 (Figure 7C). A Tukey test conducted between treatments found OL larvae to have significantly longer spicules and a higher spicule to body ratio than all other treatments, (spicule length: IH-OL difference = -13.03 [-17.918382 -8.143442], $p << 0.001$; OH-OL difference = -10.11 [-15.070580 -5152945], $p << 0.001$; IL-OL difference = -8.71 [-13.776860 -3.636646], $p = 0.0001$) (spicule to body ratio: IH-OL difference = -0.0941 [-0.12860664 -0.059651844], $p << 0.001$; OH-OL difference= -0.066 [-0.10101975 -0.031058352], $p << 0.001$; IL-OL difference = -0.0622 [-0.09801530 -0.026483768], $p = 0.0001$). All other treatments were not significantly different from one another in pairwise comparisons. For body length, OL larvae were significantly longer than OH larvae (body length: OH-OL difference = -3.25 [-

5.9445562 -0.5532508] , $p = 0.0108$). All other comparisons of body length were insignificant.

3.5 Eggs

With regard to traits of the eggs from females conditioned inside vs. outside the kelp, egg phenotype tended to be more variable among inside females compared to outside, but size and biochemical storage (lipid or protein) did not differ significantly between treatment groups. The eggs from inside females tended to be larger (92.49 ± 2.47 μm) than those from outside females (90.86 ± 2.25 μm). This difference in diameter was not statistically significant when taking into consideration variation between mothers of a given treatment as a random effect ($df = 16$, $t = -1.88$, $p = 0.0772$). There was not a significant effect of maternal treatment on lipid content per egg (Welch's T-test, $t = 0.93763$, $df = 14.23$, $p = 0.3641$) or size corrected per unit volume ($t = 0.3694$, $df = 14.302$, $p = 0.7172$). There was also not a significant effect of maternal treatment on protein content per egg ($t = -0.36869$, $df = 15.248$, $p = 0.7174$) or size corrected per unit volume ($t = -1.2298$, $df = 15.229$, $p = 0.2374$). In a PCA analysis of egg traits and female test diameter, PC1 and PC2 accounted for 37.96% and 27.48% of the variation in the data (Figure 8). Protein and lipid content per egg were negatively correlated in PCA space while test diameter and egg volume were positively correlated with one another. Overall maternal treatments did not separate in PCA space but the outside treatment encompassed less than the inside treatment.

4. Discussion

4.1 Summary

Our goal was to assess the transgenerational effects of field acclimation to natural variability in water properties associated with a kelp forest on the purple urchin, *Strongylocentrotus purpuratus*. As a foundation to the overall field experiment, we did find differences in abiotic conditions inside vs. outside the kelp forest, as has been noted by other investigators (Delille et al. 2009, Frieder et al. 2012, Hoshijima & Hofmann 2019, Hirsh et al. 2020). At our study in the Santa Barbara Channel, the environment inside the kelp forest showed generally higher pH and warmer temperatures and the variability of pH, temperature, and oxygen was more predictable inside the kelp forest, while high-frequency variation was greater outside (Figure 3). At the biological level, these physicochemical differences aligned with differences in the physiological parameters assessed in the larvae of differentially acclimatized mothers. Larvae from mothers conditioned inside the kelp displayed lower thermal tolerance, indicating potential increased vulnerability to future MHWs (Figure 5). Those larvae from inside-conditioned mothers that were raised under high pCO_2 conditions also had (insignificantly) elevated respiration rates and less skeletal development indicating possible metabolic stress (Figure 6, 7). Conversely, larvae from outside-conditioned mothers raised under low pCO_2 had the greatest skeletal development and highest thermal tolerance. Overall, the fitness implications of the observed pattern of transgenerational effects on larval traits may vary based on the compounding stressors of the larval environment at critical points in development.

4.2 Conditions inside and outside the kelp forest

The difference in average pH and temperature between inside and outside was likely due to the depth difference, the time of year, and the thermal regime during a MHW. We observed colder water offshore at the outside site likely due to the difference in water depth in addition to the flow attenuation created by the kelp (Gaylord et al. 2007). This difference may have been exacerbated by a MHW that occurred in 2018 during the first half of our deployments which seemed to extend the vertical stratification of summer in addition to elevating temperatures throughout the water column (Figure 4). This depth difference may also have contributed to the pH difference between inside and outside sites, with the inside showing elevated pH, particularly for the first half of our deployment. Previous studies have shown depth to be a large determinant of pH difference at coastal sites, exhibiting more influence than horizontal spatial variation within a site (Frieder et al. 2012, Koweek et al. 2017).

The elevated pH inside the kelp forest we observed was largely concentrated in the first half of our deployments when temperatures were highest and the kelp canopy was very dense; as high temperatures persisted the kelp began to die and the pH regimes across sites converged for the second half of the conditioning period. In a prior study in the SBC with a similar experimental setup, Hoshijima and Hofmann found that pH and oxygen were slightly higher outside the kelp forest relative to inside while the temperature was slightly lower outside (Hoshijima & Hofmann 2019). However, the reef at which their sensors were deployed was far shallower and the regime comparisons took place in Spring when upwelling is stronger in the SBC. We also saw cooler temperatures outside the kelp forest, yet the temperatures were higher at both our sites than in their study, despite the deeper depth,

indicative of the time of year but also the thermal anomaly during our experiment. Other recent studies conducted in the Monterey Bay region have seen that small differences in pH and DO associated with kelp were outweighed by site specific impacts based on wave exposure and currents and that the buffering effects of kelp were isolated to a narrow depth band at the canopy and diminished at depth, indicating that any potential pH refuge was unlikely to benefit benthic animals (Hirsh et al. 2020, Traiger et al. 2021). Our study looked only at the benthic water properties as our primary goal was to co-locate the physicochemical monitoring with our biological conditioning of the adult urchins, so we are unable to compare to the surface conditions at our site. However, the difference between our findings and those of other recent studies suggests that the differences observed may be spatiotemporally specific.

The most consistent pattern in seawater chemistry was that the inside site displayed more predictable variation, weighted towards lower frequencies, while the outside site showed less predictable, high frequency variation (Figure 3). These findings are consistent with those of Hoshijima and Hofmann at a different shallower site, Mohawk Reef, in the SBC in Spring, indicating that this pattern is likely persistent throughout the year (Hoshijima & Hofmann 2019). The extreme physicochemical variability of coastal sites across space and time necessitates improved monitoring of pH, DO, and temperature across a range of habitats and at high temporal resolution as the aggregate of all axes of variation is difficult to characterize and mimic accurately in the lab (Waldbusser & Salisbury 2014, Baumann et al. 2015). Therefore, if we are to understand the impact of variations on different scales on the biology of critical species, field-based studies that monitor and take advantage of natural heterogeneity in variation, as well as mean parameters will be critical. More studies that

employ natural variations and gradients to study transgenerational effects *in situ* will give us a better understanding of how variability will impact recruitment and fitness across generations (Murray et al. 2014, Griffiths et al. 2021).

4.3 Transgenerational effects

We raised the larvae from mothers acclimated to the physicochemical regimes inside and outside a SBC kelp forest under high and low pCO_2 treatments in order to assess how their maternal conditioning impacted their capacity to deal with environmental stress. We saw that the differences in maternal conditioning in combination with the larval environment impacted the thermal tolerance of offspring as well as their skeletal growth. These findings indicate that the natural heterogeneity in coastal environments can have effects across generations and, further, that the interaction between multiple stressors will likely impact the local success and survival of larvae of key species.

Hoshijima and Hofmann assessed an earlier larval stage than this experiment; however, they similarly found less variation in egg provisioning of proteins among outside conditioned mothers (Hoshijima & Hofmann 2019). A previous lab experiment found that eggs from mothers held at simulated static upwelling conditions (similar to the outside conditions in our field experiments) were larger when compared to eggs from mothers held under ambient conditions, though not significantly (Wong et al. 2018, Wong et al. 2019). We see the opposite pattern in comparing eggs from mothers outside the kelp forest to those from mothers inside. Hoshijima and Hofmann also did not see a significant difference in egg size (Hoshijima & Hofmann 2019). Colder temperatures have been associated with larger egg volumes in several species (Pettersen et al. 2019). However, it is worth noting that though

there was a difference in temperature between the sites, the entire region was impacted by a MHW during this experiment causing elevated temperatures both inside and outside the kelp forest.

After developing under high or low pCO_2 conditions, we found a significant effect of larval treatment on spicule development, with larvae developing under high pCO_2 displaying reduced skeletal formation. This pattern has been seen in prior studies (Yu et al. 2011, Padilla-Gamiño et al. 2013, Strader et al. 2020). This contrasts with the lab experiment of Wong et al. who found that the effect of the maternal conditioning environment impacted skeletal growth at the prism stage while the developmental pCO_2 treatment did not have a significant effect (Wong et al. 2019). In our investigation, larvae from outside mothers raised at low pCO_2 (OL) had significantly greater skeletal development and a significantly higher spicule to body ratio than all other treatment groups (IH, IL, and OH). In contrast, at the hatched blastula stage, Hoshijima and Hofmann saw that the larvae which came from outside mothers but were raised under high pCO_2 conditions (OH) had larger body size than all other treatment groups (Hoshijima & Hofmann 2019). It is interesting that in both cases the largest embryos and larvae came from outside conditioned mothers. The differences in the impact of the developmental treatment on body size between the two studies could be due the crossing design employed, or the developmental stage assessed. Larvae are generally more vulnerable to pH and temperature stress than embryos and the onset of calcification begins between the stages investigated in these two studies (Przeslawski et al. 2015). In our study, this elevated performance of OL larvae was matched with elevated thermal tolerance relative to the other treatment groups.

Within each maternal treatment group, those larvae which developed under high pCO_2 were less tolerant of an additional stressor in the form of an acute thermal exposure than those which developed under low pCO_2. These findings vary from the patterns seen by Karelitz et al. who investigated larval thermal tolerance of five echinoderm species across a geographic range and found that thermal tolerance was not diminished by low pH exposure (Karelitz et al. 2017). In red urchins, *Mesocentrotus franciscanus*, elevated temperature during development boosted thermal tolerance but developmental pCO_2 treatment did not have an impact (Wong & Hofmann 2020). However, similar to our findings, elevated pCO_2 has been seen to impact thermal stress response in red urchins at the pluteus stage (O'Donnell et al. 2009). Within each larval treatment (high and low pCO_2), those larvae which came from outside mothers had higher thermal tolerance than those from inside mothers. Adult exposure to warmer temperatures can increase developmental thermal tolerance in some species of urchins (Pecorino et al. 2013); however, in this study, the larvae whose mothers were at the deeper, cooler site displayed higher larval thermal tolerance. There was not a significant effect of maternal treatment on larval metabolic rate; however, larval high pCO_2 exposure seemed to induce elevated metabolic rate only in larvae from inside conditioned mothers. Effects of acidic conditions on larval metabolic rates vary with larval stage, feeding or non-feeding, and are mediated by other stressors (Stumpp et al. 2011, Padilla-Gamiño et al. 2013).

Development under high pCO_2 had a negative impact on larval performance: larvae which developed under high pCO_2 had lower thermal tolerance than their low pCO_2 counterparts, they showed reduced spicule growth and a lower spicule to body ratio, and larvae from inside conditioned mothers raised under high pCO_2 tended toward elevated

metabolic rates. The impacts of maternal conditioning are less clear: the metabolic rate of larvae from outside conditioned mothers was not impacted significantly by high pCO_2 exposure during development, larvae from outside conditioned mothers raised under low pCO_2 had the highest spicule growth, spicule to body ratios, and the largest body size (though not significantly). The larvae from inside conditioned mothers raised under high pCO_2 generally had the lowest performance for all metrics, with the exception of body length for which there was not a significant effect of larval or maternal treatment.

However, the implications of these elevated larval performance metrics are likely context dependent. Purple urchin larvae reach the pluteus, their feeding larval stage after around 48 hours and then have a planktonic pelagic larval duration of 29-86 days (Strathmann 1978). The oceanographic conditions during their first lecithotrophic 48 hours of initial development, or the following month(s) feeding prior to settlement will have large consequences on their survival. With the rise of MHWs and the forecasted increase in the frequency and duration of upwelling, there is an extreme range of oceanographic conditions which larvae could develop in (Feely et al. 2008, Frölicher et al. 2018). Interannual variability and slight differences in spawning time could lead to larvae recruiting under drastically different thermal, pH, and/or oxygen regimes. Okamoto et al. have shown that larval settlement patterns for the purple urchin vary based on temperature and climate variations (Okamoto et al. 2020). In the SBC, SST was negatively correlated with larval urchin settlement. In this experiment, larvae from outside conditioned mothers showed higher thermal tolerance. Therefore, if the larvae were to enter the water column during a MHW, larvae from outside mothers or those from deeper sites likely have increased probability of survival. OL larvae also had the greatest skeletal growth and body size, which

could be advantageous in avoiding predation or acquiring food in low food conditions (Allen 2008, Chan et al. 2011). In contrast, under high pCO_2 conditions, larvae from outside conditioned mothers (OH) had the smallest body size, though not significantly, which could make them more vulnerable to predation (Allen 2008). IH larvae had higher metabolic rates which could help them to develop faster to feeding and eventually settlement stage but would increase metabolic demand while reduced spicule development could inhibit their capacity to feed (Hart & Strathmann 1994, Hart 1995). Therefore, the combination of stressors experienced during key times during their development might determine the benefit of any maternal transgenerational effect (Byrne & Przeslawski 2013).

Though this experiment represents the natural variation between inside and outside a kelp forest site through *in situ* acclimation, the urchins were fed consistently throughout the experiment to control for food availability. This served to isolate the effect of the physicochemical environment but in nature the availability and composition of food would very drastically between sites having strong implications for maternal provisioning. Inside conditioned urchins would likely have higher metabolic costs due to warmer temperatures but also have access to kelp, whereas outside conditioned urchins would need to rely on other food sources such as diatoms and encrusting algae. Urchins in barrens may therefore have different, and even more diverse, microbiomes which could influence their metabolism and stress response (Marangon et al. 2021, Miller et al. 2021). The nutritional quality of kelp itself declines at warmer temperatures which would require urchins to eat more of it to meet their higher metabolic costs under MHW regimes (Lowman et al. 2022). Therefore, the balance between the abiotic environment and food availability would reshape the consequences of each acclimation regime.

Overall, isolating the physiochemical differences by site, the results of this study indicate a benefit of maternal conditioning outside a kelp forest. The relative stress of a lower pH environment outside the kelp forest may have primed the offspring of those mothers with increased resilience to environmental stress, allowing them to maintain average metabolic rates under different larval pCO_2 regimes and endure acute thermal stress. The relative predictability of the inside environment would be more likely to induce transgenerational plasticity (Burgess & Marshall 2014). However, it is also possible that the high frequency variability of the outside site may have induced epigenetic changes to enhance the plasticity of gene regulation to respond to rapid changes and these changes could have been passed down to the offspring. Though it was not intended in the design of the study, a MHW persisted through a significant proportion of the adult field acclimation. Therefore, differences in temperature across the two sites may likely have been the most important property driving transgenerational effects during acclimation. Though temperatures were elevated at both sites, the relatively lower temperature at the deeper, less sheltered, outside site may have provided a relative thermal refuge for those mothers.

4.4 Kelp, urchins, and marine heatwaves (MHWs)

One inadvertent aspect of this field experiment was that during late summer and early Fall of 2018 several MHWs occurred in our study region. Specifically, from late June to December, 5 MHW events occurred and were 7-21 days long with a total of 76 days of extreme thermal stress in bottom temperatures at our study site (see Figure 4 for examples from SST). This temperature regime likely interacted strongly with pH and DO and influenced our TGP results (Bautista & Crespel 2021). Giant kelp forests provide critical

habitat and food supply as a foundation species in the California Current, and urchins shape this ecosystem as a dominant herbivore. MHWs, such as the 2014-2016 eastern Pacific MHW, "the Blob," have been demonstrated to cause shifts in the community structure of the kelp forest in the SBC (Michaud et al. 2022). Therefore, it is essential to consider how these two pivotal species, and their ecological relationship will be impacted by future climate change (Starko et al. 2022).

Recent studies have challenged the idea that kelp forests are extremely vulnerable to warm temperatures (MHWs) (Reed et al. 2016) and indicate that the patterns of global decline seem to be largely driven by local stressors and variation in biotic interactions (Krumhansl et al. 2016, Thomsen et al. 2019, Wernberg et al. 2019, Starko et al. 2022). For instance, in an analysis of "the Blob" impacts were variable in timing and extent throughout the range of giant kelp with mortality driven largely by the absolute temperature reached, not the anomaly above normal (Cavanaugh et al. 2019). Many kelp taxa show a consistent thermal limit of recruitment at 18°C, though some species, including *Macrocystis pyrifera*, can produce sporophytes above this threshold (Muth et al. 2019). Temperature variation across depth and small spatial scales has a significant impact on kelp resilience through MHW events, yet biotic interactions such as those with urchin populations regulate the capacity for cool areas to serve as kelp refugia (Starko et al. 2022). Therefore, if water property differences associated with depth can have transgenerational effects, as seen in this study, this could exacerbate biotic feedbacks between kelp and urchin populations. Furthermore, kelp nutritional quality has been decreasing in southern California with higher temperatures (Lowman et al. 2022). Therefore, if urchins need to eat more kelp to meet the

same, or increased, metabolic demand under high temperatures, this could further prevent kelp from recovering or moving to deeper cooler areas.

MHWs can occur in the SBC during key periods of the purple urchin life cycle such as gametogenesis (Summer-Fall) as well as early larval development through metamorphosis (December-January) (Figure 4). MHWs may impact the dispersal and recruitment of purple urchin larvae, influencing gene flow and population dynamics (Byrne 2011, Okamoto et al. 2020). Where settlement is not reduced, elevated temperatures may shorten pelagic larval duration, impacting dispersal (Byrne 2011). Adult exposure to MHWs may have negative effects on gonad quality or leave populations at increased susceptibility to disease (Tajima et al. 1997, Uthicke et al. 2014). While purple urchin sperm can withstand elevated temperatures during fertilization, sperm of male urchins exposed to MHW temperatures during spermatogenesis display reduced fertilization success (Leach et al. 2021). Leach et al. calls to attention the importance of considering transgenerational impacts through both parental lines, not solely the maternal line as in this study. MHWs, therefore, may impact adult populations, have transgenerational effects influencing early bottlenecks such as fertilization, and even sub-lethal exposure at early life stages may have negative carryover effects at later life stages (Byrne 2011, Przeslawski et al. 2015). It is crucial to consider the interaction of MHWs, such as the one which occurred during the acclimation period of this experiment, with other environmental variability and gradients in factors such as salinity, pH, and DO within and across generations as these interactions are most often synergistic (Przeslawski et al. 2015) and conditions in the North Pacific are likely to support even more extreme MHWs in the future (Barkhordarian et al. 2022).

4.5 Concluding statements

Overall, in this study, larvae from mothers conditioned outside the kelp forest performed better on average when developing under high pCO_2 conditions, displaying greater skeletal development and increased thermal tolerance compared to their counterparts from inside conditioned mothers. The fitness implications of the transgenerational effects observed may vary based on the compounding stressors of the larval environment at critical points in their development. It is difficult for multi-stressor lab studies to capture the full breadth and nuance of variability experienced across space and time in complex ecosystems such as coastal kelp forests, and therefore should be complemented by field based monitoring and acclimation experiments.

Acknowledgements

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Figures & Tables

Table 1. LT50, LT25, and LT10 values for echinopluteus larvae from different maternal and larval treatments. All values given as mean ± standard deviation.

Temperature Treatment Mother - Offspring	LT_{10}	LT_{25}	LT_{50}
Inside - Low	27.4 ± 0.2	28.4 ± 0.1	29.5 ± 0.1
Inside - High	26.8 ± 0.2	27.9 ± 0.1	28.9 ± 0.1
Outside - Low	28.8 ± 0.1	29.3 ± 0.1	29.9 ± 0.1
Outside – High	27.2 ± 0.2	28.3 ± 0.1	29.3 ± 0.1

Figure 1.

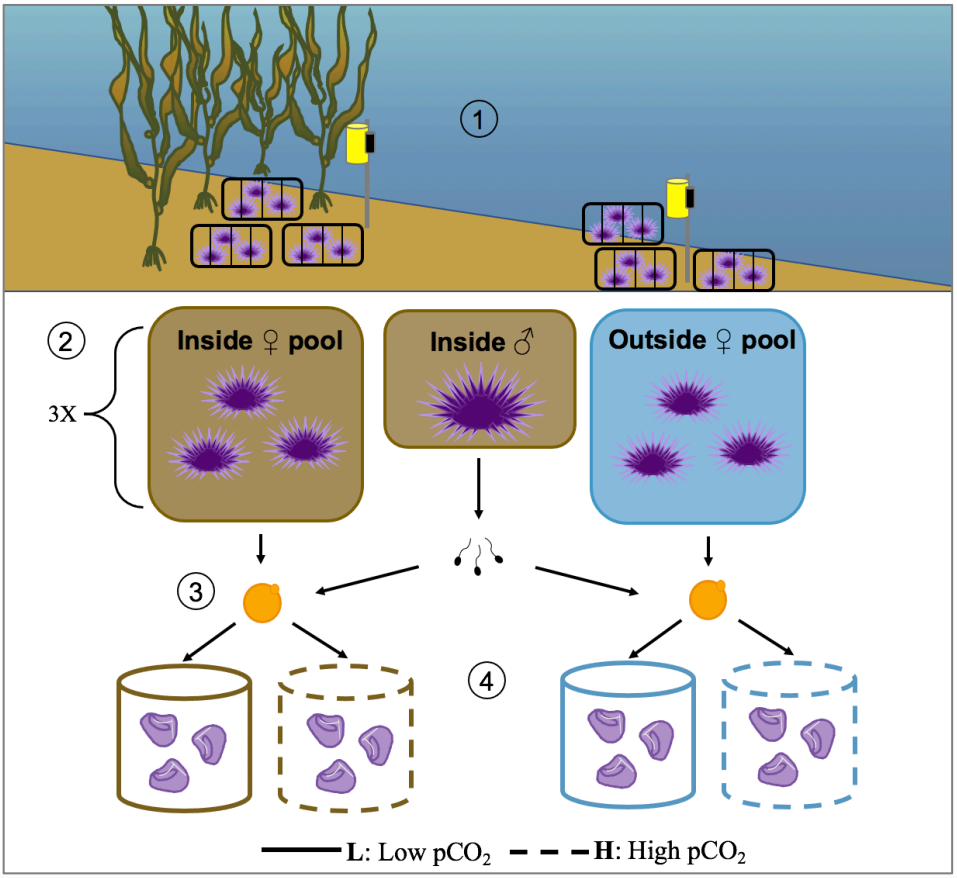
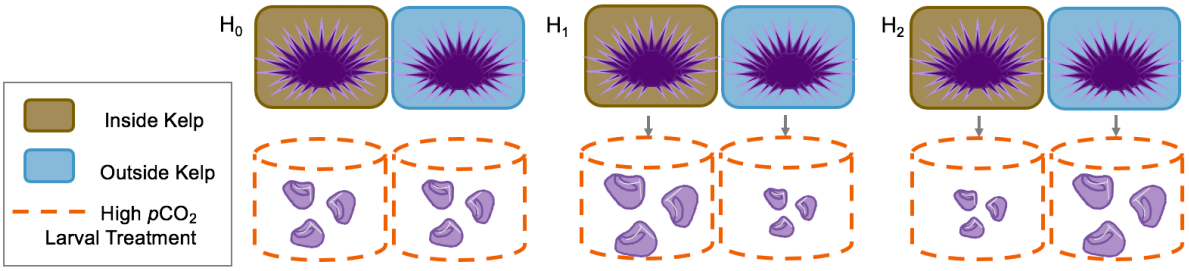


Figure 1. Experimental design. 1) Adult purple urchins, *Strongylocentrotus purpuratus*, were held in cages inside (I) or outside (O) the kelp forest for 6 months prior to spawning. pH, temperature, and dissolved oxygen sensors were deployed at both sites. 2) Eggs from $n = 3$ mothers were pooled to create $n = 3$ pools from each site. 3) Each pool of eggs ($n = 6$) was fertilized with sperm from 1 inside conditioned male. 4) Fertilized eggs from each pool were then divided into low pCO_2 (L: ~435 µatm), or high pCO_2 (H: ~1050 µatm) treatments to develop until the prism larval stage yielding four final combinations of maternal and larval treatment: larvae from inside mothers (I) raised under low pCO_2 (L), IL; larvae from inside mothers (I) raised under high pCO_2 (H), IH; larvae from outside mothers (O) raised under low pCO_2 (L), OL; and larvae from outside mothers (O) raised under high pCO_2 (H), IH.

Figure 2.



Effect	**NA**	**NA**	+	-	-	+
Support			Dupont et al., 2013, Zhao et al., 2018, Verkaik, et al., 2016, Hoshiiima & Hofmann, 2019, Uthicke et al., 2021		Hoshiiima & Hofmann, 2019, Wong et al., 2017, Wong et al., 2018, Suckling et al. 2014, Dupont et al., 2013, Clark et al., 2019, Suckling et al., 2015	

Figure 2. Possible directions for transgenerational effects of conditioning inside and outside kelp forest kelp forest conditioning on purple urchin larval development under high pCO_2 . Based on current support from the literature, transgenerational impacts may align with different hypotheses: H_o: Conditioning inside and outside a kelp forest has no transgenerational impact. H_1: Negative effect from high pCO_2 - urchins who were acclimated outside the kelp forest suffer from higher pCO_2, lower oxygen and pass down damage or are unable to provision offspring proficiently while urchins who were acclimated inside the kelp forest may have a more hospitable environment of low pCO_2 and higher dissolved oxygen allowing them provision their eggs more than those acclimated outside the kelp forest, creating more resilient larvae. H_2: Positive effect of priming- urchins who were acclimated inside the kelp forest are naïve to higher pCO_2, lower oxygen and do not confer changes to offspring upregulating pathways to help them cope while urchins acclimated outside the kelp forest who experience more acidic, low oxygen conditions may be primed by poor conditions and confer resilience to these same stressors to their offspring through maternal provisions or epigenetic modifications.

Figure 3.

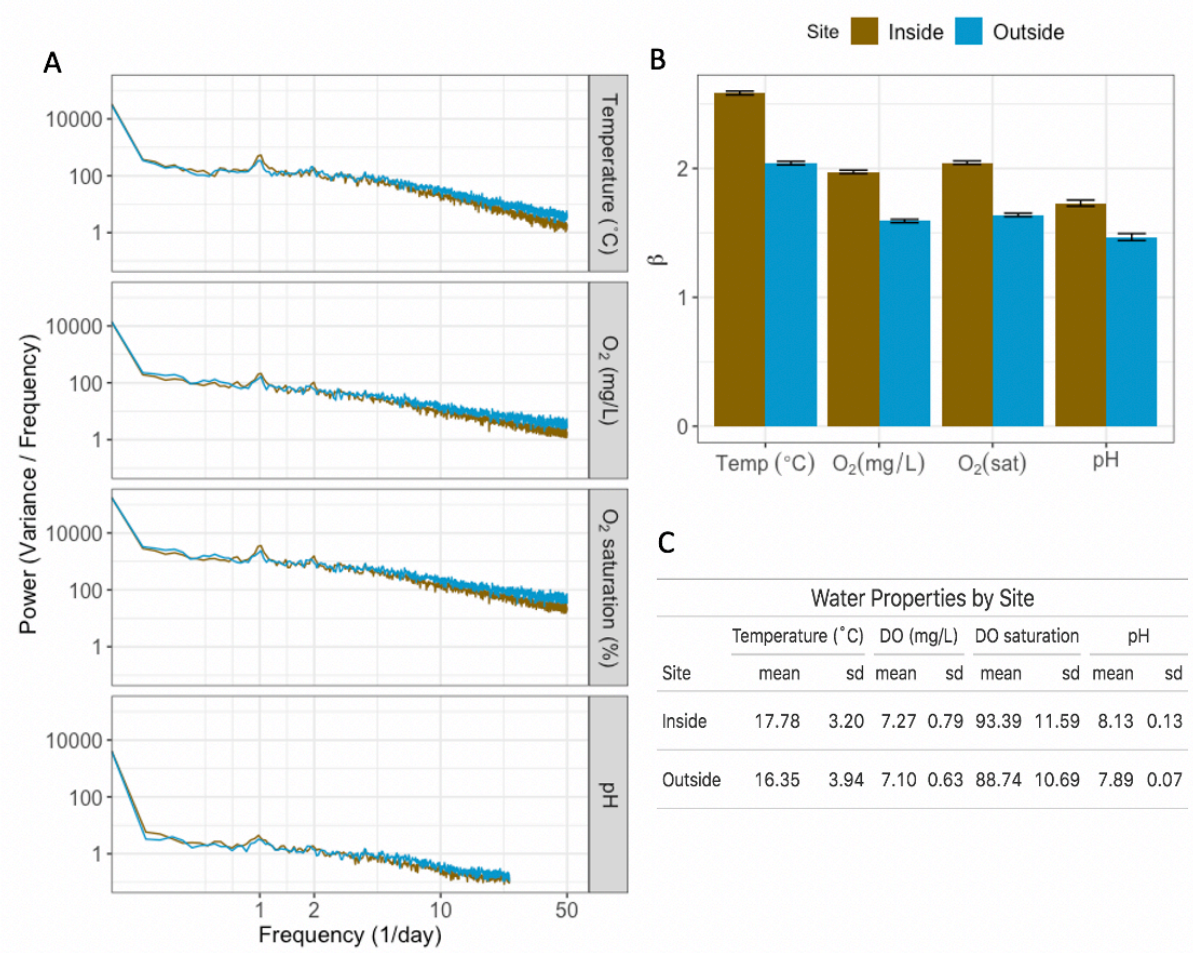


Figure 3. Temperature (°C), dissolved oxygen (concentration, mg L^{-1} and saturation, %), and pH inside (brown) and outside (blue) of a kelp forest environment, shown as A) power spectra, B) calculated β indexes (±SE), and C) mean values for the deployment (±SD).

Figure 4.

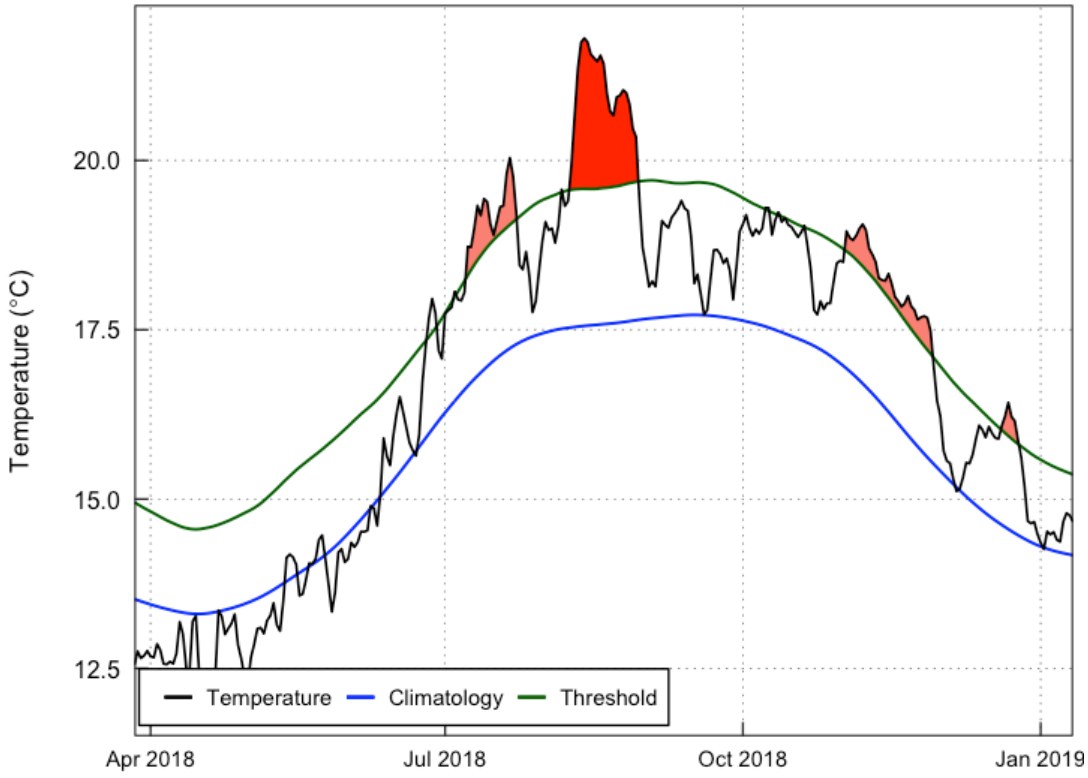


Figure 4. Marine heatwave (MHW) events during the deployment period calculated using heatwaveR. Long-term climatology (blue line), seasonal variation threshold at the 90th percentile (green line), and MHW events (red areas) were determined from sea surface temperature encompassing the region of the Santa Barbara Channel containing Arroyo Quemado Reef.

Figure 5.

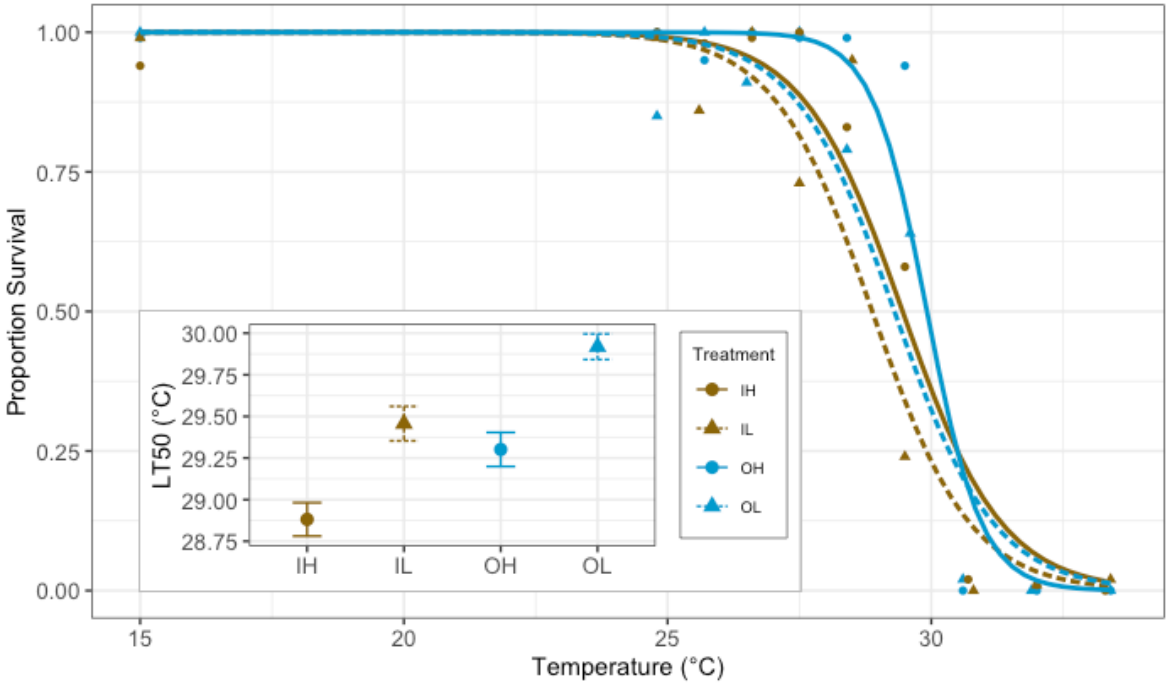


Figure 5. Effect of maternal (I: Inside or O: Outside kelp forest) and larval (H: High pCO_2 or L: Low pCO_2) treatment on *Strongylocentrotus purpuratus* prism larval thermal tolerance, measured as proportion survival after a 1-hour acute heat shock and recovery. Acute heat shock temperature is shown on the x-axis. Inset displays the calculated LT_{50} for each treatment ± standard error. Maternal group is shown by color and larval treatment is shown by pattern, IL – brown, solid line; OL - blue, solid line; IH – brown, dashed line; OH - blue, dashed line.

Figure 6.

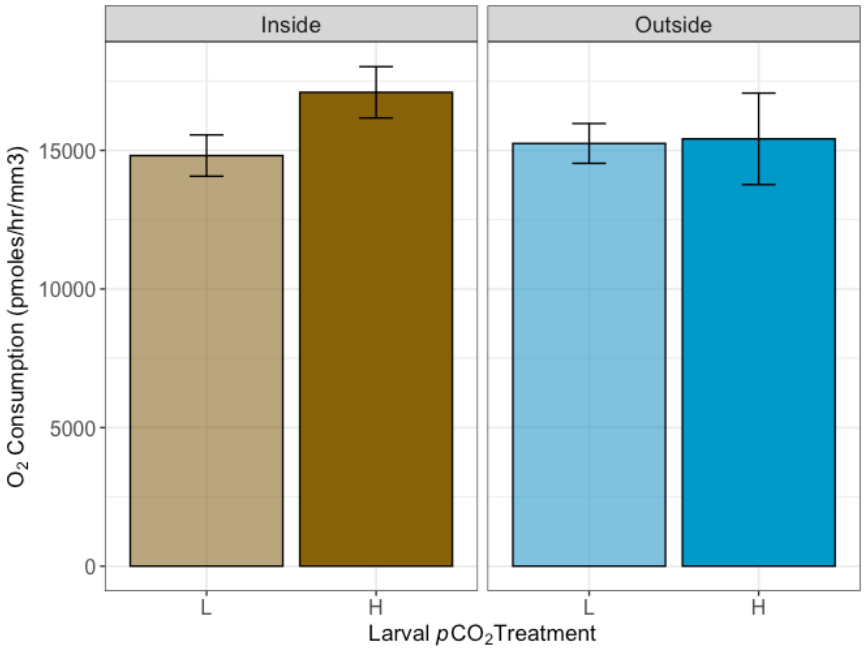


Figure 6. Effect of maternal (I: Inside or O: Outside kelp forest) and larval (H: High pCO_2 or L: Low pCO_2) treatment on size corrected oxygen consumption (picomoles hr^{-1} mm^{-3}) of *Strongylocentrotus purpuratus* at the prism larval stage. Error bars represent standard error. Maternal group is shown by color and larval treatment is shown by shade, IL – brown, light; OL - blue, light; IH – brown, light; OH - blue, light.

Figure 7.

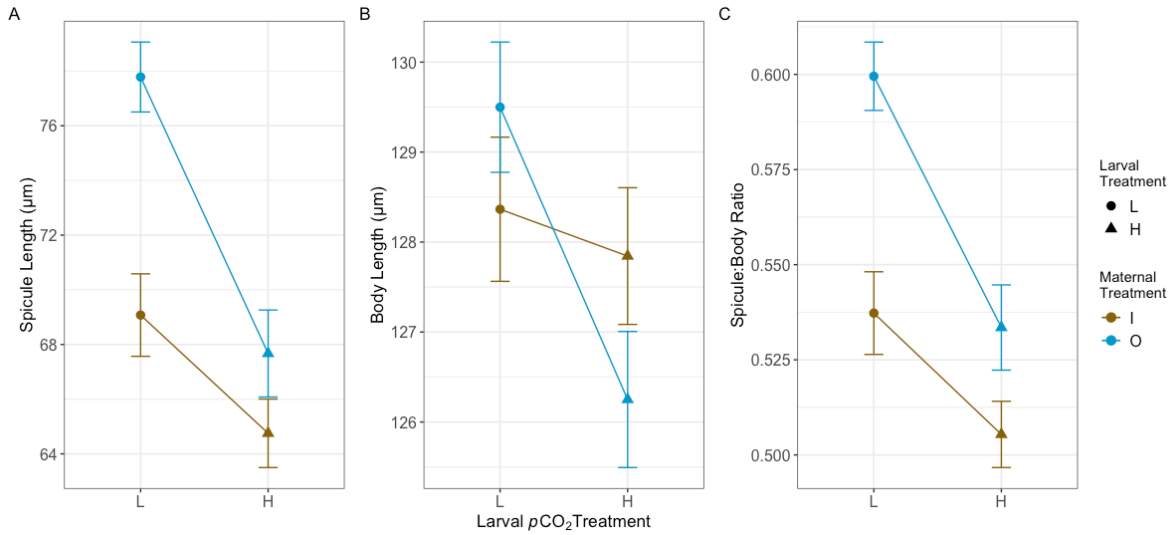


Figure 7. Effect of maternal (I: Inside or O: Outside kelp forest) and larval (H: High pCO_2 or L: Low pCO_2) treatment on A) spicule length (µm), body length (µm), and C) spicule length to body length ratio. Error bars represent standard error. Maternal group is shown by color and larval treatment is shown by shape, IL – brown, circle; OL - blue, circle; IH – brown, triangle; OH - blue, triangle.

Figure 8.

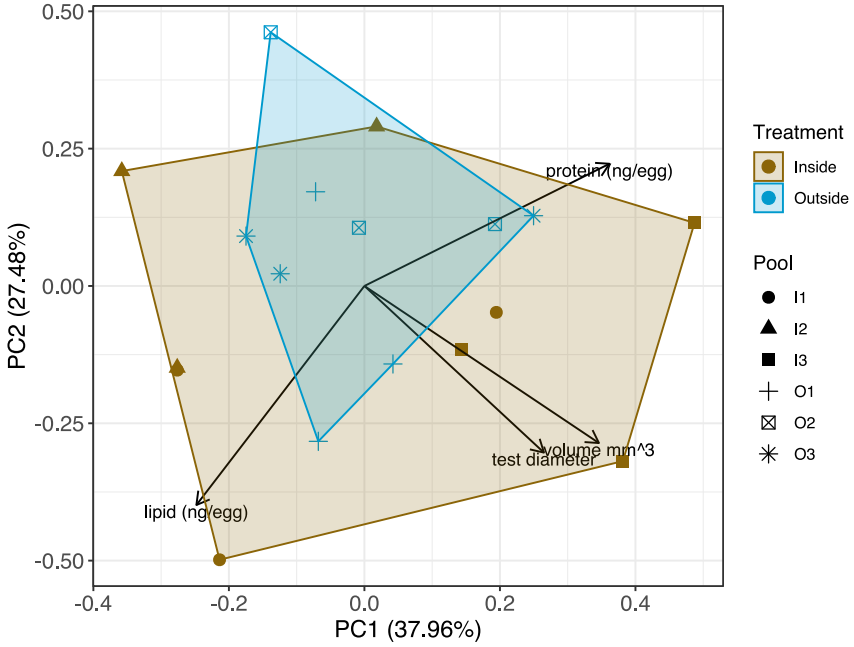


Figure 8. PCA of *Strongylocentrotus purpuratus* egg measurements including egg volume, protein, and lipid, averaged to match individual female test diameter measurements. Maternal group (I: Inside or O: Outside kelp forest) is shown by color I – brown, O – blue and egg pool is shown by symbol.

II. Paternal heat exposure affects larval development in green-lipped mussels, *Perna canaliculus*

Abstract

The green-lipped mussel, *Perna canaliculus*, is critically important to the New Zealand aquaculture industry. However, the recent rise in marine heatwaves (MHWs) poses an acute threat to this industry through summer mortality events. This study investigated the potential for paternally-mediated transgenerational plasticity to improve offspring performance under heat stress. We simulated a week-long MHW event, exposing male *P. canaliculus* broodstock to elevated (22°C) or ambient (17.5°C) temperatures immediately prior to spawning, and evaluated the effects of paternal heat exposure on successful development, size, and lethal thermal tolerance of their larvae that were also reared under ambient or elevated (20°C) temperatures through completion of the lecithotrophic trochophore stage. Elevated paternal and larval temperatures both increased abnormal development, reducing larval yield, while initial D-veliger shell length was predominantly influenced by developmental temperature with longer shells formed at 20°C. The tolerance of the larvae to an additional 1 hour heat-shock was largely conserved, though veligers from heat-exposed fathers raised under 20°C showed a small, but significant, elevation in Lethal Tolerance 50 (LT_{50}), the temperature at which 50% of the larvae are predicted to die. These results indicate that paternal thermal environment over a relatively short period can influence offspring performance in this species. While the costs of increasing paternal temperatures prior to spawning seem to outweigh the benefits for the aquaculture industry, considering the thermal environment of potential broodstock may be important for influencing larval yields. These

findings also have implications for wild spat availability as MHWs are forecasted to continue accelerating.

1. Background & Overview

Our overarching goal in this study was to examine the role of paternal effects in altering progeny thermal tolerance in the context of MHWs. Though hatchery programs in New Zealand have been rapidly expanding in recent years, the majority of the industry still relies on wild spat collected largely from a single area of coastline, 90 Mile Beach on the North Island of New Zealand. Another important source of wild caught spat comes from Golden Bay in the South Island, allowing for extended mussel harvesting periods (Alfaro et al. 2011). For hatchery production, the broodstock that are used are often collected from coastal mussel farms and brought into controlled facilities only shortly before spawning. In the South Island, green-lipped mussels display a bimodal spawning pattern in the austral spring and fall; therefore, a period of peak *P. canaliculus* gametogenesis aligns with the summer MHW events (Buchanan 2001, Alfaro et al. 2011). Both hatchery-derived and wild-collected spat supply and quality may therefore be affected by MHW events mediated through the parental generation. It is important to understand the risks of MHWs to future wild spat supply to inform policy and planning around reducing stressors that impact supply or investing more in hatcheries to sustain the industry. In a hatchery setting, it is critical to consider the roles that the environmental history of each parental line may play in offspring performance with regard to a stressor such as temperature, and whether practices should adjust to take advantage of, or counteract, any potential consequences.

The focus of this investigation was to isolate the transgenerational effects of the paternal line in order to 1) indirectly investigate the role of epigenetic modifications, and 2) consider the advantages or risks to the aquaculture industry of paternal heat exposure. To accomplish this, we simulated a MHW event in the lab, exposed male *P. canaliculus* broodstock to elevated or ambient temperatures for 1 week immediately prior to spawning, and assessed the effects of paternal heat exposure on the successful development, size, and lethal thermal tolerance of the mussel larvae.

2. Materials & Methods

2.1 Experimental design

In March 2019 mature *Perna canaliculus* were transferred from a long-line farm in Pelorus Sound (Marlborough, New Zealand) to a nearby broodstock management facility at the Cawthron Aquaculture Park (Nelson, New Zealand), where they received ambient seawater enriched with elevated natural phytoplankton levels (Ragg et al. 2010). Each individual was induced to spawn (see below) to establish sex and then allowed to restore gametes for a minimum of nine months. In November 2019, randomly-selected adult males were conditioned to either a 1 week thermal challenge at ~22°C (considered heat exposed - H) immediately prior to spawning or continued to be held at ambient temperature, ~17.5°C (considered naïve – N) (Figure 1, Table 1). The naïve (N) temperature, 17.5°C, was selected based on current ambient temperatures in November. The heat exposed (H) temperature, 22°C, was selected to simulate MHW conditions, as it is above average maximum summer SST for the region, but within the range experienced in the South Island during recent MHW events (Thomsen et al. 2019). Females (n = 8) were all held at ambient temperature

(~17.5°C) to isolate the effects of paternal temperature exposure, and pools of eggs from all females combined were each fertilized by individual males from each treatment: naïve, N, and heat exposed, H. Upon fertilization, embryos sired from each father (n = 5 per adult acclimation treatment) were divided into two larval treatments to ~17.5°C (considered ambient - A) or 20°C (considered warm - W) (Figure 1, Table 1). This warm, W, temperature, 20°C, is more commonly experienced than the paternal heat exposure and has been recorded for several weeks during recent MHWs in the Marlborough Sounds region (Broekhuizen et al. 2021). These larval temperatures yielded 4 treatment groups (Figure 1): those from heat exposed fathers (H) raised under warm temperatures (W), HW; those from heat exposed (H) fathers raised under ambient (A) temperatures, HA; those from naïve (N) fathers exposed to warm (W) temperatures, NW; and those from naïve (N) fathers exposed to ambient (A) temperatures, NA. These larvae were raised to the early D-veliger larval stage in static cultures before being assessed for shell size, abnormality, and lethal thermal tolerance to investigate the effects of both the paternal and larval thermal regime on larval performance and phenotype.

2.2 Adult mussel acclimation and spawning

During acclimation, the mussels were held in flow-through aquaria and supplied with mono-cultured algae (*Tisochrysis lutea and Chaetoceros calcitrans*, 1:1 cell ratio, ~40 cells μL^{-1}). Temperature was recorded at 10-minute intervals with *in situ* loggers (Water Temperature Pro v2 Data Loggers, Onset Hobo^{TM}) and verified by daily spot checks. On November 14, 2019, mussels were spawned by "thermal cycling", following industry practices with cycles of 12 and 25°C (Gale et al. 2016). When sperm and eggs had

successfully been collected from all groups, sperm concentrations were calculated from 5 males from each acclimation temperature (17.5 & 22°C) using a hemocytometer; egg concentrations were calculated from 8 females, all from 17.5°C, by counting three aliquots of eggs per female so that a coefficient of variance (CV) of less than 10% was reached. Eggs were visually checked for quality during counting.

2.3 Fertilizations and larval rearing

10 egg pools were made by gently combining eggs from all 8 females in approximately equal numbers to help ensure genetic diversity of the offspring. Each egg pool was then fertilized with the sperm of one father from either the 17.5°C (N) or 22°C (H) exposure treatment. Fertilizations were conducted in seawater pre-incubated with 4 mM EDTA and sperm was added at a concentration of 200 sperm per egg with 1000 eggs mL^{-1} seawater. After gently mixing the fertilization pools for 10-15 minutes, the formation of polar bodies was confirmed by checking aliquots of a few egg pools under the microscope. Fertilized eggs were loaded at a density of 50 embryos mL^{-1} into 3 replicate 400mL plastic beakers, and 6 replicate 4mL tissue culture dish (TCD) wells per treatment filled with EDTA enriched seawater incubated to ~17.5°C (A) or 20°C (W) (Table 1). Embryos from the 400mL static cultures were later used for the thermal tolerance trial at veliger stage and the embryos in the TCDs were later used to assess prodissoconch I shell size and incidence of malformation.

2.4 Larval sampling

Embryogenesis took place in EDTA-enriched seawater at the respective treatment temperatures until the first veliger shell (prodissoconch I) was formed, approximately 38 hours post-fertilization for the 20°C treatment and 41 hours p.f. for the 17.5°C treatment. As the larvae from the 20°C larval treatment developed more quickly, sampling of treatments was staggered in time, with visual assessment and thermal tolerance trials conducted first on the larvae reared at 20°C and then approximately 3 hours later for the larvae reared at 17.5°C, when they had developed complete prodissoconch I shells. When the D-hinge veliger stage was reached in the TCDs for each treatment, the larvae were fixed by the addition of 200µL of 10% formalin and stored at 4°C until they were scored and measured, within 4 days of sampling. When the D-hinge veliger stage was reached in the 400mL culture vessels, larvae were passed through a 43µm mesh to concentrate the larvae and remove dead and degrading tissue. The concentrated larvae were suspended in a uniform volume of 3mL of seawater to approximately standardize concentrations.

2.5 Thermal tolerance trial

To evaluate the effects of paternal heat exposure and developmental temperature on thermal tolerance, larvae from each treatment were subjected to an acute heat shock within a thermal gradient for 1 hour, allowed to recover at ambient temperature overnight, and then scored as alive or dead. 20mL scintillation vials of EDTA-enriched seawater were equilibrated to each of 8 temperatures across a temperature gradient from 28°C to 38°C, using an aluminium heat block. The temperature of each vial was recorded at the start of the trial. A volumetrically equal number of concentrated veliger larvae from all replicates from

each father/treatment combination were pooled together, yielding 2 treatment pools for the 20°C reared larvae: those from heat exposed parents (H) raised under warm temperatures (W), HW, and those from naïve (N) parents exposed to warm (W) temperatures, NW. Thus, the lethal temperature 50 trial (LT_{50} - the predicted temperature at which 50% of the larvae from a given treatment die) was conducted at the scale of the treatment group, not the individual father. Larvae from each pool were gently mixed to achieve a uniform distribution and then 1000 larvae were pipetted into 16 corresponding treatment vials across the temperature gradient, with each treatment having two replicate vials at the same column on the heat block. Control larvae were held in ambient scintillation vials at ~18.3°C during the 1-hour challenge period. Vials were capped, and larvae were incubated at their temperatures for 1 hour. Vials were then removed from the block and allowed to return to ambient temperature. After 24 hours the vials were gently swirled to create a uniform suspension of individuals. A ~1mL aliquot per vial was viewed through an inverted microscope and the first 100 larvae seen were scored as either alive or dead. Larvae were scored based on the signs of movement, even cilial movement regardless of normality. When the 17°C larvae (HA and NA treatments) reached the veliger stage, the pooling, incubation, and scoring process was repeated.

A generalized linear mixed-effects model was used to statistically assess differences in thermal tolerance among treatments, including vial temperature, paternal treatment, larval treatment and their interaction as fixed effects and replicate as a random effect using the lmerTest ((Bates et al. 2007, Kuznetsova et al. 2015) and lme4 packages (Bates et al. 2007) in R (version 3.5.1). A Wald chi-square test was used to assess the significance of each

factor. LT_{50} for each treatment was calculated using logistic regression with the MASS package (Venables & Ripley 2013) in R.

2.6 TCD assessments, normality and larval body size

After preservation in 0.5% formalin, larvae in the 6 TCD wells per father were scored for development and normality. Eggs and larvae were identified to be in 5 categories: egg, "splatter", less than D-hinge veliger, abnormal D-hinge veliger, and D-hinge veliger. Every individual in each well was scored (n = ~180 per well). Previous trials (NLCR – unpublished observations) suggested that early embryos tended to destabilize in the fixative, resulting in a "splatter" of unconsolidated cells. The 'less than D-hinge' and "splatter" categories were later collapsed into a single class representing arrested development. Individuals were categorized as 'abnormal D-hinge larvae' if they had reached the veliger stage but displayed shell or tissue deformities such as a concave, convex, or warped hinge shell, or protruding tissue. Veliger size was measured as the longest length of the shell parallel to the hinge (n = 35 individuals measured per well; cellSens™ image analysis software, Olympus). Statistical comparisons of incidence of normal development were conducted using a generalized linear mixed effects model considering paternal treatment, larval treatment and their interaction as fixed effects and father and assessor as random effects. The model for each stage was fitted using a logit link function. A Wald chi-square test was used to assess the significance of each factor on the proportion of larvae determined to be at a given stage. A generalized linear mixed effects model was also used to compare shell size between treatments with paternal treatment, larval treatment and their interaction as fixed effects and father as a random effect. Statistical tests were run in R using the lmerTest and lme4 packages. Post-hoc tests to

compare individual treatments were conducted in the lsmeans package (Lenth 2013) using the Tukey method to adjust for multiple comparisons.

3. Results

3.1 Summary

Male *Perna canaliculus* broodstock underwent a simulated MHW event, or remained naïve at ambient temperatures, for 1 week immediately prior to spawning, and the effects of paternal heat exposure on the successful development, size, and lethal thermal tolerance of their larvae reared at two developmental temperatures were assessed. In short, we found that 1) both larval and paternal temperature influenced successful development, with higher temperatures having a negative impact, 2) the warmer developmental temperature had a positive effect on the average size of the larvae which reached veliger stage successfully, but also increased the variation in veliger size, and 3) lethal thermal tolerance was high and tightly constrained for all treatment groups, with veligers from heat exposed fathers who were raised under warm developmental temperatures displaying a slightly higher LT_{50}. Results for each response metric are explained in further detail in the sections that follow.

3.2 Larval development: normality

Normality characterizations were performed on 6 replicate TCD well cultures per sire per treatment (NA, NW, HA, HW) (n = 120 total cultures). A higher proportion of individuals developed normally in the 17.5°C ambient (A) larval treatment as compared with the 20°C warm (W) larval treatment (Figure 2). Within each larval treatment, larvae from 22°C heat exposed (H) fathers had higher developmental abnormality than those from 17.5°C

acclimated naïve (N) fathers. Larvae from naïve fathers raised in ambient conditions (NA) had the highest proportion of normal development (87.86 ± 3.20 %), while larvae from heat exposed fathers reared under warm conditions (HW) had the lowest proportion of normal development (77.62 ± 8.75 %). Larvae from naïve fathers raised in warm larval temperatures (NW) and those from heat exposed fathers raised under ambient conditions (HA) exhibited intermediate levels of normal development (80.78 ± 5.64 and 80.32 ± 9.70 % respectively). There was a significant difference in the proportion of normal veligers in the NA treatment group compared to all other groups, and a significant difference between the proportion of normal veligers developed from heat exposed fathers depending on the larval treatment they were reared in (HW relative to HA). Paternal ($\chi^2 = 28.57$, df = 1, p = 9.05e-08) and larval treatment ($\chi^2 = 92.99$, df = 1, p < 2.2e-16) , as well as their interaction ($\chi^2 = 27.35$, df = 1, p = 1.70e-07) explained a significant amount of the variation in percentage of normal larvae.

When considering what proportion of larvae successfully reached veliger stage, regardless of normality and relative to those stalled at an earlier stage, there was a significant effect of paternal ($\chi^2 = 18.80$, df = 1, p = 1.46e-05) and larval treatment ($\chi^2 = 28.89$, df = 1, p = 7.66e-08), as well as their interaction ($\chi^2 = 15.10$, df = 1, p = 0.000102). A higher percentage (90 ± 2.59 %) of embryos from naïve fathers raised in ambient conditions (NA) reached veliger stage than from any other treatment group (86.03 ± 4.88 % of NW, 82.48 ± 9.37 % of HA, 82.78 ± 6.58 % of HW). The proportion of veliger larvae that displayed abnormalities from each treatment group varied significantly by developmental temperature ($\chi^2 = 83.96$, df = 1, p < 2e-16), but not by paternal temperature ($\chi^2 = 2.63$, df = 1, p = 0.1052) or their interaction ($\chi^2 = 2.98$, df = 1, p = 0.0845). Veligers from the warm developmental treatment (NW and HW) had a relatively higher frequency of abnormalities (6.11 ± 3.57%

and 6.43 ± 4.54 % respectively) compared to those from the ambient developmental treatment (2.38 ± 1.84 % for NA and 2.66 ± 2.67 % HA). Finally, more eggs remained undeveloped in the 17.5°C ambient larval treatment than in the 20°C warm larval treatment (χ^2 = 11.75, df = 1, p = 0.00061) (Table 2). Paternal temperature did not have a significant effect on the number of eggs remaining (χ^2 = 1.44, df = 1, p = 0.23).

3.3 Larval development: shell size

Length of the veliger shell was significantly influenced by larval developmental temperature (p < 2e-16) as larvae developing in 20°C were generally longer (89.96 ± 2.37µm for NW and 90.33 ± 2.49µm for HW) than those developing in 17.5°C (88.36 ± 2.10µm for NA , 88.98 ± 2.11µm for HA) (Figure 3). Larvae from heat exposed fathers were on average 1.34µm longer when raised under warm conditions compared to ambient, while larvae from naïve fathers were on average 1.60µm longer when raised under warm conditions compared to ambient. Veligers from heat exposed fathers tended to be longer than their naïve sired counterparts, though this effect was not significant (p = 0.0530) and the interaction between paternal and larval treatment was also not significant (p = 0.0563). Under ambient developmental conditions veligers from heat exposed (H) fathers were on average 0.62µm longer than veligers from naïve (N) fathers, while under warm developmental conditions larvae from H fathers were on average 0.37µm longer than those from N fathers. These results were consistent with a preliminary trial that manipulated temperature during early development of *P. canaliculus*, in which our results showed that veliger shell lengths increased with increasing temperature across a range from 17 to 21°C (Supp. Figure 1).

There was higher variation in veliger shell size among individual larvae from the 20°C larval treatments, NW and HW, (Coefficient of Variation: 2.63 and 2.76% respectively) compared to the 17.5°C ambient larval treatments, NA and HA (Coefficient of Variation: 2.38 and 2.37% respectively). Although the size ranges of larvae from each sire within a treatment largely overlapped, there was slightly more variation in average size of a veliger from different heat exposed sires relative to naïve sires whose larvae displayed a more constrained average size (Figure 3, inner boxplot). The mean veliger size from each heat exposed father had a variance of 0.32µm in the ambient larval treatment, and 0.36µm in the warm larval treatment, while the mean veliger size from each naïve father had a variance of 0.16 and 0.15µm in the ambient and warm larval treatments respectively.

3.4 Larval performance: thermal tolerance

In the thermal tolerance trial, paternal temperature ($\chi^2 = 5.90$, df = 1, p = 0.0151), larval temperature ($\chi^2 = 5.11$, df = 1,p = 0.0238), and the interaction between paternal and larval temperature ($\chi^2 = 4.92$, df = 1,p = 0.0265) explained a significant amount of the variation in the binomial survival data. Yet, the LT_{50} for each treatment group, the lethal temperature at which 50% of the larvae died, was tightly constrained around 36°C (Figure 4). The LT_{50} was highest for larvae from heat-exposed fathers raised under the warm developmental treatment (LT_{50} HW = 36.27 ± 0.05°C). The LT_{50}s for all other treatment groups were slightly lower, (LT_{50} NA = 36.16 ± 0.05°C, NW = 36.12 ± 0.05°C, HA = 36.15 ± 0.05°C).

4. Discussion

4.1 Summary of results

In this study, we investigated the effects of male *Perna canaliculus* broodstock heat exposure prior to spawning on larval development and performance under ambient and warm developmental temperatures. The salient findings were that 1) elevated paternal and developmental temperatures both hindered normal larval development, 2) larvae which reached veliger stage successfully grew larger on average under warm developmental temperatures, as did those from heat exposed fathers, though the paternal effect on size was not significant, and 3) veligers from heat exposed fathers raised under warm developmental temperatures had the highest LT_{50}, though lethal thermal tolerance was high and very similar for all treatment groups. Overall, the persistent increase in MHWs in New Zealand may have detrimental effects on green-lipped mussel aquaculture, as elevated temperatures negatively impact larval development in the wild and heat exposed fathers produce decreased larval yields in hatcheries.

4.2 Context of acclimation temperatures

MHWs are already an ongoing and escalating threat in New Zealand. The temperatures used to induce thermal stress in this experiment, 22°C for the fathers and 20°C for the offspring (Table 1), are within the range currently possible in the South Island, where the Marlborough Sounds encompass the largest mussel growing area in New Zealand (Heasman et al. 2020). A higher temperature was used for the paternal exposure in order to simulate an acute 1 week MHW event, around the upper limit of temperatures currently experienced during MHWs in the region. SST in sheltered regions of the South Island such as

Pile Bay, exceeded 23°C for many days in the 2017-2018 MHW (Thomsen et al. 2019). Temperature dynamics can vary significantly within the Marlborough Sounds in contrast with the open coastline, however temperatures in the Sounds remained over 20°C for several weeks during recent MHWs (Broekhuizen et al. 2021). This more common temperature, 20°C, was used for the offspring developmental warm temperature to ensure some offspring survived with the hope of being able to distinguish differences in treatment groups. Large numbers of larvae are needed for successful spat recruitment so small changes in developmental success can be significant ecologically and commercially. As green-lipped mussels tend to have a bimodal spawning behaviour in the South Island, with one period of gametogenesis in winter followed by spawning in early spring, and a second period of gametogenesis in summer preceding a spawning event in late summer - early fall (Buchanan 2001), the alignment of these MHWs with gametogenesis is likely concentrated heavily around the summer spawning period. Based on the results of this study this temporal alignment could have strong ecological consequences going forward. The North Island populations typically show a broader single spawning period from June to December as temperatures are increasing from their lowest point, which may indicate that paternal exposure to positive thermal anomalies prior to spawning to be less likely for these populations (Alfaro et al. 2001).

4.3 Effects of the larval developmental environment

Our results showed that the warm (W) developmental environment slightly but significantly increased the size of newly formed veliger larvae (Figure 3). This pattern was consistent with our preliminary trials manipulating solely developmental temperature (Supp

Figure 1). There was also higher variance in size among the larvae in the warm developmental treatment. Growth rate in bivalve mollusc larval development from veliger to spat is generally positively correlated with developmental temperature, though temperature variation also interacts with other factors of the larval environment such as salinity, food availability, and pCO_2 (Rico-Villa et al. 2009, Lazo & Pita 2012). Size at metamorphosis has been shown to be largely independent of temperature in some species and temperature-dependent in others (Pechenik et al. 1990, Rico-Villa et al. 2009, Galley et al. 2010, Lazo & Pita 2012). In these studies, there appears to be a trade-off between growth rate and size at metamorphosis with regard to temperature, with high temperature driving faster development but smaller sizes at settlement. For *P. canaliculus*, size at metamorphosis appears essentially fixed, hence larval growth rate directly influences recruitment into the juvenile population (Ragg et al. 2019). Importantly, the environmental conditions experienced during embryogenesis in *P. canaliculus* can have latent effects, with subtle differences in the size and morphology of the first D-veliger stage subsequently amplifying into extended growth, survival and recruitment differences (Ragg et al. 2019). Faster growth under warmer conditions could alter the length of the free-living larval stage and the distribution and abundance of new recruits, affecting population connectivity, the structure and function of intertidal and subtidal communities, and impact commercially important spat catching sites. Future research could productively focus on both the direct and carry-over effects of warming on *P. canaliculus* larval development and recruitment, considering the complex interactions of energetic trade-offs (Sprung 1984), disease vulnerability (Travers et al. 2015), size-dependent predation (Pechenik 1999) and, in a hatchery setting, the influence of size-grading and culling (Helm et al. 2004).

The effects of warm water temperature on incidence of abnormal development and thermal tolerance were more complicated and mediated by the paternal treatment. Fewer larvae from the warm (W) developmental treatment made it successfully to the veliger stage or formed a normal veliger shell (Figure 2). For those larvae from naïve (N) fathers, the larval temperature had a significant effect on both metrics. However, for larvae from heat exposed (H) fathers, the warm developmental temperature only had a negative effect on the number of normal veligers formed, but if malformed individuals are also considered, the total number of larvae reaching veliger stage held constant. The number of normal veligers is likely a more important distinction as the abnormal veligers are unlikely to survive through the larval bottleneck, especially in the wild (Helm et al. 2004, Ragg et al. 2019). Under warm developmental temperatures, these larvae are likely to develop a little more quickly and grow to a slightly larger size, particularly those from heat exposed fathers which could help to offset the survival costs of higher abnormality at the population level.

Some species, such as Sydney Rock Oysters, *Saccostrea glomerata,* display an optimum temperature for larval performance in terms of fertilization, survival, and normal development, with decreasing performance on either side of this optimum (Parker et al. 2009). Though the optimum for *P. canaliculus* cannot be determined from the current experimental design, negative impacts of high temperature on development may set in below 20°C. Interestingly, more individuals remained at the egg stage without developing further under the ambient (A) larval developmental treatment, regardless of paternal temperature. As fertilization was carried out under identical conditions, following robust protocols for this species (Adams et al. 2009), it was considered unlikely that the differences reflected variability in fertilization success; it is however possible that this may have been an artifact

of warmer temperatures in the (W) larval development treatment driving rapid decomposition of unfertilized eggs into a state not clearly identifiable as an egg, resulting in these individuals being categorized with the other 'less than D' embryos.

4.4 Effects of the paternal acclimation environment

Paternal heat exposure was associated with a slight (non-significant) increase in veliger size (Figure 3). However, the effects on normal development were largely negative, with a lower proportion of larvae from heat exposed (H) fathers successfully reaching veliger stage (Figure 2). As size was only assessed in larvae that successfully reached veliger, this slight size trend towards larger veliger larvae from heat exposed fathers is more likely a selective artifact than a positive transgenerational effect. Because fewer veligers successfully developed from these groups, the ones who did reach this stage to be measured may have been the ones with the most advantageous genetic makeup, resulting in a skewed sample.

Within the ambient (A) larval treatment, a lower proportion of larvae from heat exposed (H) fathers reached veliger stage or formed a normal veliger shell. These same trends were evident within the warm (W) developmental treatment but were non-significant. The fact that these negative effects of paternal acclimation environment were seen even when the offspring were raised under ambient temperature means that they could be a significant factor driving yields, even in a hatchery setting where rearing temperature can be controlled. In addition, in our study there was more variability in survival to veliger and normal development from heat exposed (H) fathers than naïve (N) fathers (Figure 2, Supp Figure 2). This observation accentuates the need to consider the interaction between underlying genetics and transgenerational effects. This same increase in variation between larvae from

different heat exposed (H) fathers in contrast to different naïve (N) fathers can also be seen in the veliger size data (Figure 3, Inner Boxplots).

The presence of negative paternal effects is consistent with some other studies. In another system exploring ecologically relevant temperatures in a MHW context, Leach et al. saw that for the purple urchin, *Strongylocentrotus purpuratus*, sperm from males acclimated to high temperatures had reduced fertilization success, despite the sperm being resilient to high temperatures during the fertilization process itself (Leach et al. 2021). In experiments manipulating the thermal environment of the marine tubeworm, *Galeolaria caespitosa*, fertilization success was largely driven by paternal environment with males from warm temperatures generally showing the lowest fertilization success regardless of the thermal environment of the female in the cross (Guillaume et al. 2016). Larvae from warm-acclimated fathers also had higher rates of arrested larval development similar to our results.

4.5 Caveats of LT50 and disagreement with previous studies

The combination of elevated paternal and larval temperatures did correlate with the highest larval LT_{50}, 36.27 ± 0.05°C, a mean increase of 0.11-0.15°C (Figure 4). This is much higher than the LT_{50} of *P. canaliculus* larvae in the literature, which was previously found to be 32.9-33.9°C (Dunphy et al. 2013). The substantial discrepancy to the prior LT_{50} with that seen in the treatments in this study is likely driven by methodological difference. In Dunphy et al., 2013, stationary larvae were considered dead if they displayed uncontrolled gaping, gut extrusion through valves, or a loss of defined internal structures, whereas in the present study larvae were only scored as dead if they exhibited no movement, even of cilia. In a study of *Mytilus californianus* larvae which used similar death metrics to the present study (no

movement or dull opaque shells), LT_{50} reached up to 35.17°C, indicating support that methodology may help to explain the discrepancies (Waite & Sorte 2022). Therefore, though the treatment pattern observed in our experiment is still interesting, 32.9-33.9°C is a more ecologically relevant LT_{50} range. Dunphy et al. found no latitudinal gradient along the South Island in LT_{50} of F2 veligers (Dunphy et al. 2013). In combination with this study, which saw little variation in LT_{50} induced by paternal and larval acclimation, with the exception of a small increase with combined paternal and larval heat exposure, larval LT_{50} may be largely fixed for these populations, resistant to variation driven by acclimation or local adaptation. Based on either study, this early life stage exhibits a large thermal safety margin with regard to even extreme current temperatures, at least for short-term exposure. This is consistent with the assertion that sub-lethal chronic stress may be a better predictor of future performance and distributions of species and populations, as species with similar upper thermal limits can have different stress responses which influence their ecological interactions. This theory was put forth by Dunphy et al. with respect to *P. canaliculus* larvae and Tagliarolo and McQuaid with respect to adult mussels, *Perna perna* and *Mytilus galloprovincialis* (Dunphy et al. 2013, Tagliarolo & McQuaid 2015). The survival bottleneck which mussels, and many other marine invertebrates, undergo during early stages due to the challenges of predation and finding suitable settlement habitat may make sublethal stress which reduces the likelihood of survival particularly impactful. Adult *P. canaliculus* have a lower LT_{50} than other marine mussel species in NZ, so if this tracks with sublethal stress as well, this species may be more vulnerable than other, less commercially valuable species (Sorte et al. 2019).

In this study, for the three phenotypic metrics assessed (successful development, LT_{50} and size) negative, positive, and non-significant paternal effects were observed. This

accentuates the need for further studies into the mechanisms driving paternal effects and careful consideration when interpreting results in order to determine which phenotypic differences may be most ecologically significant or industry-relevant.

4.6 Takeaways for wild spat

Currently the New Zealand aquaculture industry relies on both hatchery-derived and wild-caught *P. canaliculus* spat, so we must consider the implications of these results for both supplies in the future. Wild recruitment, particularly for the summer/fall spawning period, is likely to be significantly influenced by MHWs going forward. Hayden (1995) found that the supply of early larvae and settlers is the primary driver of the number of wild *P. canaliculus* recruits in the Marlborough Sounds 8 weeks after settlement, after which point other factors begin to play a more significant role (Hayden 1995). So a decrease in larval supply, either by adult mortality (Li et al. 2020), or arrested larval development due to paternal effects or larval environment could drastically reduce spat supply. Temperature has been incorporated into spat forecasting models, with positive temperature anomalies one month prior having a positive correlation with settlement at some South Island sites, specifically when coupled with other environmental factors associated with La Niña episodes, and large tidal swings (Atalah & Forrest 2019). However, a positive temperature anomaly was considered when the observed temperature was warmer than the 25 yr monthly average and did not examine the effects of MHW explicitly. For Ninety Mile Beach, daily and seasonal water temperatures have not been shown to influence the timing or the scale of spatfall events and the natural gametogenesis and spawning cycle for the North Island falls within the winter/spring months and so is less likely to be affected by MHWs in the same

way as the South Island populations (Alfaro et al. 2010). As MHW events become more prevalent it will be critical to track their imprint on wild spat recruitment over time.

4.7 Takeaways for hatchery aquaculture

In a hatchery setting, temperatures through early development can more easily be controlled. However, the results of this study indicate that breeders should carefully consider the thermal conditions of their broodstock immediately prior to spawning to support the desired results. In the present study we found that paternal heat exposure as short as 1 week had negative impacts on the successful early development of larvae. Adjusting for this may involve bringing broodstock into a controlled hatchery setting earlier during a MHW and holding them under lower temperatures prior to spawning, though further studies would be required to see if this type of recovery period would have the desired effect, and the duration of recovery required.

If broodstock are to be brought in and spawned immediately, thermal variation across space and depth of the farms where they are sourced could be leveraged for a similar result. Temperatures in the Marlborough Sounds can vary significantly over short scales, across space and depth. In peak summer, late January 2020, between 1m and 10m depth at the same site in Clova Bay, the temperature varied by up to 4.59°C (Supp Figure 3). This depth difference spans the depth range of many mussel ropes. The maximum temperature reached at 1m depth during this period was 22.13°C, the heat exposure used in the paternal challenge reported here. Between the middle of October to early March, the temperatures across 9 sites throughout the Marlborough Sounds varied by a maximum of 5.14°C and an average of 2.17 ± 0.81°C at any given time (Delorme, South, unpublished data). This natural thermal

variation provides the ability to select broodstock from more desirable thermal environments, across sites or depth within one site depending what is available to the operation in question. Knowledge of local thermal variation and biological understanding of within and across generation carryover effects from thermal exposure, through studies such as this, can be coupled with improved MHW forecasting in order to proactively expand options for farms to maintain yields under extreme conditions (Holbrook et al. 2020, Jacox et al. 2022).

4.8 Necessary future work

The results of this study are compelling in that they indicate a negative paternal effect induced by a relatively short thermal exposure, of the order possible in a MHW scenario. However, there is much work to be done to untangle the mechanisms and consequences of paternal effects and how they interact with other factors such as maternal effects, underlying genetics, and different timescales.

It is critical to consider each parental line when investigating transgenerational effects and their interaction, but few studies have successfully done so to date. Previous work does indicate that transgenerational effects can vary within the same species when transmitted through the maternal or paternal lineage (Lane et al. 2015, Venkataraman et al. 2019). For two marine tubeworms, both paternal and maternal thermal experience influenced offspring performance in *Galeolaria caespitosa*, with paternal effects playing a larger role (Guillaume et al. 2016); while for *Hydroides elegans* maternal and paternal low pH exposure had both opposite and additive effects (Lane et al. 2015). It is possible that time scale of exposure may also influence the two parental lines differently given the offset in the length of gametogenesis for each sex in most species.

The parental capacity to mobilize a transgenerational acclimatory response can also be dependent upon their underlying genetics, influencing both the magnitude and direction of effects (Parker et al. 2012, Parker et al. 2015, Goncalves et al. 2016). Previous population genetic studies of *P. canaliculus* have found a distinct break between the North and South Island populations at the Cook Strait (Apte et al. 2003, Wei et al. 2013). Wei et al. (2013) found that sea surface temperature explains more of the genetic variation between populations and individuals than any other geographic or environmental variable considered, suggesting a selective force of temperature acting upon this species. This combination of reduced gene flow and selective pressure could provide the impetus for local adaptation to warmer temperatures. The combination of this natural genetic variation in wild populations along with industry selective breeding programs could strongly influence the effects of parental conditioning in this species.

Furthermore, it is important to consider the timing, duration, and predictability of environmental stressors when considering transgenerational effects (Burgess & Marshall 2014). Guillaume et al. saw that a reduction in offspring performance driven by paternal effects was especially severe in environments that varied compared with those that were stable (Guillaume et al. 2016); thus, the strength of the effects observed in our experiment may vary if the treatments had been held constant for longer. However, in another species of marine tubeworm, *Hydroides diramphus,* Jensen et al. noted that gamete phenotype was determined by the salinity conditions each parent experienced immediately prior to reproduction (Jensen et al. 2014). In the case of the green-lipped mussel, effects may manifest differently between the spring and summer/fall spawning periods as the match between paternal experience and larval environment could be drastically different if a MHW

precedes spawning or arises while larvae are in the water column. A week of simulated heat wave exposure is sufficient to bring about significant changes in bivalve gonad morphology (Vázquez et al. 2021) and influence subsequent embryogenesis (current study); it is therefore important to determine whether even more acute thermal challenge, including the thermal cycling used to induce spawning in *P. canaliculus* (Gale et al. 2016), may also influence offspring phenotype.

Finally, considering the entire life history may also be valuable. For instance, high temperature exposure during adulthood led to reduced sperm count and lower sperm quality in crickets, while the effects on sperm were reversed if the crickets were exposed earlier in development, prior to adulthood (Gasparini et al. 2018). In *Drosophila*, sire temperature had a slightly negative effect on overall fecundity of their offspring (Huey et al. 1995). In a hatchery setting, tracking the same F1 mussels beyond early development through adulthood, or even their F2 progeny, could reveal if there are carryover effects in fecundity or grandparental effects (Parker et al. 2015). This was unfortunately not feasible within the constraints of the current study.

4.9 Conclusions

Overall, our study raises a critical concern that paternal effects can influence offspring phenotype in *P. canaliculus* and that negative consequences for larval development can be induced after just 1 week of paternal heat exposure. In addition, elevated larval temperatures also negatively impact larval developmental success. Therefore, for green-lipped mussels, the negative impacts of MHWs which are rapidly becoming a persistent threat in New Zealand, will be felt not only within the generation of exposure but across generations through the

paternal line. Both wild populations and industry yields will likely decline in the coming years as MHWs pose an increasing threat world-wide, including New Zealand. Though New Zealand may suffer less in terms of average temperature increase relative to tropical regions, the last decade has shown that these acute heat wave events are likely to shape the future of aquaculture in the country. Warmer temperatures already have harsh effects both directly on organismal growth and performance but also indirectly through food chain disruption and higher incidence of disease and toxins (Lake et al. , KPMG 2020, Salinger et al. 2020). Aquaculture will be critical to meet our future global protein needs; however, the effects of climate change and MHWs will vary greatly based on the spatial extent, geographic region, and species considered and urgently require dedicated research and consideration going forward (Weatherdon et al. 2016, Barange et al. 2018, Holbrook et al. 2020, Oyinlola et al. 2020). This study highlights the need to consider the transgenerational implications of these MHW events when attempting to disentangle their consequences for the industry.

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I am grateful to my collaborators, Drs. Gretchen Hofmann, Nikki Hawes, and Norman Ragg. This work was supported by the Cawthron Shellfish Aquaculture Research Platform funded by the New Zealand Ministry for Business, Innovation and Employment. The authors are grateful to the Cawthron Aquaculture Park team for their support, especially Joanna Copedo for generously sharing her knowledge and time. LK was supported by a National Science Foundation Graduate Research Fellowship and this research was supported by an award from the Schmidt Family Foundation to LK. I am grateful to Dr. Erika Eliason and Dr. Halley Froehlich for feedback in the writing of this chapter.

Figures & Tables

Table 1
Temperatures for adult conditioning and larval development of *Perna canaliculus* measured every 5 minutes using Water Temperature Pro v2 Data Loggers (Onset Hobo™).

		Temperature (˚C)
Adult treatment	Heat Exposed (H)	22.04 ± 0.68
	Naïve (N)	17.51 ± 0.05
Larval treatment	Warm (W)	19.78 ± 0.29
	Ambient (A)	17.63 ± 0.29

Table 2
Mean percentage and standard deviation of embryos from each treatment that were found in each state of development and normality. Different letters among treatments represent statistically significant differences for a particular state of development ($p < 0.05$) after a Tukey multiple comparisons test. Treatments are as follows: NA - larvae from naïve (N) sires raised in ambient (A) larval temperatures, NW- larvae from naïve (N) sires raised in warm (W) larval temperatures, HA - larvae from heat exposed (H) sires raised in ambient (A) larval temperatures, HW- larvae from heat exposed (H) sires raised in warm (W) larval temperatures.

Treatment	***% D-hinge veliger***	***% abnormal D-hinge veliger***	***% less than veliger***	***% Egg***
NA	87.86 ± 3.20^{a}	2.14 ± 1.58^{a}	9.27 ± 2.55^{a}	0.73 ± 0.66^{a}
NW	80.78 ± 5.64^{b}	5.25 ± 3.10^{b}	13.75 ± 4.91^{b}	0.23 ± 0.38^{b}
HA	80.32 ± 9.70^{bc}	2.16 ± 2.24^{a}	16.42 ± 8.94^{b}	1.10 ± 1.13^{a}
HW	77.62 ± 8.75^{c}	5.16 ± 3.50^{b}	17.04 ± 6.61^{b}	0.18 ± 0.30^{b}

Figure 1

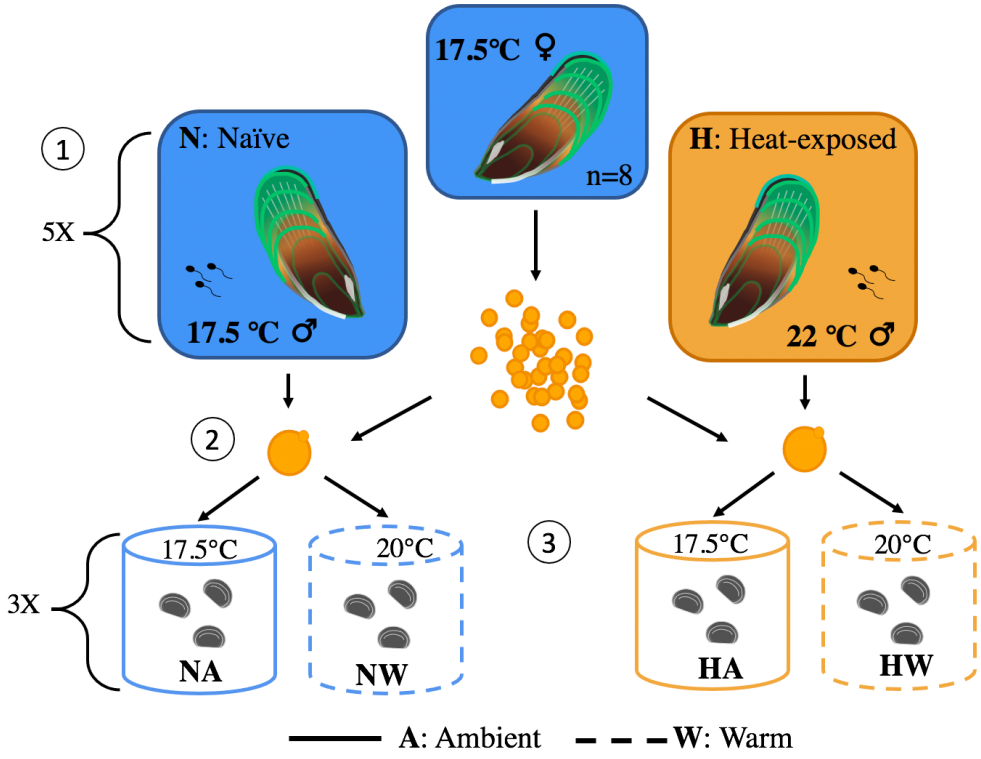


Figure 1. Experimental design. 1) Male *Perna canaliculus* mussels were held at 17.5°C, naïve (N), or 22°C, heat-exposed (H) for one week prior to spawning. 2) The conditioned fathers (n=5 per treatment) were individually crossed to pooled eggs from 17.5°C mothers. 3) Fertilized eggs from each of the 10 crosses were then divided into 17.5°C, ambient (A), or 20°C, warm (W) treatments to develop until the veliger larval stage (n=3 larval culture replicates per combination of father and developmental temperature). There are four final combinations of paternal and larval treatment: larvae from heat-exposed fathers (H) raised under warm temperatures (W), HW; larvae from heat-exposed (H) fathers raised under ambient (A) temperatures, HA; those from naïve (N) fathers exposed to warm (W) temperatures, NW; and those from naïve (N) fathers exposed to ambient (A) temperatures, NA.

Figure 2

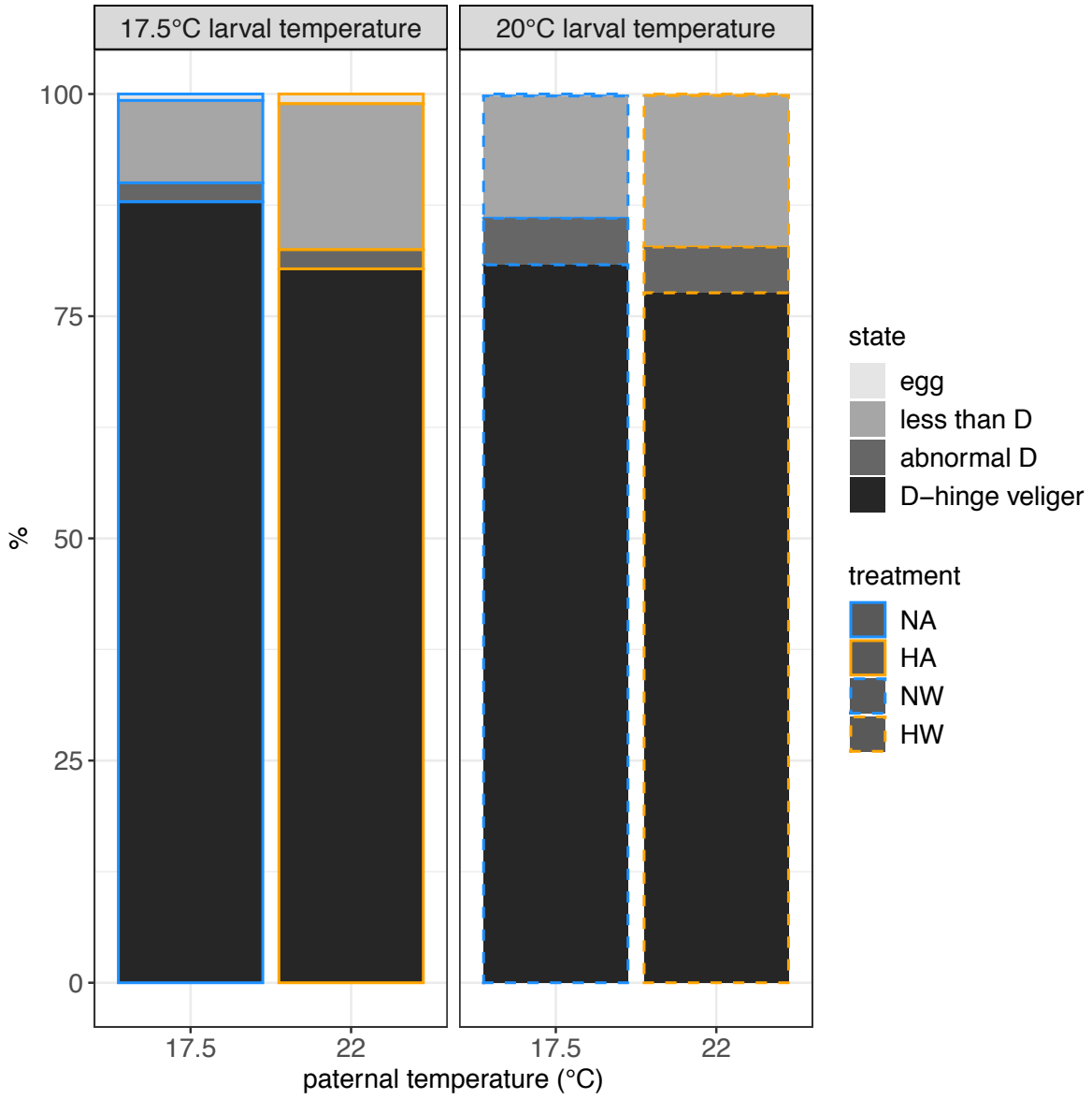


Figure 2. Effect of paternal (H: heat-exposed or N: naïve) and larval (A: ambient or W: warm) treatment on larval development and normality of *Perna canaliculus*. Percentage of embryos in a given state is shown by internal bar shading. Paternal group is shown by color and larval treatment is shown by pattern, NA – blue, solid line; HA - orange, solid line; NW – blue, dashed line; HW - orange, dashed line.

Figure 3

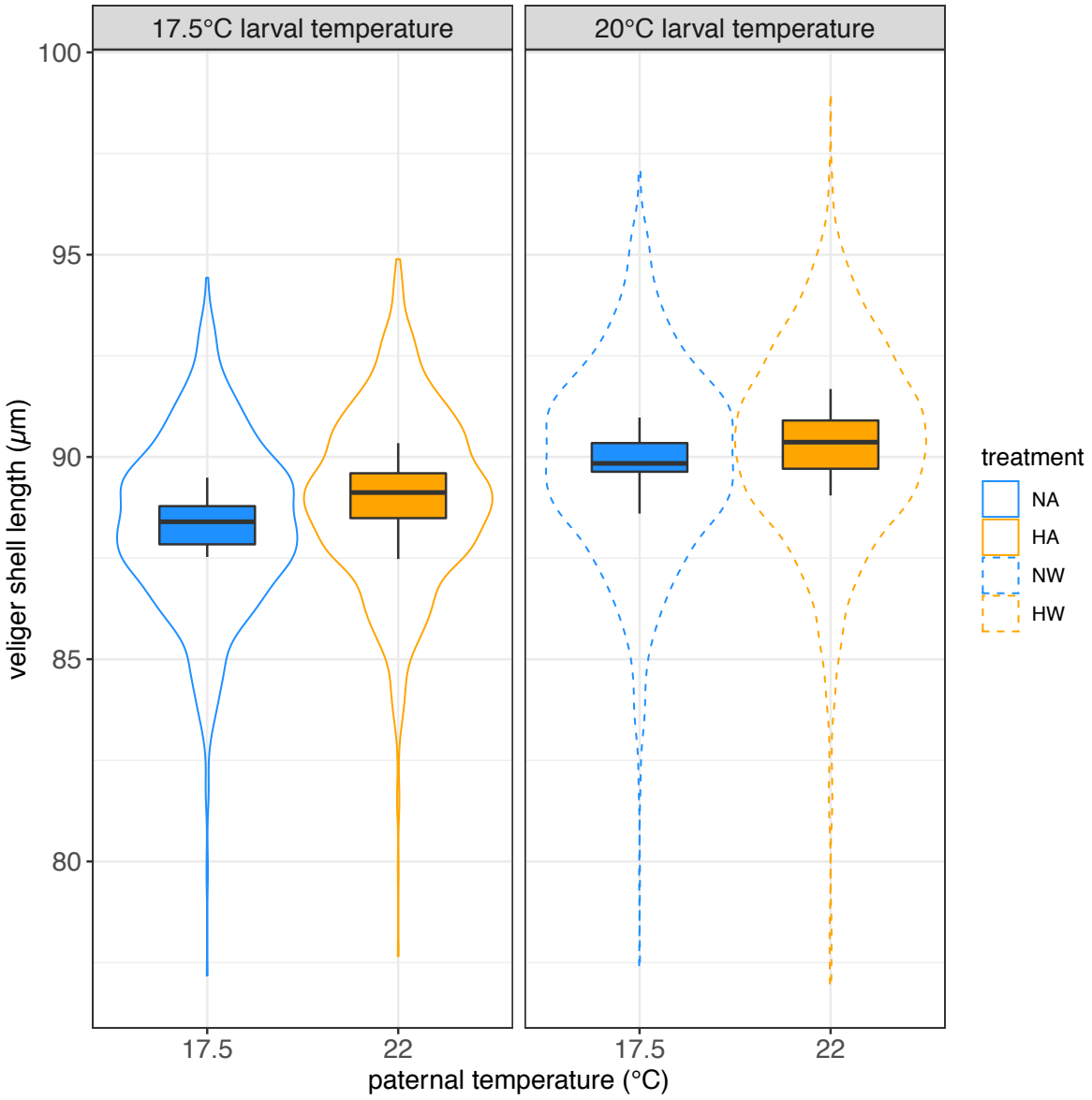


Figure 3. Effect of paternal (H: heat-exposed or N: naïve) and larval (A: ambient or W: warm) treatment on first D-hinge veliger shell length (µm) of *Perna canaliculus*. Inner boxplots display the variation between average veliger size calculated for each replicate for each father per treatment, whereas the outer violin plot shows the variation between each individual larvae in a treatment. Paternal group is shown by color and larval treatment is shown by pattern, NA – blue, solid line; HA - orange, solid line; NW – blue, dashed line; HW - orange, dashed line.

Figure 4

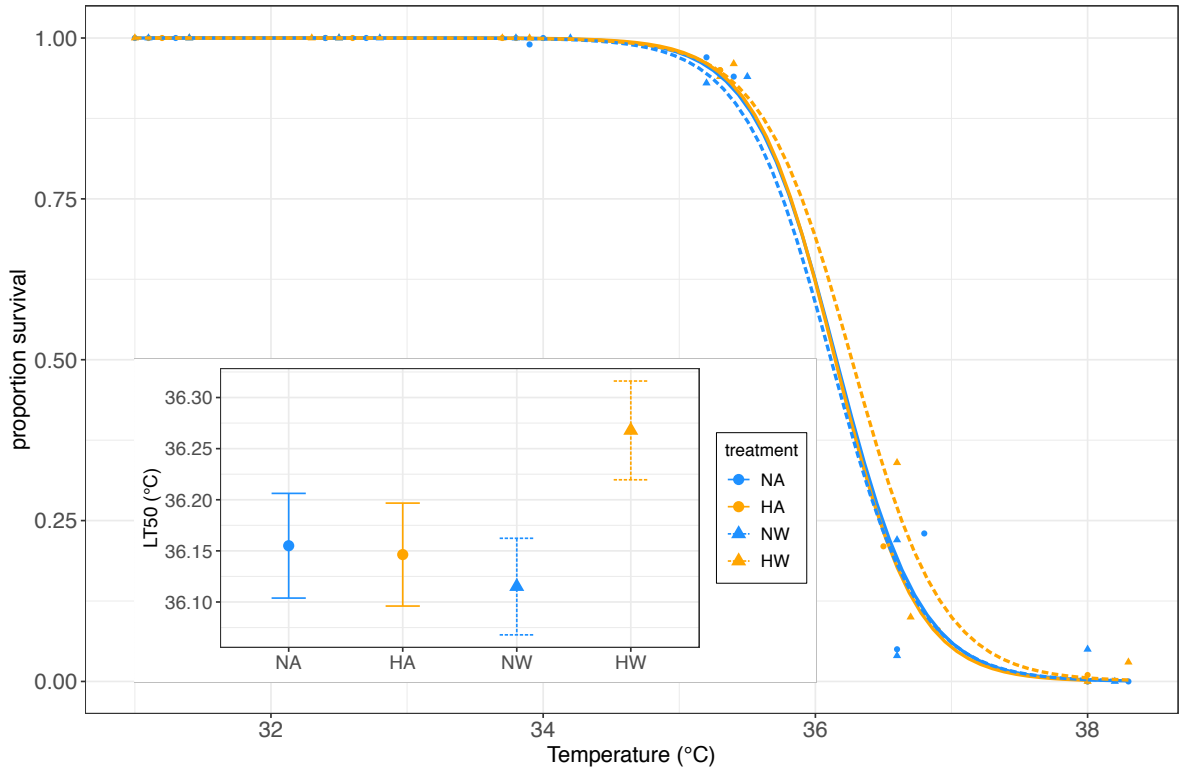


Figure 4. Effect of paternal (H: heat-exposed or N: naïve) and larval (A: ambient or W: warm) treatment on veliger thermal tolerance of *Perna canaliculus*, measured as proportion survival after 1 hour acute heat shock and recovery. Acute heat shock temperature is shown on the x-axis. Temperatures below 31°C have been removed from the graph for ease of visualization. Inset displays the calculated Lethal Temperature 50 (LT_{50}) for each treatment, error bars represent mean ± standard error. Paternal group is shown by color and larval treatment is shown by pattern, NA – blue, solid line; HA - orange, solid line; NW – blue, dashed line; HW - orange, dashed line.

Figure 5

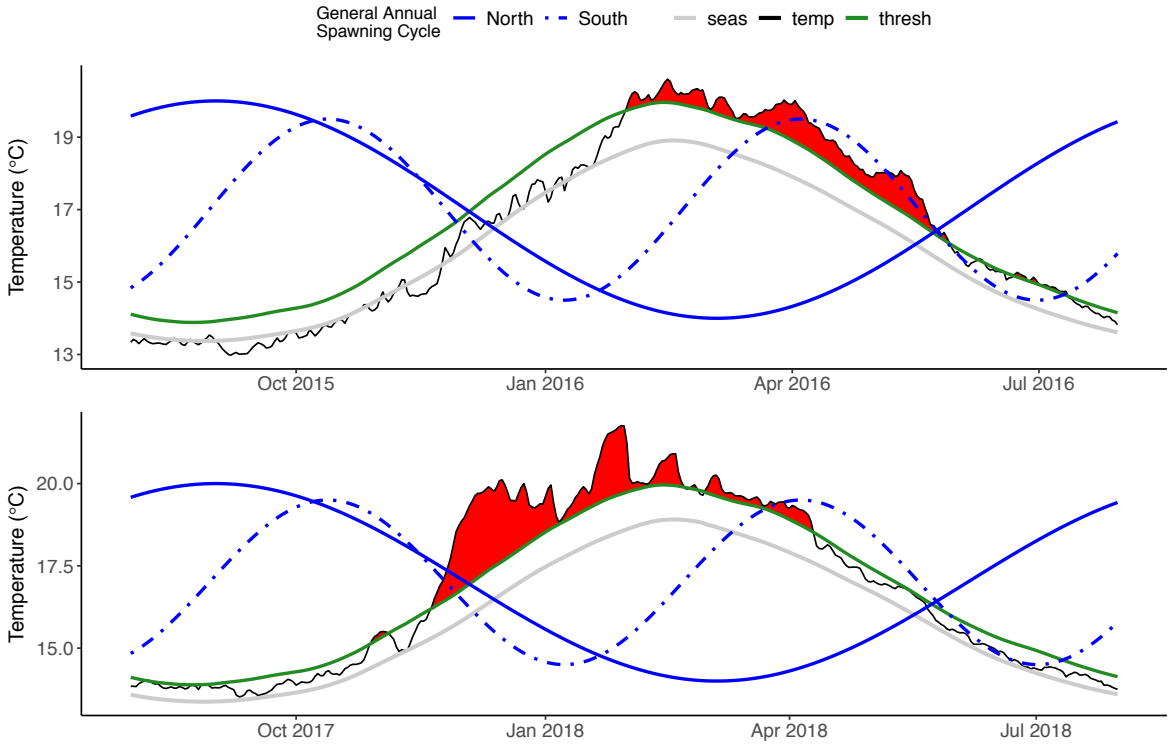
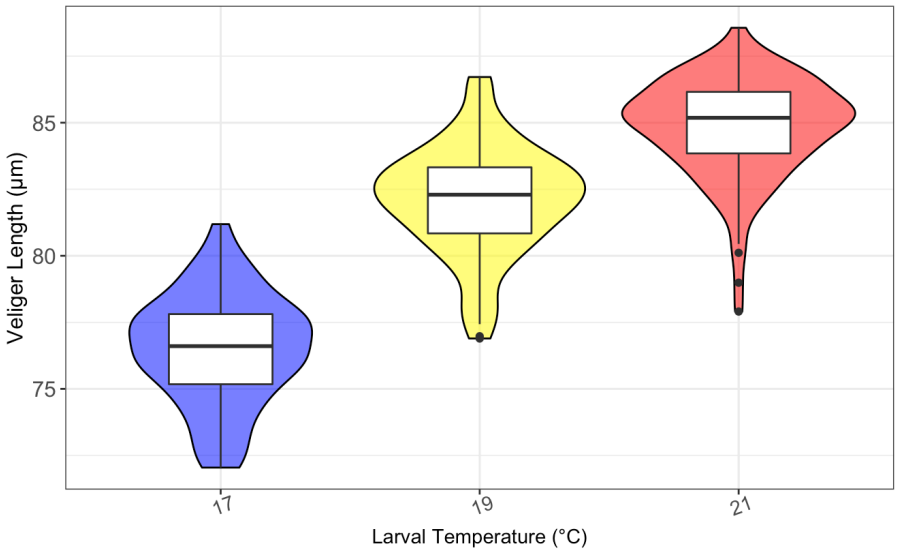


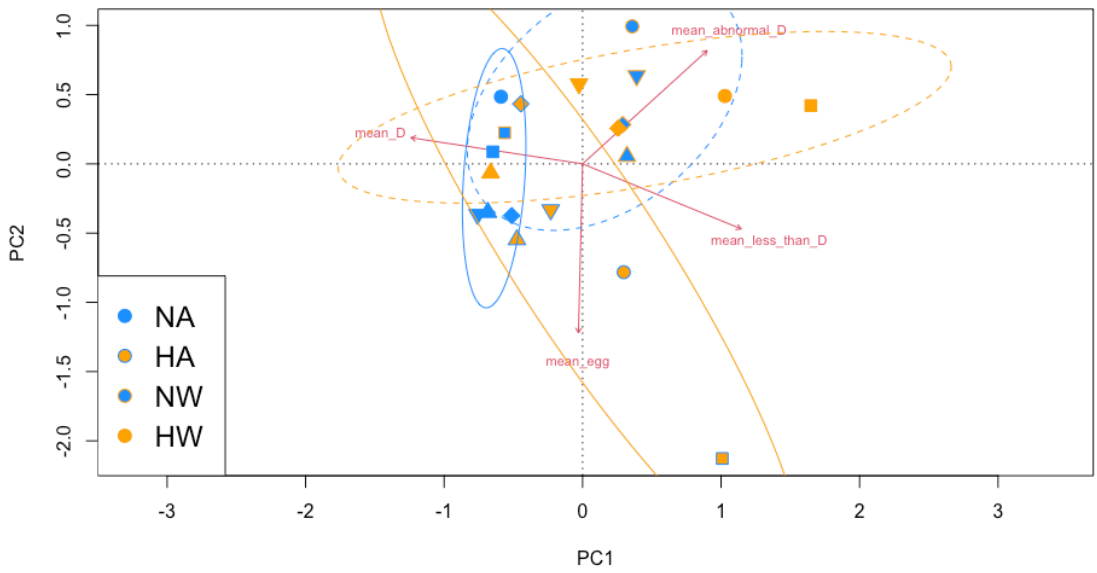
Figure 5. Conceptual diagram depicting general spawning cycle for *Perna canaliculus* populations on the North Island (solid blue line) and South Island (dashed blue line) of New Zealand alignment with two recent marine heatwaves (MHWs) (Alfaro et al. 2011). The upper and lower panel display two examples of representative marine heatwaves impacting the Tasman Sea in 2016 and 2018 visualized in heatwaveR (v 0.4.5), as events persisting about the 90th percentile threshold (green) above the seasonal climatology (green) for least 5 days (Schlegel & Smit 2018) using NOAA 1/4° Daily Optimum Interpolation Sea Surface Temperature (OISST) data (Huang et al. 2021) for the region between -45 and -35 degrees latitude and 165 and 175 degrees longitude.

Supplementary Figure 1

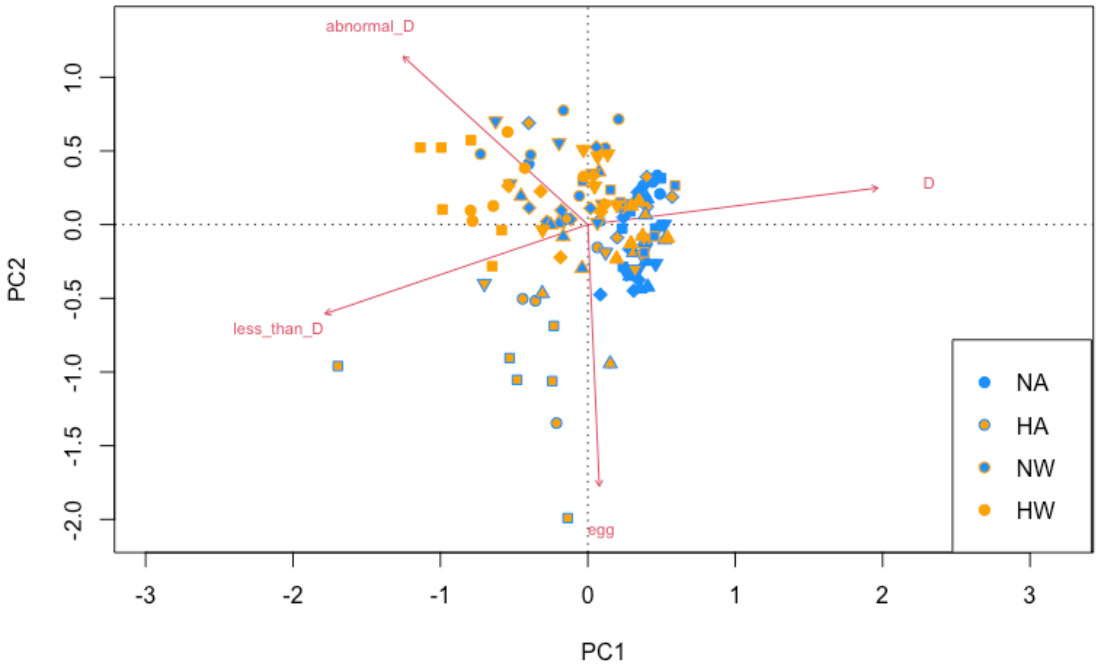


Supplemental Figure 1. Veliger shell length (µm) of *Perna canaliculus* larvae from preliminary trial, manipulating only developmental temperature (°C).

Supplemental Figure 2
A.

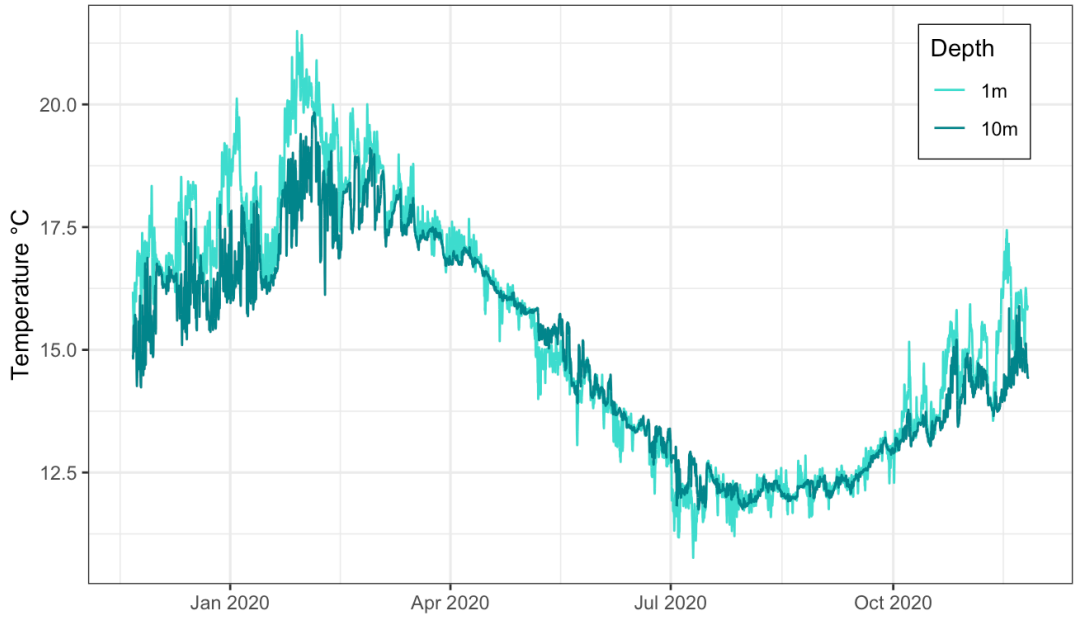


B.



Supplemental Figure 2. RDA of all normality data with A) each father reduced to a mean of his corresponding replicates or B) all replicates shown. Inner color represents paternal temperature, border color represents larval temperature, ellipses (A) are 95% confidence range. Ellipse color represents paternal temperature (orange – heat exposed (H), blue – naïve (N)), line type represents larval temperature (solid – ambient (A), dashed – warm (W)). Shape represents father number.

Supplementary Figure 3



Supplemental Figure 3. Temperature (°C) at 1m and 10m depth in Clova Bay. Moving average filter applied.

III. Interaction between selective breeding and paternally mediated transgenerational effects under simulated marine heat wave conditions in green-lipped mussels, *Perna canaliculus*

Abstract

Marine heatwave (MHW) events are forecasted to continue increasing in the Tasman Sea region around New Zealand with devastating consequences for the New Zealand aquaculture industry. This study investigated the how chronic (1 month) exposure to marine heatwave conditions impacts paternally-mediated transgenerational effects in the green-lipped mussel, *Perna canaliculus* and whether these impacts vary based on whether or not the fathers originate from families selectively bred for thermal tolerance. We simulated a month-long MHW event, exposing male *P. canaliculus* broodstock from selectively bred and wildtype lineages to elevated (21°C) or ambient (17°C) temperatures immediately prior to spawning, and evaluated the effects of paternal heat exposure on successful development and size of their larvae that were also reared under ambient or elevated temperatures through completion of the lecithotrophic trochophore stage. Elevated paternal and larval temperatures both increased abnormal development, reducing larval yield and negative paternal effects were observed in both selectively bred and wildtype lineages. We also analysed MHW occurrences from SST data from the region around the top of the South Island where the majority of mussel farms are located and detected a trend towards longer marine heatwaves with greater cumulative intensity. These results indicate that *P. canaliculus* farms are likely to experience longer periods of chronic heat stress in the future and that selective breeding may not alleviate negative paternal effects of this exposure.

1. Background & Overview

Marine heatwaves (MHWs) are increasing not only in frequency but also intensity and duration around New Zealand (NZ), with forecasts indicating that MHWs could become a persistent state in this region in the near future (Salinger et al. 2020, Behrens et al. 2022). Therefore, prolonged heat exposure, such as the month exposure used in this experiment, is an escalating threat for both wild green-lipped mussels (*Perna canaliculus*) and brood-stock in grow-out operations. This is particularly true for the late summer to early fall spawning period for wild populations and farms on the South Island (Buchanan 2001). It has been demonstrated that the paternal environment can impact offspring success through as little as 1 week of exposure (see previous chapter) or even the conditions immediately prior to reproduction (Jensen et al. 2014). As paternal effects are still understudied, it is critical to probe how duration of exposure mediates paternal effects. Longer exposures have been shown to result in both negative and adaptive paternal effects in purple urchins over a month (Leach et al. 2021, Leach 2022) or in the dampening of negative effects when the environment is stable for tube worms (Guillaume et al. 2016).

Due to the devastating consequences of MHWs on industry yields in NZ, companies are investing in selective breeding programs focused not only on traditional success metrics such as growth but on thermal tolerance specifically. Proprietary *P. canaliculus* families selectively bred for heat tolerance resulting from a collaboration between Cawthron, SPATnz, and BreedCo were used in this experiment. There is some support in the literature for selective breeding moderating the magnitude of transgenerational effects in the case of CO_2 stress (Parker et al. 2012, Goncalves et al. 2016). Observations across naturally heterogeneous pH and salinity environments in the field further highlight the importance of

considering the interaction between selection and plasticity in mussels, *Mytilus edulis*, and oysters, *Crassostrea virginica*, respectively (Eierman & Hare 2015, Stapp et al. 2017, Thomsen et al. 2017).

In this chapter, I investigated the interaction between selective breeding and paternal effects driven by heat exposure in the commercially and culturally important New Zealand aquaculture species, *P. canaliculus*. In my previous chapter, I found that a relatively short, 1-week, paternal heat exposure to 22°C immediately prior to spawning had detrimental effects for larval development in this species. Following up on this study, I hypothesized that this pattern might shift if the exposure was more chronic, providing more time for a paternal lineage to mount a transgenerational response. Furthermore, I hypothesized that the direction and magnitude of paternal effects might be influenced by the sire's underlying genetics. Therefore, in collaboration with Cawthron and SPATnz scientists, I designed this experiment to be conducted in parallel using sires from families selectively bred for their heat tolerance alongside "wildtype" sires from non-selectively bred families collected from farms in the Marlborough Sounds. To test these hypotheses, sires from each lineage type underwent a chronic, stable 1-month paternal acclimation to either ambient (17°C) or heat exposed (21°C) conditions designed to mimic either baseline or prolonged marine heatwave (MHW) conditions in the Marlborough Sounds region of NZ, where the majority of green-lipped mussel farms are located. Embryos from these genetically distinct and differentially acclimated fathers were then reared reciprocally under ambient (17°C) or heat exposed (21°C) temperatures to the veliger stage in order to assess the interaction between paternal lineage and paternal heat exposure on their thermal tolerance.

We found that larval and paternal heat exposure negatively influenced successful development and size of the veliger larvae. Selectively bred lineages performed better than wildtype under larval heat exposure though this effect was diminished when the sires were also heat exposed. These results indicate that chronic heat exposure, which is increasingly likely under future MHW scenarios, will be detrimental to the wild recruitment and hatchery yields of *P. canaliculus* larvae through negative paternal effects.

2. Materials & Methods

2.1 Experimental design

In March 2017, as part of a selective breeding program operated by Cawthron, SPATnz, and BreedCo, 3 full-sibling families were created by crossing parents known to have high temperature tolerance. These families were raised in the commercial SPATnz hatchery and deployed to a mussel farm in the Marlborough Sounds. In mid-October, 2019, individuals from these families along with non-selectively bred mussels (referred to as "wildtype" from this point on) from a long-line farm in the Marlborough Sounds (Marlborough, New Zealand) were collected and transferred to a nearby broodstock management facility at the Cawthron Aquaculture Park (Nelson, New Zealand), where they received ambient seawater enriched with elevated natural phytoplankton levels and were held at 17°C (after (Ragg et al. 2010)). In February 2020, half of the selectively bred and "wildtype" mussels were transferred to slowly brought up to 21°C (considered heat exposed – H) over 5-6 days and held at that temperature for ~1 month until March 11th, 2020 while the rest remained at 17°C (considered ambient – A) (Figure 1).

A pool of eggs from n = 6 wildtype females from ambient temperature (~17°C) was generated in order to isolate the effects of paternal temperature exposure. Aliquots of the ambient wildtype egg pool were each fertilized by 12 individual males: One male from each of 3 selectively bred families from each adult acclimation temperature (17 or 21°C) and 6 different wild type individuals, 3 from each adult acclimation temperature (17 or 21°C). After fertilization, the pool of embryos sired by each father was divided into reciprocal larval treatments ~17°C (ambient – A) or ~21°C (heat exposed – H) (Figure 1). This design resulted in 8 treatment groups: embryos from selectively bred (S) or "wildtype" (W) fathers, acclimated to ambient (A) or heat exposed (H) paternal conditioning temperatures and then raised under ambient (A) or heat exposed (H) developmental temperatures (Figure 1). For instance, a larval culture from a selectively bred father acclimated to warm temperature but developing under ambient temperature would have the treatment group SHA, whereas a larval culture from a "wildtype" father acclimated to ambient temperature and developing under heat exposure would have the treatment group WAH. These larvae were raised to the early D-veliger larval stage in static cultures at which point they were assessed for shell size and abnormality in order to investigate the interaction between selective breeding and the paternal and larval thermal regime on larval phenotype.

2.2 Adult mussel acclimation and spawning

During acclimation, the mussels were held in flow-through aquaria and supplied with mono-cultured algae (*Tisochrysis lutea and Chaetoceros calcitrans*, 1:1 cell ratio, ~40 cells μL^{-1}). Temperature was recorded at 10-minute intervals with *in situ* loggers (Water Temperature Pro v2 Data Loggers, Onset Hobo™) and verified by daily spot checks. On

November 14, 2019, mussels were spawned by “thermal cycling”, following industry practices with cycles of 12 and 26°C (Gale et al. 2016). When sperm and eggs had successfully been collected from all groups, sperm concentrations were quantified for 6 males from each acclimation temperature (17 & 21°C) using a hemocytometer; egg concentrations were quantified for 6 females, all from 17°C, by counting three aliquots of eggs per female so that a coefficient of variance (CV) of less than 10% was reached. Eggs were visually checked for quality during counting.

2.3 Fertilizations and larval rearing

12 egg pools were made by gently combining eggs from all 6 females in approximately equal numbers to help ensure genetic diversity of the offspring. Each egg pool was then fertilized with the sperm of one selectively bred (S) or wildtype (W) father from either the 17°C (A) or 21°C (H) paternal exposure treatment. Fertilizations were conducted in seawater pre-incubated with 4 mM EDTA and sperm was added at a concentration of 200 sperm per egg with 1000 eggs mL^{-1} seawater. After gently mixing the fertilization pools for 10-15 minutes, the formation of polar bodies was confirmed by checking aliquots of a few egg pools under the microscope. Fertilized eggs were loaded at a density of 50 embryos mL^{-1} into 6 replicate 4mL tissue culture dish (TCD) wells per treatment filled with EDTA enriched seawater incubated to ~17°C (A) or 21°C (H) (Figure 1). Six replicate TCD well cultures per sire per paternal and larval temperature treatment resulted in n = 144 total cultures.

2.4 Larval sampling and TCD assessments (normality and larval shell size)

Embryogenesis took place in EDTA-enriched seawater maintained at the respective treatment temperatures until the first veliger shell (prodissoconch I) was formed, approximately 37 hours post-fertilization for the 21°C treatment and 41.5 hours p.f. for the 17°C treatment. As the larvae from the 21°C larval treatment developed more quickly fixing of TCDs was staggered in time. When the D-hinge veliger stage was reached in the TCDs for each treatment, the larvae were fixed by the addition of 200µL of 10% formalin and stored at 4°C until they were scored and measured, within 4 days of sampling. Larvae were scored for development and normality and measured following the methods outlined in chapter 2.

2.5 Marine heatwave (MHW) characterization

To quantify MHW events, NOAA 1/4° Daily Optimum Interpolation Sea Surface Temperature (OISST) data (Huang et al. 2021) was used for the region covering the top of the South Island of New Zealand, including Golden Bay, Tasman Bay, and the Marlborough Sounds region, between -41.25 and -40.25 degrees latitude and 172.5 and 174.5 degrees longitude. OISST data for the region spanned January 1st, 1982 to August 30th, 2022. MHW events were calculated using heatwaveR (v 0.4.5), as events persisting about the 90th percentile for least 5 days (Schlegel & Smit 2018). The distribution of maximum MHW event intensity throughout the year was calculated using month long bins and assigning each heatwave to the month of its date of peak intensity.

3. Results

3.1 Summary

To assess the effect of paternal heat exposure and selective breeding on offspring thermal tolerance, male *P. canaliculus* broodstock from either wildtype families or families selectively bred for heat tolerance underwent a simulated MHW event at 21°C for one month, or remained at ambient temperature (17°C), for one month immediately prior to spawning. The impact of paternal heat exposure, selective breeding, and their interaction was assessed by measuring successful development and shell size of their larvae reared at 17 and 21°C. In short, we found that 1) larval heat exposure negatively influenced successful development, as did paternal heat exposure to a lesser degree; 2) selectively bred lineages performed better than wildtype under larval heat exposure though this effect was diminished when the sires were also heat exposed; 3) larval and paternal heat exposure negatively impacted the average size of the larvae which reached veliger stage successfully, and increased the variation in veliger size; and 4) the negative effect of larval heat exposure on shell size was larger for wildtype lineages, though overall variation in shell size was still minimal. Results for each response metric are explained in further detail in the sections that follow.

3.2 Marine heatwaves (MHWs)

In our analysis of OISST for the region at the top of the South Island, encompassing Golden Bay, Tasman Bay, and the Marlborough Sounds, MHW events appeared to be increasing in duration with peak intensities aligning with key periods in the reproductive cycle for *P. canaliculus* populations in the area. The duration of MHW events has

significantly increased in this time period along (linear model: duration ~ year, slope = 0.62 ± 0.23, p = 0.00961), with a corresponding increase in their cumulative intensity (linear model: cumulative intensity ~ year, slope = 0.9856 ± 0.4193, p = 0.0214) (Figure 4). While the majority of MHW events we detected in the region were less than 18 days, 11 events (14% of the total events calculated) have exceeded 1 month (28 days) in duration 5 of which occurred since 2020, indicative of the trend towards longer MHW events. Throughout the year, MHW maximum intensity correlates with the gametogenesis cycle of *P. canaliculus* on the South Island as described by Buchanan (Buchanan 2001), particularly the summer gametogenesis period preceding the fall spawn and the fall spawning period when larvae are developing (the time period investigated in this experiment) (Figure 5). Average SST across the region exceeded 21°C (the heat-exposure temperature used in this study) on 9 days in this OISST dataset. Average SST exceeded 20°C on 107 days, 78% of which occurred since 2015, including a continuous 30 day streak during the MHW in summer 2018.

3.3 Larval development: normality

Larval normality assessments revealed a negative impact of larval and paternal heat exposure, which was larger in wildtype lineages. Normality characterizations were performed on 6 replicate TCD well cultures per sire per paternal and larval temperature treatment (n = 144 total cultures). Overall the vast majority of individuals (~96.3%) developed normally in the 17°C ambient larval treatment (Table 1, Figure 2), regardless of paternal temperature or family type. Under the heat exposed 21°C larval treatment (H), larvae from 21°C heat exposed (H) fathers had higher developmental abnormality than those from 17°C acclimated ambient (A) fathers (Table 1, 2, Figure 2). Within the wildtype lineages, paternal heat

exposure was associated with an increase of 7.02% in the percentage of abnormal larvae raised at 21°C. In contrast, within the selectively bred lineages, paternal heat exposure was associated with an increase of 14.01% in the percentage of abnormal larvae raised at 21°C. Under the ambient 17°C larval treatment, larvae from both family types saw slightly higher levels of normal development when their fathers were exposed to 21°C but this increase was nominal and insignificant (1.05 and 1.45% for selectively bred and wildtype families respectively). Within the 21°C larval treatment, larvae from selectively bred fathers acclimated to ambient temperature (SAH) had the highest proportion of normal development (71.73 ± 6.72%), while larvae from wildtype fathers that underwent heat exposure (WHH) had the lowest proportion of normal development (53.26 ± 6.75%). Larvae from selectively bred fathers that underwent heat exposure (SHH) and those from wildtype fathers acclimated to ambient temperature (WAH) exhibited intermediate levels of normal development (57.72 ± 8.22 and 60.28 ± 9.74% respectively). There was a significant difference in the proportion of normally developed larvae in ambient larval temperature groups compared to the heat exposed larval temperature, and a significant difference between the proportion of normally developed larvae from selectively bred fathers acclimated to ambient temperature compared to all other paternal groups heat exposed as larvae (Table 1). Family type, paternal, and larval treatment, all contributed significantly to the variation in the percentage of normal larvae as well as the interactions between each factor, with the exception of the interaction between paternal temperature and family type ($p = 0.054$) (Table 2).

When considering what proportion of larvae successfully reached veliger stage, regardless of normality and relative to those stalled at an earlier stage, there was a significant effect of paternal ($\chi^2 = 76.98.$, df = 1, p = <2.2e-16) and larval treatment ($\chi^2 = 35.01$, df = 1, p

= 3.29e-09), as well as their interaction (χ^2 = 88.75, df = 1, p = <2.2e-16), but family type was not significant (χ^2 = 0.08, df = 1, p = 0.77). Approximately 96.21% of embryos reared under ambient temperature reached veliger stage. Under larval heat exposure, embryos from fathers acclimated to ambient temperature reached veliger stage at higher rates (86.24 ± 4.53% for selectively bred fathers, and 87.01 ± 6.12% for wildtype fathers). In contrast, under larval heat exposure, embryos from fathers that underwent heat exposure had slightly lower rates of reaching veliger stage (78.74 ± 5.02% for selectively bred fathers, and 74.29 ± 7.64% for wildtype fathers). The proportion of veliger larvae that displayed abnormalities from each treatment group varied significantly by developmental temperature (χ^2 = 528.64, df = 1, p < 2e-16), and paternal temperature (χ^2 = 63.56, df = 1, p = 1.55e-15), and the interaction of family type with either temperature treatment (paternal*family type: χ^2 = 17.51, df = 1, p = 2.86e-05; larval*family type: χ^2 = 7.51, df = 1, p = 0.006), but by family type alone (χ^2 = 0.019, df = 1, p = 0.89). Under ambient developmental temperatures, >99% of larvae which reached veliger stage formed normal shells. Under developmental heat exposure, veligers from selectively bred fathers acclimated to ambient temperatures had the highest proportion of normal shells (83.16± 5.95%), while veligers from wildtype fathers acclimated to ambient temperatures had the lowest (68.99 ± 8.17%). Veligers from selectively bred and wildtype fathers that underwent heat exposure showed intermediate levels of normal shell formation (73.2 ± 8.35 and 71.93 ± 7.56% respectively), though the fewest larvae made it to veliger at all from these heat exposed groups. Finally, very few eggs (<1%) remained undeveloped across all treatments.

3.4 Larval development: shell size

In terms of morphometrics, larval shell size was mainly impacted by larval developmental temperature. Length of the veliger shell was very similar across all treatment groups, with mean shell length ranging 1.94 µm from 87.75 ± 2.29 µm for WHH larvae to 89.68 ± 2.04 µm for SAA larvae (Figure 3). Despite the small scale of variation, differences in shell size were significantly influenced by larval developmental temperature ($p = 0.011$) as larvae developing in 21°C were on average ~0.66 µm shorter than those developing in 17°C. Larvae from heat exposed fathers were on average ~0.78 µm shorter when raised under warm conditions compared to ambient, while larvae from ambient fathers were on average ~0.54 µm shorter when raised under warm conditions compared to ambient.

The interaction between family type and larval temperature was also significant ($p = 0.0323$). Larvae from selectively bred fathers were on average ~0.46 µm shorter when raised under warm conditions compared to ambient, while larvae from wildtype fathers were on average ~0.86 µm shorter when raised under warm conditions compared to ambient. Veligers from heat exposed fathers tended to be shorter by ~0.57 µm than their ambient sired counterparts ($p = 0.00021$). There was higher variation in veliger shell size among individual larvae from the 21°C larval treatments, (Coefficients of Variation: SAH=7.78%, SHH 8.52%, WAH 8.26%, WHH 8.27%) compared to the 17°C ambient larval treatments (Coefficients of Variation: SAA=4.65%, SHA 4.65%, WAA 4.77%, WHA 3.87%) (Figure 3).

4 Discussion

4.1 Summary of results

In this study, we investigated the transgenerational effects of prolonged heat exposure (1 month) prior to spawning on male *P. canaliculus* broodstock from either wildtype families or families selectively bred for heat tolerance. We assessed the impact of paternal heat exposure, selective breeding, and their interaction by quantifying normal development and shell size of their larvae reared under ambient and elevated developmental temperatures. The most important findings were that paternal heat exposure negatively impacted larval development and although larvae from selectively bred lineages demonstrated a comparative advantage over wildtype under larval heat stress this difference was diminished when the sires were also heat exposed. Our results do not indicate selective breeding producing underlying genetics capable of reversing the direction of paternal effects from negative to adaptive. Furthermore, the diminished advantage of larvae from selectively bred lines following paternal heat exposure hints at a potential trade-off between father and offspring which could be further investigated. Our analysis of SST indicates that prolonged heatwave events on the order of a month do occur in the region at the top of the South Island of NZ and that MHW duration is increasing over time consistent with broader studies of MHW trends. Overall, this trend is threatening to aquaculture, as longer MHWs can have negative effects both within and across generations for the green-lipped mussel regardless of whether those mussels have been selectively bred for thermal tolerance.

4.2 Effects of the larval developmental environment

Exposure to elevated temperatures (21°C) during development resulted in higher levels of abnormal development and slightly smaller shell sizes for veliger larvae in this experiment. The trend of higher levels of abnormal development at higher temperatures was consistent with the findings of the previous chapter. Increased variation in shell size under higher temperatures was also observed in both studies. The impact on normal development was larger in this study likely because the larval temperature differential was higher (17 vs. 21°C instead of 17.5 vs. 20°C). The larger impact of developmental temperature on normality in this experiment could also have been exaggerated by the larvae being overall healthier or higher quality in this experiment exemplified by higher baseline levels of normal development under ambient temperatures (>95% vs. >80% normality). The trend in body size, however, reversed between experiments. In the previous chapter, shell size showed a slight increase with temperature, in keeping with other studies of molluscan larvae (Rico-Villa et al. 2009, Lazo & Pita 2012) (Ch 2, Figure 3, S1). In contrast, shell size decreased slightly with elevated developmental temperature in this study. However, the differences in shell size were extremely small in both experiments. One factor that may have contributed to the reversed direction of the trend could be that the two studies were conducted in different spawning periods for the species which span the summer months, the first in late Spring and the second in early Fall. This raises a question of whether plasticity in thermal response could be programmed cyclically with seasonal cycles for larvae of this species, which could be potentially interesting to investigate. Variation in spawning timing has been tied to larval thermal plasticity in Atlantic cod, *Gadus morhua*, with consequences for short term adaptation to environmental change (Oomen & Hutchings 2015).

4.3 Paternal transgenerational effects

4.3.1 Mechanisms of paternal effects

This study sought to characterize the transgenerational effects of prolonged heat exposure prior to spawning, specifically through the male line. The mechanisms behind paternal effects are still largely unclear. However, paternal environment can influence sperm morphology. For instance, temperature has been shown to influence sperm size in different directions for various taxa, such as a decrease in sperm length with temperature for the land gastropod, *Arianta arbustorum*, (Minoretti et al. 2013, Marshall 2015). Changes to sperm morphology can have downstream effects on fertilization success, particularly in an environment with sperm competition as would be relevant for wild populations of many marine invertebrates, including mussels. In a hatchery setting, sperm competition can be largely eliminated if desired, but sperm morphology is capable of influencing offspring phenotype later on and the direction of effects may not be the same, i.e. modifications which increase fertilization success may not necessarily correlate with positive changes in offspring performance (Bilde et al. 2009, Crean & Bonduriansky 2014). Sperm may transfer epigenetic modifications such as DNA methylation or histones, as well as possibly RNAs which could influence offspring gene regulation and the activity of transposable elements; proteins transferred via sperm could also influence function such as early cell divisions (Immler 2018). In various species of flies, paternal effects can even be mediated by properties of the seminal fluid itself rather than modifications to the DNA contained within the sperm (Chapman et al. 2001, Crean et al. 2014, Crean et al. 2016).

4.3.2 Trends

In this study, exposing male *P. canaliculus* to 21°C for 1 month prior to spawning had a negative impact on normal development of their larvae when those larvae were raised under 21°C as well. This pattern was in keeping with the previous chapter, which also saw negative impacts on normality resulting from an acute 1-week paternal exposure. They also align with findings of reduced larval thermal tolerance in another mussel species, *Mytilus californianus*, associated with chronic parental heat exposure across a natural gradient in the intertidal (Waite & Sorte 2022). The negative impacts of the 1-month chronic paternal heat exposure on larval normality under high temperatures were more pronounced than in the previous acute exposure study. This pattern is in contrast to findings a previous study in tube worms which saw that reduction in performance driven by negative paternal effects was less severe when the paternal environment was stable for a longer period of time (Guillaume et al. 2016).

The paternal effects observed in this study were negative; however, paternal effects of heat exposure can vary in degree, and direction, depending on the phenotypic metric considered (Leach 2022). In this study, size and larval yield (here measured with the proxy of % normality) were picked as response metrics to assess paternal effects because they are highly industry relevant. However, given the forecasted escalation of marine heatwaves, it may be pertinent to investigate carryover effects of parental heat exposure in other industry relevant phenotypic metrics such as disease susceptibility or stress response. Though the takeaways from this study for paternal effects as a mitigation strategy are not positive, it is not a comprehensive assessment for the role of TGP in MHW resilience for *P. canaliculus*. Maternal effects were beyond the scope of this study and it is likely that, barring controlled

hatchery settings or intentionally varied broodstock site selection, MHW conditions are likely to impact both parental lines in parallel. In the pH literature, transgenerational effects can vary through the maternal vs. paternal line and even offset each other (Lane et al. 2015, Venkataraman et al. 2019). Therefore, it would be pertinent to pursue investigations into TGP through the maternal line and whether this offsets or exacerbates the patterns seen here.

4.3.3 Selective breeding and possible trade-offs

Selective breeding for thermal tolerance is expanding in aquaculture to combat climate change threats (Ineno et al. 2005, Sae-Lim et al. 2017, Tan et al. 2020, Acquafredda et al. 2021). Therefore, in this study, we investigated how selective breeding for thermal tolerance would interact with paternal effects of heat exposure. In the case of CO_2 stress, selective breeding has been shown to modulate transgenerational effects, modifying the magnitude and even direction of response (Parker et al. 2012, Parker et al. 2015, Goncalves et al. 2016). In this study, paternal heat exposure had a negative impact on larval response to elevated temperatures for offspring of both wildtype fathers and fathers from families selectively bred for heat tolerance. When comparing larvae from fathers that were conditioned to ambient temperature (17°C), those from selectively bred fathers performed better under high temperatures (shown by larger shell sizes and higher levels of normal development) compared to those from wildtype fathers (Figure 2, 3). However, when the fathers were conditioned under elevated temperature (21°C) for 1 month prior to spawning, the larvae from selectively bred and wildtype fathers performed more similarly. This is concerning from an industry standpoint because the benefit of selective breeding appears to be partially diminished by negative paternal effects. Biologically, this is interesting because the larvae from the selectively bred fathers do show some resilience to the larval heat

exposure when the fathers are not heat stressed first. This could indicate a potential trade-off happening between the performance of the father and his offspring when the fathers are heat exposed.

The reasons behind a potential trade-off are unclear. It is possible that the sperm itself is incurring damage from the heat exposure in the sire's gonad in the weeks before spawning that diminishes any benefit of the selective breeding. Through environmental stress, sperm can incur damage to DNA, or accompanying RNA, hormones, and proteins in seminal fluids (Immler 2018, Evans et al. 2019). Properties of the seminal fluid may also have been impacted by the paternal environment (Fraser et al. 2016, Patlar et al. 2019) with potential impacts on offspring phenotype (Kekäläinen et al. 2020). Alternatively, although any underlying genetics will be passed from father to offspring, if some portion of the heat tolerance targeted in the selective breeding process was epigenetically driven, it is possible that these marks might not be passed to the offspring under heat stress as stress can prematurely degrade epigenetic machinery such as genome wide methylation states (Horvath et al. 2014, Bonduriansky & Crean 2018). Furthermore, fitness consequences of any epigenetic change or paternal effect do not always act in the same direction at all life history stages and may vary based on the phenotypic metric assessed (Marshall 2008, Kekäläinen et al. 2015, Crean & Immler 2021, Leach 2022). Lastly, it is possible that the temperature used in this experiment, 21°C, exceeds the threshold to which the selectively bred fathers are tolerant.

4.4 The MHW landscape

4.4.1 Future conditions for the top of the South Island

MHW events around the top of the South Island are on track to worsen in the coming years, with potentially devastating consequences for *P. canaliculus* farming. The Tasman Sea region has warmed consistently 0.2-0.3°C per decade over the past ~40 years (Sutton & Bowen 2019) and is no exception to the global trend in longer, more frequent, and more intense MHWs in the future (Oliver et al. 2018, Behrens et al. 2022). OISST data for the region at the top of the South Island analysed in this study displays the pattern of MHW events significantly increasing in duration and cumulative intensity. Recent MHWs in the Tasman Sea such as that of 2015-2016 have lasted almost a year, while others, including that of summer 2017-2018, have been shorter but more acute (Perkins-Kirkpatrick et al. 2019, Salinger et al. 2019, Oliver et al. 2021b). These different MHW phenologies will create different stress and acclimation environments for *P. canaliculus* broodstock. However, this study, and the previous chapter, indicate that both shorter more acute events and longer more chronic events may have negative transgenerational impacts through the paternal line. In our analysis of regional OISST, events exceeding 1 month in duration (the duration used in this study) were heavily weighted towards recent years. While prolonged periods of SST exceeding 20-21°C were uncommon, nearshore temperatures where mussels farms are abundant are generally higher than these open water offshore averages detected by satellite; one of the factors currently driving aquaculture offshore (Heasman et al. 2020).

For the area around NZ, Behrens et al. found that peak probability for MHWs is reached in middle of February, the middle of austral Summer, aligning with peak MHW intensity (Behrens et al. 2022). Our analysis similarly saw that for the annual cycle of MHW

intensity, maximum intensity was highest in the summer aligning with when male *P. canaliculus* are redeveloping their gonads and remaining high through March when peak spawning occurs and larvae will be developing in the water column. Furthermore, the forecasts of Behrens et al. indicate that the peak of high probability for MHWs will broaden in the future, extending the MHW season particularly into the austral Fall (Behrens et al. 2022). Therefore, the summer into fall spawning period for *P. canaliculus* in the South Island is under greater threat than the late spring spawning period. However, while future MHW intensity will increase most drastically around the North Island, under high emission scenarios the Tasman Sea and the coastal South Island region are at risk of a persistent year round MHW state relative to baseline seasonal cycles and a continual acclimation regime of elevated temperatures for *P. canaliculus* farms in the area.

4.4.2 Takeaways for aquaculture

The forecasts for higher probability of MHWs and longer duration of events will have industry wide consequences for *P. canaliculus* and NZ as well as globally (Smith et al. 2021, Smith et al. 2023). As harvest management for wild fisheries needs to become more flexible and reactive to MHWs (Caputi et al. 2016, Caputi et al. 2019), aquaculture practices in NZ and beyond are also forced to adapt in the face of this and other environmental stressors (Heasman et al. 2020). Interventions being investigated include selective breeding along with species switching, shifting of farm sites, and nutritional mitigation strategies (Heasman et al. 2020, Maulu et al. 2021, Islam et al. 2022).

This study indicates that when considering the advantages of selective breeding for traits such as heat tolerance it will be valuable to evaluate the responses of not only the adult animals but also the traits of their offspring if adult heat exposure occurs during key periods.

Selective breeding programs are costly and may not be lucrative if the benefits are diminished through transgenerational effects when adults are exposed to high temperatures in grow-out operations before becoming broodstock. However, the selectively bred offspring in this study did perform better under larval heat stress when their fathers were shielded from it, which does allow for proactive measures to be taken with improved forecasting as discussed below. Selective breeding for traits other than heat tolerance, but compounded by heat stress, such as disease resistance or feed conversion efficiency may still be beneficial (Sae-Lim et al. 2017). In the global context, a recent studies have found New Zealand to have comparatively high capacity to adapt to the effects of climate change on aquaculture in order to protect livelihoods (Handisyde et al. 2017), and comparatively high acknowledgement of current climate impacts on aquaculture in popular media (Froehlich et al. 2022) which could bode well for support for innovative adaptation of the industry. For instance, the Moana Project is investing in improved monitoring and regional forecasting of MHWs alongside investigation of the larval connectivity of key NZ seafood species such as the green-lipped mussel with mātauranga Māori at its core (https://www.moanaproject.org/).

4.4.3 Future directions with improved forecasting

As MHW forecasting continues to improve in its spatial and temporal resolution (Holbrook et al. 2020, Jacox et al. 2022), knowledge of the biological impacts of MHW exposure across generations can be leveraged by farmers to avoid the worst of negative effects. Physiology and life history informed harvest strategies are showing value for flexible management interventions of wild fisheries stocks if MHW events can be identified early on (Caputi et al. 2016, Caputi et al. 2019). In an aquaculture context, if farmers know a MHW will be coming before a planned spawn they can potentially bring broodstock into the

hatchery earlier than normal to buffer them from the effects or harvest broodstock from a different farm location deeper or further offshore (Heasman et al. 2020). If broodstock cannot escape a MHW event entirely, even a limited recovery period in a hatchery prior to spawning may help to mitigate negative paternal effects (Jensen et al. 2014). Furthermore, as MHWs are likely to threaten the late summer/fall spawning period more, South Island hatcheries could potentially shift the peak of their breeding season operations more to the smaller Spring spawning period, though this timing would need to be balanced against exposing juveniles to additional heat stress and spat loss (Delorme et al. 2020, South et al. 2022).

Regional forecasts of fine spatial resolution will also be critical for predicting and adapting to fluctuations MHWs may cause in wild spat recruitment. A recent study inferring paternal areas of juvenile *P. canaliculus* for the region at the top of the south island found that the majority of parental distances were <7.5km but varied significantly between regions (eg. Inner Pelorous Sound vs. Tasman Bay) (Atalah et al. 2022). Overall, this indicates that larvae and early juveniles are likely to experience the same MHW state as their parents as durations increase, with compounding effects as indicated in this study. Thus, finer scale predictions and circulation modelling will allow for improved recommendations of where to place both farms and spat catchers. Though MHWs are a growing threat to *P. canaliculus* larvae through both within-generation and transgenerational effects, improved forecasting and investment in studying the life history and physiology of this species can arm farmers with information as to how best to adapt in a changing thermal landscape.

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Figures & Tables

Table 1
Mean percentage and standard deviation of embryos from each treatment that were found in each state of development and normality. Different letters among treatments represent statistically significant differences for a particular state of development ($p < 0.05$) after a Tukey multiple comparisons test.

Family Type	Temperature Treatment (Paternal-Larval)	D-hinge Veliger	Abnormal D-hinge Veliger	Less than Veliger	Egg
Selectively Bred	17°C - 17°C	94.97 ± 2.12^{a}	0.44 ± 0.48^{a}	4.17 ± 1.94^{bcd}	0.43 ± 0.65
	17°C - 21°C	71.73 ± 6.72^{b}	14.51 ± 5.33^{c}	13.51 ± 4.46^{e}	0.25 ± 0.37
	21°C - 17°C	96.42 ± 1.23^{a}	0.53 ± 0.48^{a}	2.94 ± 1.32^{abc}	0.11 ± 0.37
	21°C - 21°C	57.72 ± 8.22^{c}	21.02 ± 6.41^{b}	21.04 ± 4.85^{f}	0.23 ± 0.42
Wildtype	17°C - 17°C	95.37 ± 1.53^{a}	0.46 ± 0.54^{a}	3.65 ± 1.72^{acd}	0.52 ± 0.43
	17°C - 21°C	60.28 ± 9.74^{c}	26.73 ± 6.14^{b}	12.20 ± 5.27^{e}	0.79 ± 1.01
	21°C - 17°C	96.42 ± 1.58^{a}	0.21 ± 0.34^{a}	3.11 ± 1.40^{abd}	0.26 ± 0.32
	21°C - 21°C	53.26 ± 6.75^{c}	21.03 ± 7.33^{bc}	25.17 ± 7.63^{f}	0.53 ± 0.71

Table 2
Statistical output (Wald chi-square test) from generalized linear mixed effects model used to analyze the effect of family type, paternal temperature, and larval temperature on the proportion of normally developed larvae.

Source	χ^2	*df*	*p*
(Intercept)	4.58	1	*0.032
Larval Temperature	9.80	1	**0.002
Paternal Temperature	41.71	1	***$1.06e^{-10}$
Family Type	5.27	1	*0.022
Larval Temperature * Paternal Temperature	50.77	1	***$1.04e^{-12}$
Larval Temperature * Family Type	6.91	1	**0.009
Paternal Temperature * Family Type	3.73	1	0.054
Larval Temperature * Paternal Temperature * Family Type	4.85	1	*0.028

Figure 1

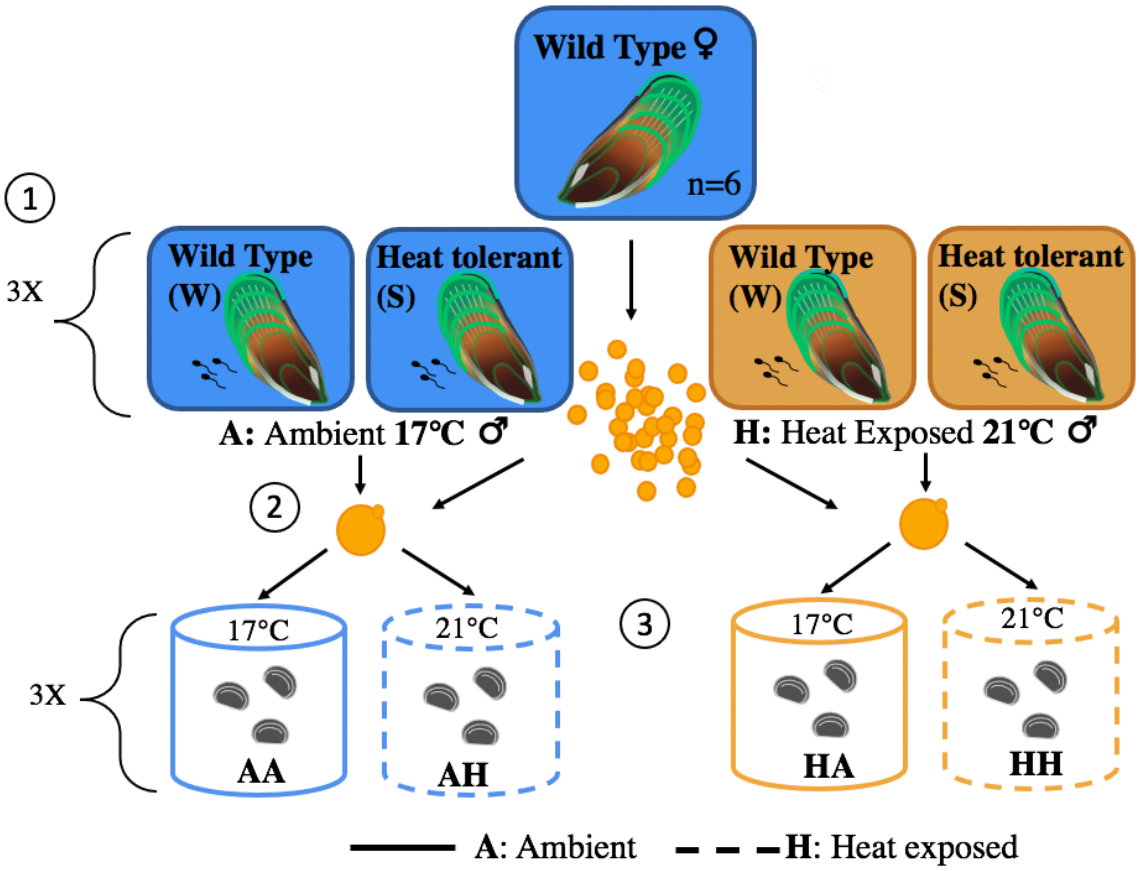

Figure 1. Experimental design. 1) Male *Perna canaliculus* mussels either from families selectively bred (S) for heat tolerance or "wildtype" (W) families were held at 17°C, ambient (A), or 21°C, heat-exposed (H) for one month prior to spawning. 2) The conditioned fathers (n=3 per family type and acclimation treatment) were individually crossed to pooled eggs from 17°C wildtype mothers. 3) Fertilized eggs from each of the 12 crosses were then divided into 17°C, ambient (A), or 21°C, heat-exposed (H) larval treatments to develop until the veliger larval stage (n=3 larval culture replicates per combination of father and developmental temperature). There are eight final combinations of family, paternal, and larval treatment - Family Type: S or W x paternal acclimation treatment A or H x larval development treatment A or H.

Figure 2

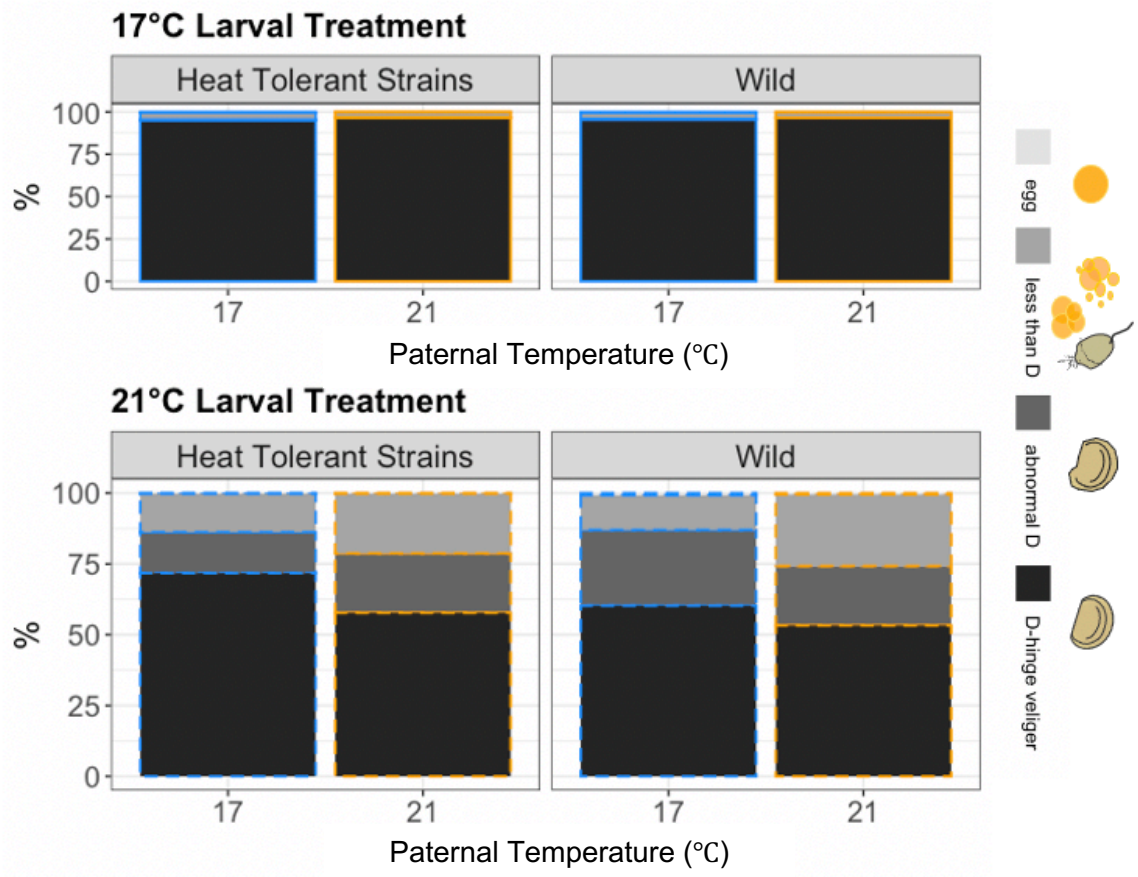

Figure 2. Effect of paternal acclimation temperature (17 or 21°C, x-axis) and larval developmental temperature (17 or 21°C, top and bottom panels) treatment on larval development and normality of *Perna canaliculus* from families selectively bred for heat tolerance (left panels) and wildtype families (right panels). Percentage of embryos in a given state is shown by internal bar shading. Paternal treatment is shown by color (blue - 17°C, orange - 21°C) and larval treatment is shown by pattern, (solid - 17°C, dashed - 21°C).

Figure 3

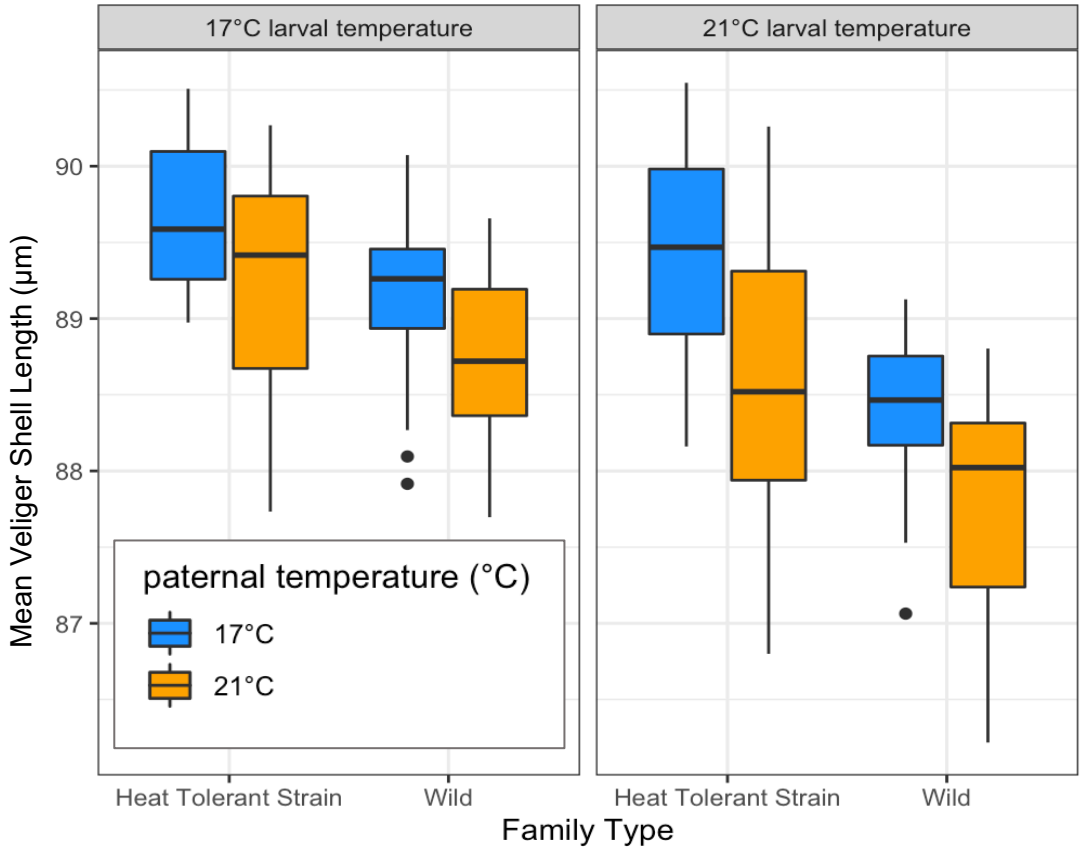

Figure 3. Effect of paternal acclimation temperature (17 or 21℃, color) and larval developmental temperature (17 or 21℃, left and right panels) on first D-hinge veliger shell length (µm) of *Perna canaliculus* from different family types (selectively bred for heat tolerance and wildtype families, x axis). Boxplots display the variation between average veliger size calculated for each replicate (n=6) for each father and treatment combination.

Figure 4

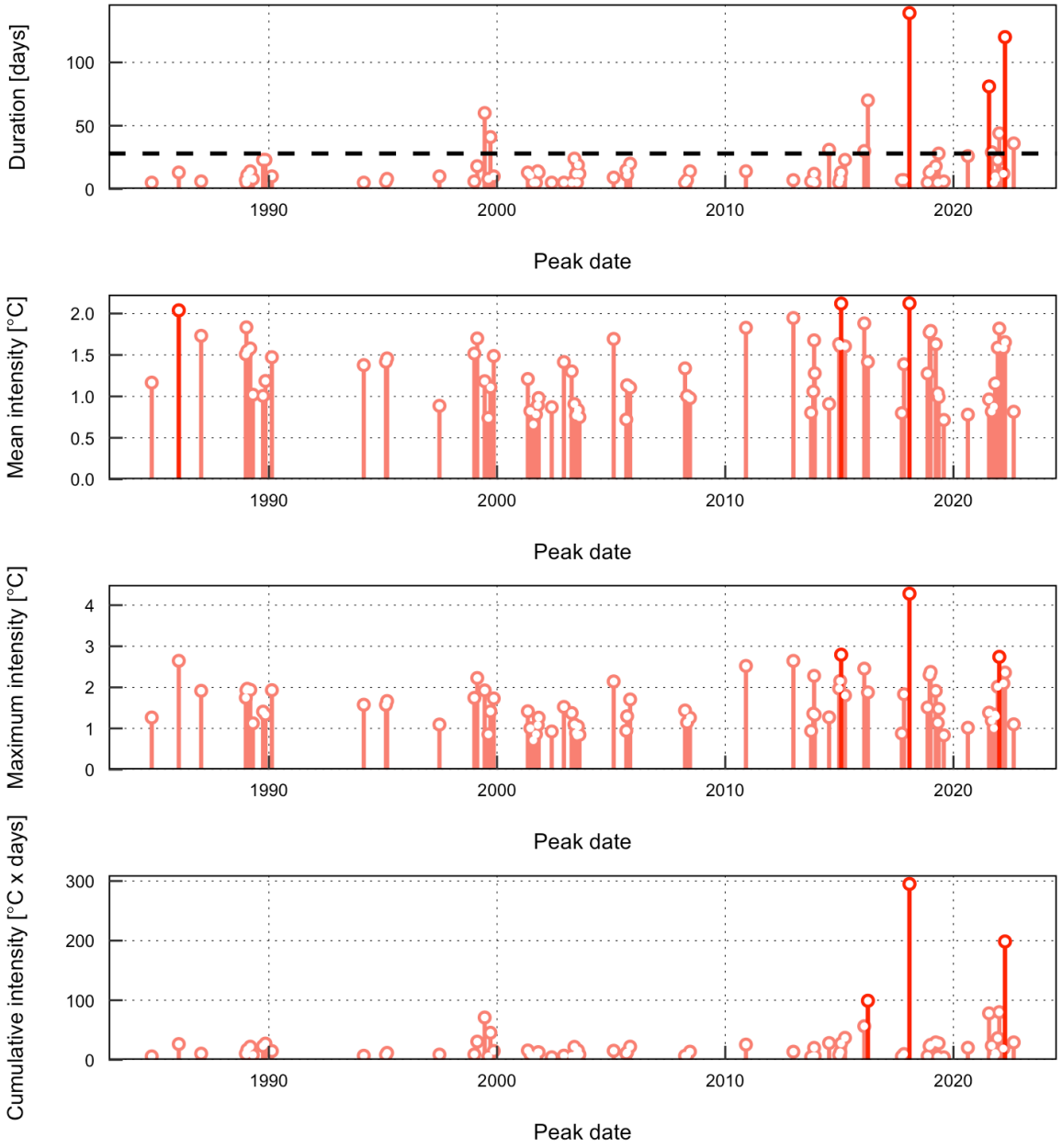

Figure 4. Marine heatwaves (MHW) impacting the region covering the top of the South Island of New Zealand, including Golden Bay, Tasman Bay, and the Marlborough Sounds region visualized in heatwaveR (v 0.4.5). MHWs are characterized as events persisting about the 90th percentile threshold above the seasonal climatology for least 5 days (Schlegel & Smit 2018) using NOAA 1/4° Daily Optimum Interpolation Sea Surface Temperature (OISST) data (Huang et al. 2021) for the region between -41.25 and -40.25 degrees latitude and 172.5 and 174.5 degrees longitude from 1982-2022. Panels show duration, mean and maximum intensity (in ℃ above the seasonal climatology) , and cumulative intensity (℃ x days). Black dashed line in the duration panel denotes events at or exceeding a threshold of one month (28 days).

Figure 5

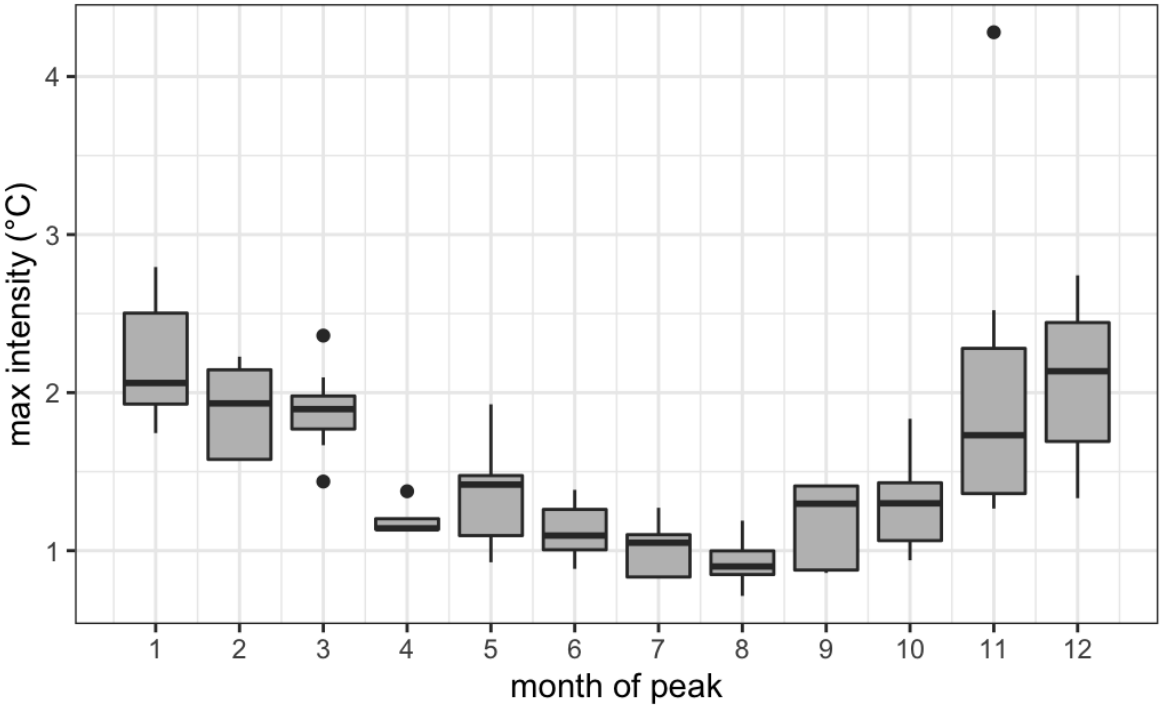

Figure 5. Distribution of marine heatwave (MHW) intensity throughout the year for the region covering the top of the South Island of New Zealand, including Golden Bay, Tasman Bay, and the Marlborough Sounds region calculated using heatwaveR (v 0.4.5). MHWs are characterized as events persisting about the 90th percentile threshold above the seasonal climatology for least 5 days (Schlegel & Smit 2018) using NOAA 1/4° Daily Optimum Interpolation Sea Surface Temperature (OISST) data (Huang et al. 2021) for the region between -41.25 and -40.25 degrees latitude and 172.5 and 174.5 degrees longitude from 1982-2022. Graph displays a boxplot of maximum intensity in ℃ above the 90th percentile above the seasonal climatology against the month of the peak date of the events.

Conclusions

Climate change poses an imminent threat to marine species and ecosystems through stressors such as ocean acidification and warming as well as an increase in extreme events such as marine heatwaves (MHWs). In order to predict how these stressors will impact the future of critical species and ecosystems it is necessary to consider impacts not only within generations but across them through transgenerational effects. A great variation in environments already exists across time and space, providing acclimatory regimes which may influence the adaptive capacity of populations. Gaining a deeper insight into how this environmental heterogeneity impacts the biological response of organisms to climate change stressors will allow for the informed management of species and ecosystems under global change. Ecosystem managers may use this information to protect areas which may serve as refuges from particular stressors but also environments characterized by periodic stress which may drive rapid adaptation through TGP, creating a diverse conservation portfolio. Fisheries managers can use information on reproductive consequences to inform seasonal closures. Finally, in aquaculture, farmers can use knowledge of the transgenerational impacts of mounting stressors such as marine heatwaves to mitigate the most detrimental effects or even leverage desirable ones to protect future yields.

Natural variation across space and time can have transgenerational effects (Murray et al. 2014, Rosa et al. 2014, Hoshijima & Hofmann 2019, Waite & Sorte 2022). TGP can have different effects when exposure is mediated through the maternal or paternal line. Consequences can be adaptive, negative, or neutral. In this thesis, I sought to explore how pertinent environmental variation across space and time influences the response of

ecologically and commercially important species to global change stressors. In the first chapter I used existing environmental variation associated with kelp forest habitat in the Santa Barbara Channel to investigate maternal effects on purple urchin, *Strongylocentrotus purpuratus*, larval response to pCO_2 and temperature stress. In the remainder of the thesis, I explored how MHW events plaguing mussel farms in New Zealand's Marlborough Sounds region will impact green-lipped mussel, *Perna canaliculus*, larval thermal tolerance through paternal effects dependent upon the duration and severity of the paternal exposure and their underlying genetics.

From this research five main takeaways and directions for future research are discussed below.

1. MHW events will impact marine invertebrate populations through transgenerational and developmental effects on larvae

Marine heatwave (MHW) events are predicted to continuing increasing in frequency and duration in both of the study systems investigated in this dissertation (Barkhordarian et al. 2022, Behrens et al. 2022), and worldwide (Frölicher et al. 2018, Holbrook et al. 2020). These MHW events have ecosystem level consequences, driving local extinctions of foundational species (Thomsen et al. 2019), shifting community composition (Michaud et al. 2022) and species ranges (Sanford et al. 2019), and exacerbating disease epidemics (Harvell et al. 2019). It is critical to understand the biological consequences of these marine heatwave events not only in terms of large scale patterns but also how they may shape population persistence of key species physiologically through sublethal effects, tradeoffs, and transgenerational plasticity.

Chapter II and III of this dissertation sought to explicitly investigate transgenerational effects of marine heatwaves through the paternal line and the effects were mostly negative. Both acute (chapter II) and chronic (chapter III) paternal exposure reduced successful development of green-lipped mussel larvae under high temperatures. Acute paternal exposure (chapter II) also had a negative impact when the larvae did not experience heat stress themselves and chronic exposure (chapter III) had a negative impact on shell size. Negative effects of exposure to MHW temperatures as larvae were also seen in normality rates (chapter II and III) and shell size (chapter III). Furthermore, development rate is highly temperature dependent (see chapter II, III methods) and can therefore impact dispersal distances. Overall, it seems that MHWs will be a threat to *Perna canaliculus* farms not only through limiting the availability of wild spat but also through reducing larval hatchery yields if the broodstock are affected by a MHW events before they are brought into a hatchery to spawn.

Though this was not the intended design of the experiment, a MHW event dominated much of the field acclimation period of the purple urchin in chapter I as well, exemplifying the present threat of these events for the California Current System and the Santa Barbara Channel. Assessments of the thermal tolerance of larvae from the differentially acclimated mothers raised under high or low *p*CO2 conditions showed that these two stressors were compounding and larvae raised under high pCO_2 were less tolerant of an additional thermal stress, indicating that these two climate change stressors could exacerbate one another as MHWs are forecasted to increase along with ocean acidification. It is possible that the abnormally high temperatures associated with the MHW which occurred during acclimation

caused the deeper, slightly cooler outside site to become a relative thermal refuge, contributing the higher performance of larvae from mothers conditioned outside the kelp.

The timing of MHW events, dominant in the summer and early fall, aligns with critical periods of both species' reproductive cycles. MHW events such as the one which occurred during the field acclimation in chapter I overlap with the gametogenesis period of *S. purpuratus* while the bimodal spawning period of *P. canaliculus* on the top of New Zealand's South Island spans the austral summer, indicating that offspring from late spring spawning events are likely to experience MHW events as larvae and juveniles, while offspring from spawning in late summer and early fall are likely to experience high temperatures as larvae, and/or come from parents who endured MHW events as well. MHW events are therefore likely to impact larval yields, recruitment, and dispersal for both species.

In the context of green-lipped mussel aquaculture, negative impacts of MHWs on early life stages from paternal carryover effects and developmental exposure may push the industry further towards hatchery production rather than wild spat collection. Hatchery breeding opens the potential for selecting broodstock from cooler areas, deeper or further offshore, and sheltering adults and larvae from high temperatures prior to reproduction and during development. However, increased manipulation of wild stocks through such intervention comes with a tradeoff of increased time in captivity, where altered food supply and lack of exposure to other types of environmental variation may have consequences for overall fitness in the natural environment once the juveniles are seeded onto grow-out operations.

2. Parental effects through both the maternal and paternal line can shape species response to environmental stress

While it is generally accepted that parental history can influence offspring performance, the majority of experiments have focused on maternal effects in laboratory settings (Mousseau & Fox 1998, Burgess & Marshall 2014, Donelson et al. 2018). However, as more studies explore the role of epigenetics in plasticity and non-genetic inheritance, interest is growing in the underexplored importance of the paternal environment (Gavery & Roberts 2010, Marshall 2015, Eirin-Lopez & Putnam 2019, Feiner et al. 2022). In addition, a small body of work has explored *in situ* carryover effects (Murray et al. 2014, Rosa et al. 2014, Hoshijima & Hofmann 2019, Waite & Sorte 2022), and highlighted the need for accurate characterizations of environmental variability in studies of TGP (Burgess & Marshall 2011, Burgess & Marshall 2014, Donelson et al. 2018). The research described in this dissertation contributes to the larger TGP research area by expanding the body of research investigating effects transgenerational in the field and the body of research targeting paternal effects specifically.

In Chapter I, I observed that *in situ* acclimation inside or outside a kelp forest environment had transgenerational effects through the maternal line. In one example, mothers conditioned outside the kelp forest produced larvae more capable of withstanding acute thermal stress. This experiment varied from some of the other TGP studies exploring field acclimation in that the element of food availability was controlled between the two maternal acclimation groups, helping to isolate the abiotic effects of the environment. The combination of the field acclimation with a controlled breeding design and larval culturing

experiment in lab allowed us to isolate a field driven transgenerational effect through strictly the maternal line.

In the Chapter II and III, transgenerational effects of simulated marine heatwave events were isolated through the paternal line. This adds to a growing body of work exploring the contribution of paternal effects (Jensen et al. 2014, Shama & Wegner 2014, Marshall 2015, Guillaume et al. 2016, Leach et al. 2021). The paternal effects observed here were predominately negative, indicating that paternal thermal exposure could have important consequences for population persistence as marine heatwaves become more frequent and severe.

3. Conclusions about transgenerational effects vary based on the metric assessed

Whether or not a transgenerational effect appears adaptive can vary widely depending on the alignment of the parental and offspring environment, the predictability of the environment, and the balance between parental and offspring fitness (J. Marshall & Uller 2007, Reed et al. 2010, Bonduriansky et al. 2012, Burgess & Marshall 2014, Shama & Wegner 2014). Furthermore, fitness consequences do not always act in the same direction at all life history stages and may vary based on the phenotypic metric assessed (Marshall 2008, Kekäläinen et al. 2015, Bautista & Crespel 2021, Crean & Immler 2021). The research presented in this thesis exemplifies this complicated phenotypic landscape.

In chapter I, the acclimation regime experienced by the mothers was complex, encompassing differences in the magnitude, variation, and predictability of variation in environmental factors including temperature, dissolved oxygen, and pH. The sites were located at different depths and inhabited by different species associated with the presence or

absence of kelp. Therefore, it is unsurprising that the results of the maternal exposure appear different depending on the phenotypic metric assessed. For instance, clear differences were observed in larval thermal tolerance, while respirometry measurements showed more context dependence of the larval environment. Between morphometric measures, different patterns were observed when considering body size versus skeletal growth through calcification. Only by considering both morphometric measures within a life history stage could you see potential trade-offs between skeletal growth and overall size; as larvae from outside mothers attained smaller sizes overall but were able to allocate proportionally more to spicule growth.

Similarly, in chapter II the effects of acute paternal heat exposure varied between the phenotypic metric assessed. When considering the LT_{50} of the larvae, there appeared to be an adaptive paternal effect where the larvae showed the highest thermal tolerance when the fathers underwent heat exposure and the larval environment aligned with that of the father. However, assessments of developmental normality revealed a different pattern, where paternal heat exposure yielded negative carryover effects in both larval environments. Conclusions about the impacts of paternal conditioning on successful development would even vary for the experiments in chapter II and III if one only assessed whether the larvae successfully reached veliger stage (chapter II, figure 2; chapter III, figure 2) without considering whether the veliger shell formed normally (an indicator of future survival). Lastly, if one only assessed veliger larval size they would detect no significant paternal effect of heat exposure.

These distinctions are important for how we design future experiments assessing transgenerational effects (Donelson et al. 2018). These different phenotypic patterns could not only lead us to broadly different conclusions but have implications for the survival, and

even dispersal, of the larvae in a natural setting (Burgess & Marshall 2011). For this reason, I would argue for assessing more than one relevant metric when possible. This may reveal potential trade-offs (Bilde et al. 2009, Crean & Bonduriansky 2014) or help to illuminate the underlying mechanisms behind transgenerational effects. When it is necessary to limit the number of metrics assessed, I would argue for including ones which most directly correlate to fitness (ie. developmental normality), in order to truly detect an effect of the parental acclimation. If one only assesses the "winners" after a majority have died, then without conducting a thorough genetic analysis any differences observed will be confounded by the question of the role of selection on underlying genetic variation of the offspring (Burgess & Marshall 2014, Donelson et al. 2018). Measures of the survival of the offspring can help to place any other interpretations in context. Finally, given the variation in results, in order for findings to have the most commercial or ecological relevance one should select the metrics used based on knowledge of the local environment and stressors and/or on the most industry relevant parameters (Diaz et al. 2018). For instance, whether or not the remaining larvae have a slight increase in thermal tolerance after paternal and larval conditioning may be less relevant in a hatchery if it comes at the cost of a 10% reduction in larval yield (chapter II).

4. The consequences of transgenerational effects can be mediated by interactions with other factors

Similar to the previous point, if one seeks to understand how transgenerational effects will play out in the actual environment, it is important to acknowledge that there are many factors which may influence both their physiological effects and the fitness outcomes. This should be taken into account when designing experiments but also interpreting the results.

From the adult acclimation perspective, factors modifying the acclimation regime may impact results. While conducting the maternal field acclimation outlined in chapter I, a major MHW event dominated a significant portion of the gametogenesis period for *S. purpuratus*. This reflects an increasing threat for urchins, and the kelp forest itself, and therefore is an extremely relevant part of the acclimation regime. However, this unexpected event likely shifted the relative importance of other aspects of the experimental setup, including depth and therefore must be incorporated into the interpretation of the results. During the adult urchin acclimation, urchins were fed ad libitum at both sites to control for food availability. In a fully natural comparison, however, food availability and nutritional content of food would vary drastically between both sites; with major consequences for transgenerational effects. Well-fed inside mothers might produce more well provisioned eggs, or outside mothers may not have the energetic surplus to mount an acclimatory response, offsetting the positive TGP effects of the physicochemical environment outside the kelp forest observed in this experiment. However, metabolic rates will also increase with temperature, underscoring the importance of considering environmental factors in tandem. Even comparing between two kelp forest sites with different depths, and therefore different temperatures, food quality may influence maternal provisioning as kelp nutritional quality has been shown decrease under high temperatures, potentially compounding metabolic stress for urchins at shallower sites (Lowman et al. 2022).

Additional factors, such as length of captivity, may have contributed to the different patterns in paternal effects observed between chapter II and III. The mussels used in the chapter II study were in held in a hatchery for much longer (over a year), and had lower baseline levels of larval normality, compared to those from chapter III. Mussels held in a

hatchery undergo far less variability and have a more constrained diet. The effects of prolonged captivity are important to consider for aquaculture because interventions such as selective breeding and parental conditioning may have to contend with the tradeoffs of removing animals from the wild for longer which may have implications for disease resistance, and holistic tolerance to other environmental stressors.

On the larval side, the CCS is an incredibly dynamic system in which MHWs are forecasted to become more persistent (Barkhordarian et al. 2022), but the spatial extent and intensity of upwelling is also predicted to increase (Diffenbaugh et al. 2004, Feely et al. 2008). Therefore, the adaptive capacity of purple urchins is likely to be shaped by the interplay and tradeoffs between multiple environmental stressors and their ecological feedbacks. This argues for assessing multiple phenotypic metrics and how parental acclimation impacts larval response to multiple stressors (Rosa et al. 2014). Performance metrics should also be informed by the local ecological and abiotic conditions. For instance, the fitness impacts of a transgenerational effect impacting body size may have different implications if the offspring are likely to go into an environment that is food limited, versus an environment with high predation stress, or high likelihood of thermal stress. Some phenotypic metrics may be more relevant to dispersal while others may be important in the context of competition.

5. Phenology of MHWs may impact transgenerational effects and evolution of reproductive traits

Differences in the phenology of an acclimation regime, may modify the severity of a transgenerational effect, as seen between chapter II and III and other studies (Suckling et al.

2014). Longer more chronic exposures led to more detrimental paternal carryover effects than shorter term more acute stressors. However, these differences may also be correlated to the season of spawning varying between the two experiments, an unintended but ecologically relevant factor in the experimental design. For green-lipped mussel populations on the South Island, thermal plasticity could be programmed cyclically with seasonal cycles. Plasticity across spawning seasons with regard to environmental stressors has been observed in other species including the European Squid, *Loligo vulgaris*, and Atlantic Cod, *Gadus morhua* (Rosa et al. 2014, Oomen & Hutchings 2015). In chapter II, the larvae from spring spawning showed lower baseline levels of normality but grew larger under warmer temperatures, (consistent with a preliminary study described in chapter II also conducted in the spring spawning period). In contrast the larvae from the fall spawning in chapter III had smaller size at higher temperatures and showed more extreme negative paternal carryover effects only when raised under high temperatures. In addition to factors such as duration in captivity, these differences could be impacted by the seasonal plasticity associated with spawning season: larvae from the Spring spawning season are likely to develop in the water column as temperatures are increasing into the peak summer months while larvae from the Fall spawning season come from parents who have been experiencing heat stress but temperatures are likely to decrease during their larval development.

The timing of MHWs during gametogenesis may modulate their effects, and even a short recovery period could temper or reverse transgenerational effects (see below in future directions). The duration of recovery needed is likely to vary between the two sexes given the differences in gametogenesis time between females and males. Probability of MHWs varies throughout the year with events most commonly occurring in the Summer and Fall months

(Sen Gupta et al. 2020). Around New Zealand, MHWs are forecasted to increase in duration and the peak period of MHW probability will widen in the future particularly weighted toward the Fall (Behrens et al. 2022). Considered together, if MHWs induce negative parental carryover effects and are increasingly prevalent in the late Summer and Fall, this may induce a new selective pressure towards increased Spring spawning or driving the second spawning peak later into the Fall, particularly if recovery is able to relieve or reverse negative parental effects. MHWs may also induce a shift towards later gametogenesis in the purple urchin because MHW events are particularly severe in the summer and fall. However, any shift in spawning can create a misalignment where larval development is offset from planktonic food availability.

Future directions

In the field of TGP, controlled lab experiments can be designed to isolate particular environmental factors for a more mechanistic understanding (Guillaume et al. 2016), while field acclimations can be used to encompass all the variability that occurs naturally (Hoshijima & Hofmann 2019). Phenotypic metrics can be chosen to investigate a particular physiological response (Waite & Sorte 2022), or a downstream ecological consequence (Burgess & Marshall 2011). Despite the complication around designing TGP experiments that are both ecologically relevant and mechanistically robust (Burgess & Marshall 2014, Donelson et al. 2018, Bautista & Crespel 2021), aquaculture is a field where this complication can be embraced and manipulated. Though the transgenerational effects of paternal heat exposure observed in this thesis were predominantly negative, the information gleaned from them can be used to improve outcomes for hatcheries in the face of future

MHWs. In the case of aquaculture, where breeding choices are controllable, experiments can leverage all the complications discussed in the points and chapters above. Broodstock can be selected from different sites further offshore or taken from deeper portions of lines to avoid the worst of negative carry-over effects, and the two sexes can be selected from different sites or conditioned separately. By considering the interactions between factors such as seasonality, duration of exposure, or which genetic line broodstock come from, hatcheries can make more informed decisions to improve outcomes. However, the studies in this dissertation are not comprehensive for determining the transgenerational effects of MHWs, and thermal conditioning, on *Perna canaliculus*. Two directions which I think would be particularly advantageous to pursue are testing different acclimation combinations between the two sexes, and testing different timing of exposure, or the manipulation of a recovery period prior to spawning.

In studies of transgenerational effects of pH and temperature different effects have been observed through the two parental lines across different phyla (Shama & Wegner 2014, Lane et al. 2015, Jonsson & Jonsson 2016, Venkataraman et al. 2019). Therefore, it is pertinent to consider transgenerational effects of relevant stressors through both parental lines and in combination when possible. This can be combined with selective breeding (Parker et al. 2012, Goncalves et al. 2016) or testing of existing genetic variation (Wei et al. 2013). Selective breeding did not offset negative effects of paternal heat exposure in this study (chapter III) but results may vary if both parents come from selectively bred lines as has been manipulated in other studies (Parker et al. 2012, Goncalves et al. 2016).

The role of recovery is understudied in the TGP literature. The majority of TGP studies utilize an exposure period directly prior to spawning or reproduction, in order to overlap with

gametogenesis but far fewer have induced a recovery period after the exposure prior to reproduction. From studies varying the timing of exposure, we know that duration can regulate transgenerational effects with longer exposure time erasing or even reversing negative transgenerational effects in studies of urchins (Dupont et al. 2013, Suckling et al. 2014). In studies varying the timing of exposure throughout life history, there is evidence that timing can modulate the strength of a maternal effect (Radersma et al. 2018) or the direction of a paternal effect (Gasparini et al. 2018). In both instances, the more advantageous result occurred when the parent was exposed to a given stressor earlier in life instead of after sexual maturity. Over a shorter timescale, switching paternal acclimation temperatures for 14 days prior to reproduction drove reduced larval survival relative to stable paternal temperatures, regardless of the order in which the two temperatures (15.5 and 21.5°C) were experienced (Guillaume et al. 2016). There is much still to learn about whether "recovery" can shift the impact of transgenerational effects, which is particularly relevant in the context of aquaculture where relevant findings can be employed in hatcheries to mitigate the impacts of negative parental carryover effects or harness positive effects .

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