

RESEARCH ARTICLE

Latitudinal comparison of thermotolerance and HSP70 production in F2 larvae of the greenshell mussel (*Perna canaliculus*)

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SUMMARY

We report the first measures of thermotolerance (recorded as percentage mortality and induced HSP70 production) for pelagic larvae of three populations of the New Zealand greenshell (green-lipped) mussel *Perna canaliculus*. Our goal was to determine whether distinct populations of *P. canaliculus* were more susceptible to predicted climate change than others, and whether such patterns of susceptibility were either genetically controlled (local adaptation of populations) or simply reflect the acclimatory capacity of this species. F2 larvae from three *P. canaliculus* populations (D'Urville Island, Banks Peninsula and Stewart Island) were subjected to an acute thermal challenge (3 h exposure to a fixed temperature in the range 20–42°C). No latitudinal patterns in either percentage mortality or HSP70 protein production were apparent. For all populations, larval mortality (LT₅₀) was between 32.9 and 33.9°C, with significant amounts of HSP70 induction only occurring in those individuals that experienced temperatures of 40°C or greater. The data presented therefore do not support the hypothesis that genetic adaptation of *P. canaliculus* to distinct thermal environments will be reflected by a corresponding difference in acute heat tolerance. In fact, the apparently vulnerable veligers show a surprisingly wide thermal safety margin. To develop a comprehensive understanding of the effects of ocean warming upon this species, subsequent studies should consider the impacts of sub-lethal stress upon fitness in addition to chronic thermal challenge and, crucially, the response of sedentary juvenile and adult stages.

Key words: percentage mortality, acute heat shock, climate change, local adaptation.

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INTRODUCTION

Global sea surface temperatures (SSTs) and the frequency and magnitude of 'extreme' events are predicted to increase with climate change (Bindoff et al., 2007; Rahmstorf and Coumou, 2011). Such a situation will threaten the current distribution of many marine taxa as the range of a species often reflects its physiological ability to withstand maximal environmental temperatures (Hofmann and Todgham, 2010). For intertidal environments, traditional models have assumed a continuous latitudinal gradient of temperature stress in which those populations residing at the edges of a species distribution are more at risk of localised extinction than those located at the centre (Sagarin and Somero, 2006). However, recent work has shown the situation to be much more complex, with a mosaic of thermal stress acting on both local and regional scales (Helmuth, 2009). Furthermore, counter-intuitive latitudinal patterns of vulnerability to increased temperatures have also been reported; i.e. cold-adapted high latitude populations being more heat tolerant than low latitude warm-adapted populations (Kuo and Sanford, 2009). This may be due to warm-adapted populations often living closer to their absolute tolerance limits and, accordingly, having less acclimation potential (Somero, 2011). Such intriguing findings have sparked great interest in mapping the effects of climate change on intertidal organisms, particularly in terms of the identification of those species that will be 'winners' under predicted climate change scenarios (Somero, 2010).

For intertidal marine invertebrates, intense interest is now being focused on the heat shock response (HSR) as a predictor of

organism and community performance under heat stress (Brown et al., 2004; Dong et al., 2008; Hamdoun et al., 2003; Lee and Boulding, 2010; Sagarin and Somero, 2006; Sorte et al., 2011; Tomanek, 2010; Tomanek and Somero, 1999). The heat shock response is highly conserved among taxa and consists of the expression of a suite of heat shock proteins (HSPs) (Parsell and Lindquist, 1994). These molecular chaperones assist in the stabilisation of protein structure not only under conditions of heat stress but also in response to other proteotoxic stressors, e.g. hypoxia, anoxia and salinity (Parsell and Lindquist, 1993). Importantly, the expression of HSPs, especially inducible HSP70 (hereafter called HSP70 and not to be confused with the constitutive form), in marine invertebrates has been used as an indicator of sensitivity to environmental stress gradients, be they on local (e.g. HSR of intertidal *versus* subtidal congeneric limpets *Chlorostoma*, formerly *Tegula*) or regional scales (e.g. biogeographic differences in expression of HSP70 for *Mytilus galloprovincialis*) (Dutton and Hofmann, 2009; Tomanek and Somero, 1999).

The use of whole-system bioassays, such as assessing mortality in response to acute thermal challenges, has also proven a robust comparative tool and provided excellent insights into inter- and intra-specific responses of invertebrates to heat stress (Kuo and Sanford, 2009; Pandolfo et al., 2010). By pairing these whole-organism assays with the HSR, an integrative understanding of the impact of thermal stress upon the organism is afforded. Selection of study organisms is crucial, however, and preference is given to those that exhibit a wide geographic distribution, and variation in physiological

responses to abiotic factors (Mykles et al., 2010). In this regard, marine mussels are an excellent study organism as they are often a dominant component of near-shore and intertidal ecosystems with explicit responses to increasing temperature, for example contractions of biogeographic range and extreme temperature-induced mortality events (Jones et al., 2010; Somero, 2012; Sorte et al., 2011). Furthermore, pronounced sub-lethal effects are evident in mussels exposed to temperature stress, with both northern (*M. edulis*) and southern hemisphere (*P. canaliculus*) mussel species exhibiting reduced growth and reproductive output in response to acute temperature challenges (Petes et al., 2007; Petes et al., 2008). Additionally, short-term responses are also evident, with freshwater mussel species increasing heart beat frequency when experiencing thermal stress (Pandolfo et al., 2010).

A key limitation to any studies of thermotolerance in marine invertebrates is an inability to resolve whether the patterns observed are genetically based, i.e. local adaptation to specific environmental conditions of a site, or a reflection of the phenotypic plasticity of a species (Sanford and Kelly, 2011). The performance of wild-sourced stock will inevitably be influenced by life history and acclimation, factors that may also influence the F1 offspring as a result of differing maternal investment in egg production (Phillips, 2007). Unbiased assessment therefore requires the use of F2 larvae of F1 parents that have been raised under the same conditions. Such experiments are rare because of the logistics involved in maintaining such organisms (Kuo and Sanford, 2009). However, a potential source of F2 larvae, hitherto untapped, is that of aquaculture hatcheries, where extensive breeding programmes are often undertaken and where the parentage and site of collection of broodstock individuals can be assured.

In the present study, we tested the thermotolerance of F2 larvae of the greenshell mussel (*Perna canaliculus* Gmelin 1791) that had been sourced from three populations from the South Island of New Zealand. This species is widely distributed throughout New Zealand, from the Kermadec Islands (30°S) to its southern limit at Stewart Island (46°S), where it often forms dense beds within the subtidal and intertidal zones. This species not only is ecologically important but also is New Zealand's largest source of aquaculture export revenue; thus, there is considerable commercial interest in selective breeding programmes (Fitzpatrick et al., 2013). Larval development of *P. canaliculus* takes up to 6 weeks and during this time it is thought that larvae may be dispersed up to several hundred kilometres (Hayden, 1995; Jeffs et al., 1999). Whilst such a distance could reduce the likelihood of local adaptation of populations occurring, recent evidence suggests that dispersal of marine invertebrate populations may not be as 'open' as once thought (Becker et al., 2007). Thus far, genetic analyses have been able to identify the large-scale haplotype structure for *P. canaliculus*, resolving three distinct groups; a northern group (encompassing the North Island and top of the South Island), a southern group (remainder of the South Island) and those populations residing at Stewart Island (Star et al., 2003). However, fine detail regional genetic structure remains to be uncovered (Apte and Gardner, 2002).

We therefore sought to identify potential biogeographic patterns in thermotolerance for *P. canaliculus* apparent in induced HSP70 protein levels or larval mortality (LT₅₀). The hypothesis was that larval *P. canaliculus* derived from parents sourced from warmer, northern latitudes would show greater resilience (i.e. lower percentage mortality and HSP70 production) to acute thermal stress than stock from colder southern sites; and that with the use of F2 larvae we would be able to discern whether any observed patterns in thermotolerance were a result of local adaptation or indicative of phenotypic plasticity for this species.

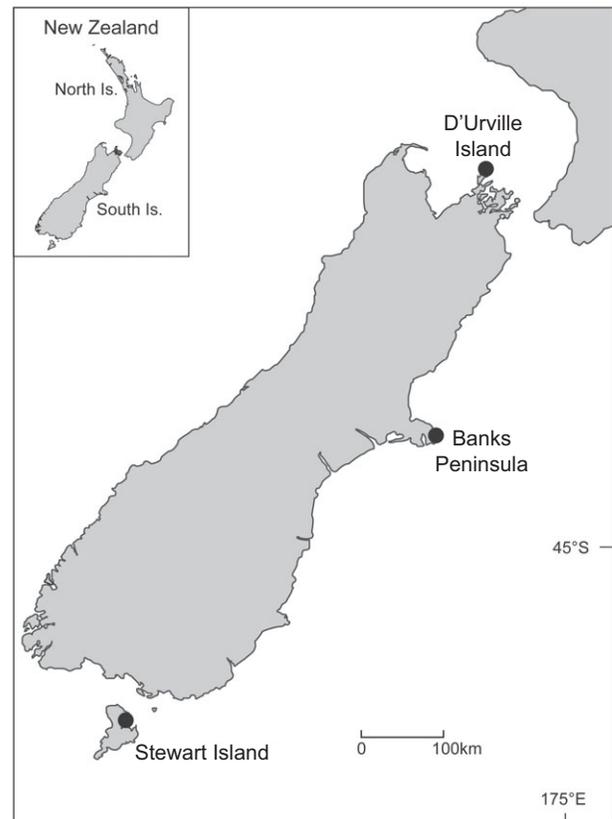


Fig. 1. Field sites for collection of adult *Perna canaliculus* used to generate F1 and F2 offspring for this study.

MATERIALS AND METHODS

Site locations and thermal regimes

In February of 2009, broodstock *P. canaliculus* were collected from three sites within the South Island of New Zealand: D'Urville Island (40°45', 173°48'), Banks Peninsula (43°42', 173°08') and Stewart Island (46°54', 168°14') (see Fig. 1). These sites are separated by a distance spanning approximately 930 km of coastline (or 7° of latitude) and consequently have differing thermal ranges. To quantify the exact thermal range of each site, SST readings were obtained from the SST 50 km (SST50) dataset available from NOAA's Comprehensive Large Array Stewardship System (CLASS, <http://www.nsof.class.noaa.gov/>). This dataset provides twice weekly SST measurements (°C), which we then averaged for each month for the period of 2007–2011 to give monthly SST values at each site.

Animal spawning, rearing and production of F2 larvae

Spawning of *P. canaliculus* broodstock and rearing of all larvae was completed in accordance with the standard methods used by the industry (Ragg et al., 2010). For our purposes, spawning of mussels was *via* thermal shock and gametes collected from mussels of the same site were mixed to provide 'site-pure' F1 larvae. Several full-sib crosses from individual male–female pairings were created in this way. Juveniles were reared to sexual maturity under identical conditions in the Cawthron Institute's shellfish nursery, receiving seawater at ambient temperature (18.1±0.6°C mean ± s.d.) from eutrophic algae ponds, allowing *ad libitum* feeding (Pilcher, 2003). Eight F1 adults from each family were spawned in January 2011 and gametes from different site-pure families crossed to create a single F2 half-sib family from each of the three geographic populations.

Once fertilised, embryogenic and trochophore stages were maintained for 48 h in static 18°C water containing EDTA. Once larvae had reached the veliger stage, they were transferred to 20°C flow-through systems for the remainder of larval rearing. Here, larvae were fed a mixture of *Isochrysis galbana* (T-Iso clone) and *Chaetoceros calcitrans* (forma *pumilum*) at a cell ratio of 1:2 and a concentration of 40 cells μl^{-1} . Measurements of shell length were obtained by capturing digital images of larvae and using ImageJ software as previously (Fitzpatrick et al., 2010). To ensure that only live larvae were used in experiments, larvae were collected from the top of the rearing tanks, i.e. still swimming, thus ensuring that measures of mortality were not inflated.

Acute heat shock treatment

To map the impact of an acute thermal shock on *P. canaliculus* larvae, samples of 7 day old larvae (~120 μm shell length) from each of the three sites were exposed to a thermal challenge for 3 h. To achieve this, a sand bath was set up, which consisted of an insulated polystyrene box (1000×600×200 mm) filled with fine, dry sand. Heat exchange coils were buried at opposing ends of the bath and received thermostatically controlled water, one at 15°C, the other at 60°C, thus providing a temperature gradient ranging between 20 and 43°C (see Table 1 for temperature measurements of each section). Larvae to be monitored for survival were stocked at 5 larvae ml^{-1} in 10 ml of seawater within 18 ml acid-washed glass liquid scintillation vials. For each population, a total of 18 vials were used to assess survival of larvae (see Fig. 2 for a schematic diagram of the experimental design). Three replicate vials from each population were randomly distributed along each isothermic row; a total of six rows provided exposure to a representative range of temperatures across the gradient. Water within each vial equilibrated to the surrounding sand temperature within 5 min of insertion. Individual vial temperatures were measured after 2 h using a thermocouple thermometer (Sper Scientific, model 800008, Scottsdale, AZ, USA) to allow a precise thermal response curve to be constructed.

Larvae were exposed to an acute thermal shock by being held in the temperature bath for 3 h before being allowed to recover for 18 h at 20°C. Following the recovery period, live and dead larvae were quantified by the addition of 0.7 ml of 85% ethanol (EtOH) to each vial as a sedative, causing larvae to cease swimming and drop to the bottom of the vessel for enumeration. Dead larvae were deemed to be those showing two or more of the following: uncontrolled gaping, extrusion of gut through gaping valves and/or a loss of internal structures.

Heat shock protein (HSP70) quantification

In order to map the production of the inducible form of HSP70 in *P. canaliculus* following acute heat exposure, a second

Table 1. Water temperatures of experimental vials recorded at each of the six divisions within the sandbath used in the acute heat shock challenge of *Perna canaliculus* larvae

Section in sandbath	Temperature (°C)
1	20±0.04
2	24±0.03
3	29±0.04
4	35±0.55
5	40±0.53
6	42±0.25

Temperature is given as mean ± s.e.m.

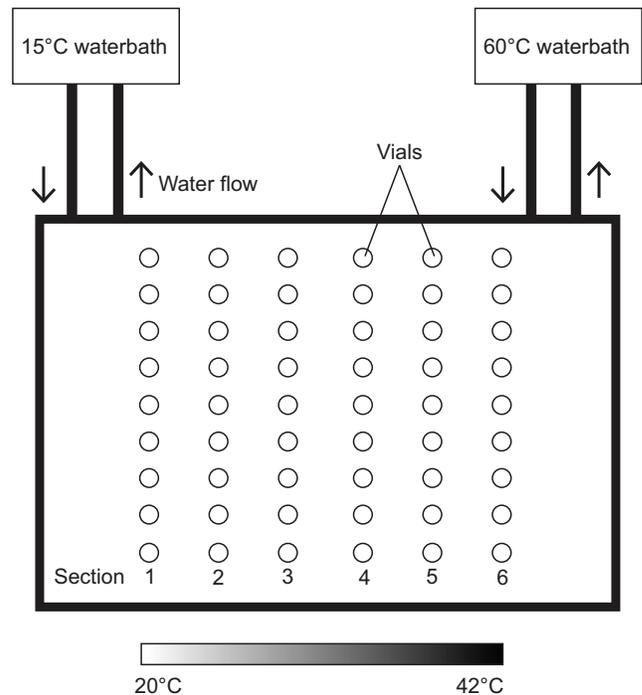


Fig. 2. Schematic diagram of the sand bath used to quantify mortality level and HSP70 expression in larval *P. canaliculus*. Three replicate vials for each geographic family were randomly distributed across each isothermic row. Heat exchange from the water baths was carried out *via* coils of 7 mm diameter polyethylene tubing buried in the sand.

experiment was run. Protocols followed those described above except that a volume of 15 ml water was used in vials and a density of 75 larvae ml^{-1} to ensure sufficient protein was obtained for analysis.

The inducible form of HSP70 was quantified using an HSP70 ELISA kit (EKS-700B Enzo Life Sciences, New York, NY, USA). Replicates of larvae were homogenised using polypropylene pestles (Raylab, Auckland, New Zealand). Extraction buffer used for homogenisation consisted of the 5× extraction buffer supplied with the HSP70 ELISA kit to which a broad spectrum protease inhibitor (SIGMAFAST, Sigma-Aldrich, St Louis, MO, USA) was added to prevent protein catabolism.

The resulting protein samples were diluted using sample diluent and aliquoted into the 96-well plates along with the diluted recombinant HSP70 standard. Following incubation for 2 h, the contents were aspirated, and the wells washed four times with wash buffer. Wells were then incubated with HSP70 antibody for 1 h. The washing procedure was repeated, horseradish peroxidase conjugate was then added and the plate was incubated again for a further 1 h. Following this, the chromophore was developed with tetramethylbenzidine (TMB) substrate and a blue colour developed in proportion to the amount of captured HSP70. The addition of an acid stop solution turned all solutions within the wells yellow and prevented further colour development. Endpoint colour intensity was measured at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA) held at 25°C.

Levels of HSP70 were expressed as $\text{ng } \mu\text{g}^{-1}$ total protein, with protein levels in the mussel tissue extracts quantified *via* the bicinchoninic acid assay (Pierce BCA kit, 23225, Rockford, IL, USA). Bovine serum albumin (BSA) was used as a protein standard.

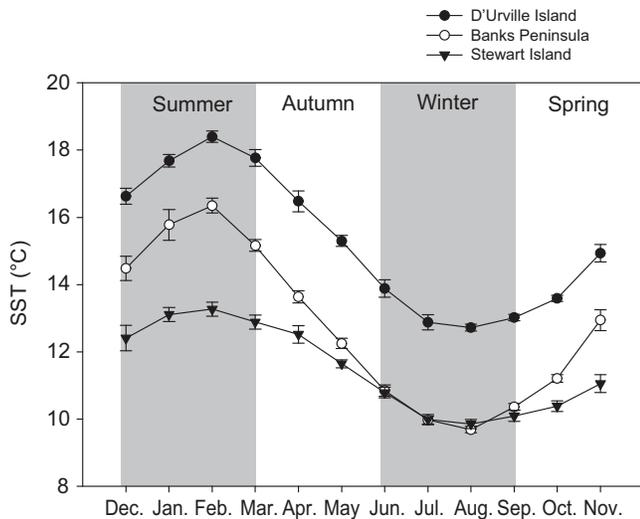


Fig. 3. Monthly sea surface temperature (SST, mean \pm s.e.m.) of the near-shore environment of source population sites averaged for the years 2007–2011. Austral seasons are superimposed above their respective months (data from NOAA).

Statistical analyses

Comparisons of seasonal SST data among sites were made using a two-way ANOVA with the interaction site \times season as the main factors. Normality and homogeneity of variance were tested with Shapiro–Wilk's and Levene's tests, respectively. Estimation of the acute lethal temperature at which 50% of the mussel larvae died (LT_{50}) followed previous methods (Kuo and Sanford, 2009). Namely, a logistic binomial regression was fitted to the number of live and dead larvae at each temperature, and LT_{50} then estimated using the inverse prediction function (based on maximum likelihood estimates) in JMP (version 9.0, SAS Institute, Cary, NC, USA). Logistic regression analyses were run with, and without, an interaction term between temperature and site. Because of a lower Akaike's information criterion (AIC) value for the 'no interaction model' (840.07 interaction model *versus* 842.91 no interaction model), we did not include an interaction term in our logistic regression (Agresti, 2007). Using the model parameters site, temperature and site \times temperature, the effect of temperature on the level of HSP70 production among sites was tested using least-squares multiple regression (Zar, 2010).

RESULTS

Thermal regimes among sites differed in an expected pattern, with average monthly temperatures decreasing with increasing latitude, i.e. D'Urville Island recording the highest SST for any season, followed by Banks Peninsula and Stewart Island, respectively (see Fig. 3). Strong seasonal differences were found within and between sites, with the exception of Banks Peninsula and Stewart Island during the winter, where no significant temperature difference was detected. This result is reflected in the significant site \times season interaction effect evident in Table 2. Additionally, differences in amplitude of the thermal regime were observed among sites, with the difference between winter and summer temperatures being 6°C for both Banks Peninsula and D'Urville Island compared with 3°C for Stewart Island (Fig. 3).

Population differences in size were evident after 1 week of larval culture; D'Urville and Stewart Island showed similar mean (\pm s.e.m.)

Table 2. Results of two-way ANOVA comparing seasonal thermal regimes among D'Urville Island, Banks Peninsula and Stewart Island sites for the years 2007–2011

Source of variation	d.f.	SS	MS	F	P
Season	3	504.1	168.0	212.0	<0.001
Site	2	444.6	223.3	280.5	<0.001
Season \times site	6	29.7	5.0	6.2	<0.001
Residual	168	133.1	0.8		
Total	179				

Data from NOAA.

shell lengths (158.1 ± 1.3 and 158.8 ± 1.3 μ m, respectively). Larvae sourced from Banks Peninsula broodstock showed a small, but significant increase in size (163.1 ± 1.64 μ m; one-way ANOVA, d.f. 2,170; $F=3.89$, $P<0.05$).

Mortality of larvae from the three sites following a 3 h temperature challenge and an 18 h recovery period are shown in Fig. 4. With regards to percentage mortality, the temperature band of 32–37°C represents a key response region for temperature-related mortality in this species, with a rise in mortality from <10% to >90% over these temperatures (see Fig. 4A). An inverse prediction model showed that, for a given temperature, individuals sourced from Stewart Island exhibited slightly higher levels of mortality compared with individuals sourced from either D'Urville Island or Banks Peninsula (Fig. 4B). However, estimates of LT_{50} were not significant among the populations tested, with 33.6 ± 0.8 , 34.0 ± 0.6 and 33.0 ± 0.7 °C confidence interval recorded for D'Urville Island, Banks Peninsula and Stewart Island, respectively (see Fig. 5, Table 3).

Levels of HSP70 production in *P. canaliculus* larvae did not correlate with site of origin (see Table 4, Fig. 6), with only temperature having a significant effect on the production of HSP70 in this species. However, the relationship between temperature and HSP70 production was not strong, e.g. r^2 of 0.30, with low levels of HSP70 in larvae held at or below 35°C followed by an abrupt, albeit variable, increase in HSP production in larvae held above 40°C.

DISCUSSION

Measures of acute thermotolerance and HSP70 production in larval *P. canaliculus* veligers are reported here for the first time. A key aim of the study was to characterise intraspecific thermotolerance patterns for F2 larvae of parents gathered from differing sites in southern New Zealand. It was hypothesised that *P. canaliculus* larvae derived from parents sourced from warmer, northern latitudes would show greater resilience (i.e. lower percentage mortality and HSP70 production) to acute thermal stress than stock from colder southern sites.

However, when challenged with temperatures ranging from 20 to 42°C, F2 *P. canaliculus* larvae from the three populations demonstrated very similar mortality and HSP70 responses. This is despite the SST profiles of the sites from which the original broodstock were collected conforming to an expected latitudinal gradient of warmer SSTs in the north *versus* colder SSTs in the south. Interestingly, winter SSTs at two sites (Banks Peninsula and Stewart Island) were similar, probably because of the dominance of the Southland Current pushing up the east coast of the South Island (Sutton, 2003). Further complicating the hydrodynamic situation in this area are alternating influxes of either cold sub-antarctic or warmer sub-tropical surface waters that occur near Banks

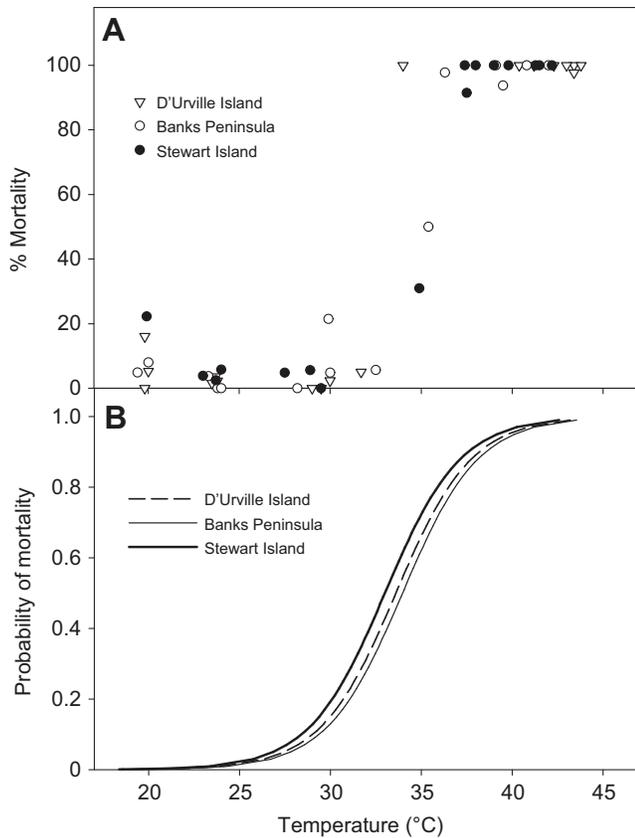


Fig. 4. Mortality of larval *P. canaliculus* from three southern New Zealand populations that were exposed to an acute thermal shock for 3 h and allowed to recover for 18 h at 20°C. (A) Percentage mortality of thermally challenged *P. canaliculus* larvae. (B) Mortality response curves fitted using logistic regression and inverse prediction, which describe the probability of 'x' mortality for a given temperature.

Peninsula (Chiswell, 1994; Shaw and Vennell, 2000). This situation of alternating hydrodynamic features may account for the similarity of winter SSTs between these two sites and the dissimilarity of summer SSTs.

Despite being raised under identical husbandry conditions, there were differences in size among the larvae used in our experiments. Banks Peninsula larvae exhibited higher rates of growth compared with their conspecifics from D'Urville and Stewart Island sites. This presumably reflects the genetically distinct nature of the larvae used; however, such size differences had little impact on thermotolerance capability among these populations. Lee and Boulding reported similar results for thermotolerance trials using the intertidal gastropod *Littorina keenae* (Lee and Boulding, 2010). In this northern hemisphere species, body size exhibited a latitudinal cline, with larger individuals found at colder, northern sites, yet no distinct latitudinal cline in thermotolerance to temperatures of 30°C and above was observed (Lee and Boulding, 2010).

For *P. canaliculus*, increases in larval mortality were only observed once water temperatures exceeded 29°C, with 100% mortality of larvae that experienced water temperatures of 42°C. Such findings reflect those observed for F1 larvae of the freshwater mussel species *Lampsilis siliquoidea*, *Potamilus alatus* and *Ligumia recta*, all of which exhibited 100% mortality when acutely exposed to temperatures of 42°C (Pandolfo et al., 2010). To the best of our knowledge, the study of Kuo and Sanford is the only other study

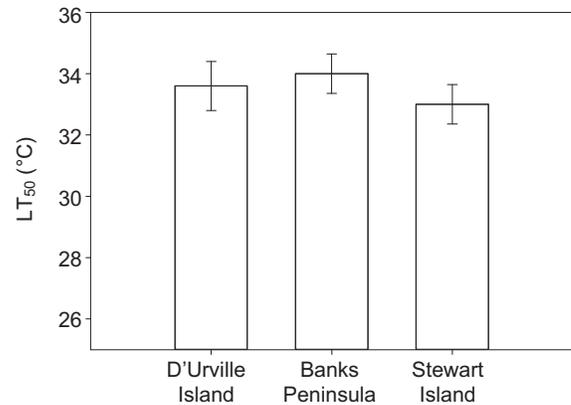


Fig. 5. Estimates of larval mortality (LT₅₀, ±95% confidence interval) for larval *P. canaliculus* from three sites in southern New Zealand that were exposed to an acute thermal shock for 3 h and allowed to recover for 18 h at 20°C.

to utilise F2 marine invertebrate larvae in thermotolerance trials (Kuo and Sanford, 2009). They found distinct regional differences in LT₅₀ for *Nucella canaliculata* larvae sourced from cool and warm water sites (Oregon versus California), indicating a strong level of local adaptation in this species. Despite using similar scales of latitudinal separation, we did not observe differences in larval *P. canaliculus* LT₅₀ among sites in the present study. A key explanation may lie in the differing larval dispersal strategies of our respective study organisms. Direct developing organisms (such as *N. canaliculata*) are thought to have greater potential for local adaptation than planktonic larvae (e.g. *P. canaliculus*), which can disperse over large distances (Parsons, 1998).

Using expected climate change scenarios, increases in SST for the New Zealand Exclusive Economic Zone are expected up to a maximum of 4°C over the next 100 years (Boyd and Law, 2011). For the most northern population we tested (D'Urville Island), this would equate to average summer temperatures peaking at 22°C, well within the acute LT₅₀ temperature of 33.5°C calculated here for *P. canaliculus*, suggesting a wide thermal safety margin. However, before *P. canaliculus* can be classified as a 'winner' under predicted climate change scenarios, the ontogeny of thermal susceptibility of this species needs to be characterised for all life stages. Thermotolerance trials using adult *P. canaliculus* observed 100% mortality, 24 h after a 3 h exposure to 35°C (B.J.D. and N.L.C.R., unpublished data). Furthermore, during 2005, wild adult populations of *P. canaliculus* at Banks Peninsula suffered high rates of mortality during a 3 day heat wave event where air temperatures reached 36°C (Petes et al., 2007). Such thermally induced mortality is not restricted to elevated temperatures, with sudden drops in water and air temperature producing significant mortality in adults and juveniles of the green mussel, *P. viridis* (Firth et al., 2011; Urian et al., 2011). Temperature variations are predicted to increase in magnitude and frequency with the progression of climate change (Rahmstorf and Coumou, 2011) and further highlight the complex interplay between biotic (e.g. reproduction, competition) and abiotic (e.g. temperature, desiccation) factors that determine the physiological susceptibility of a species (Tomanek and Helmuth, 2002). Thus, it may be that the eurythermal intertidal life stages (juvenile and adult) of *P. canaliculus* are actually more thermally sensitive than the larval stages developing in stenothermal subtidal environments. Recent studies with a terrestrial ectotherm (the mealworm *Tenebrio molitor*) demonstrate that adults were less

Table 3. Results from binomial logistic regression describing the relationship between temperature and probability of mortality of *P. canaliculus* larvae from three sites after experiencing an acute thermal challenge

Whole-model test				
Model	–log likelihood	d.f.	Chi square	Prob>Chi square
Difference	1030.98	3	2061.97	<0.0001
Full	416.03			
Reduced	1447.01			
AIC	840.10			
Parameter estimates				
Term	Estimate	s.e.m.	Chi square	Prob>Chi square
Intercept	–16.07	0.70	440.83	<0.0001
D'Urville Island	–0.04	0.13	2.74	0.78
Banks Peninsula	–0.21	0.15	3.84	0.10
Stewart Island	0.25	0.12	29.4	0.06
Temperature	0.48	0.02	521.41	<0.0001

AIC, Akaike's information criterion.

thermally resilient than larvae (Belén Arias et al., 2011). Such observations broadly agree with a general hypothesis advanced by Pörtner and Farrell, who suggest that the thermotolerance window will increase through the gamete and larval stages to maximum values in juveniles, declining again as sexual maturity is reached (Pörtner and Farrell, 2008).

Furthermore, chronic sub-lethal thermal stress may be of greater relevance to the pelagic larvae of mussel species. For example, Buchanan observed high mortality in *P. canaliculus* larvae reared at 24°C, compared with apparent optima at 16–19°C (Buchanan, 1998). Ragg and colleagues also note that the effect of subtle stress may not be immediately detectable in *P. canaliculus* larval survival and growth, but become manifest as reduced pediveliger competency to metamorphose and settle (Ragg et al., 2010). Thus, for marine ectotherms, quantifying thermotolerance may be necessary for all life stages, particularly for those species where larval and adult forms exist in very different environments, i.e. stenothermal subtidal larvae versus intertidal eurythermal adults.

It should be noted that the 3 h immersed thermal shock administered in the current trials does not represent a simple challenge. Young veligers of the brown mussel, *Perna perna*, were found to consume ~2 ng O₂ individual^{–1} h^{–1} at 24°C (Lemos et al., 2003). Veligers of *P. canaliculus* may maintain an even higher metabolic rate, with preliminary measurements suggesting an oxygen consumption rate of ~5 ng O₂ individual^{–1} h^{–1} under our

Table 4. Results of multiple regression comparing levels of HSP70 production of *P. canaliculus* veligers and temperature among sites

ANOVA					
Source	d.f.	SS	MS	F	Prob>F
Model	3	0.002	0.0007	7.9775	0.0003
Error	42	0.003	0.00009		
Total	45	0.006			
Parameter estimates					
Term	Estimate	s.e.m.	t-ratio	Prob>t	
Intercept	0.0039594	0.006394	0.62	0.5391	
Site	–0.002987	0.001702	–1.75	0.0866	
Temperature	0.0007485	0.00017	4.41	<0.0001*	
Site × temperature	–0.000323	0.000211	–1.53	0.1344	

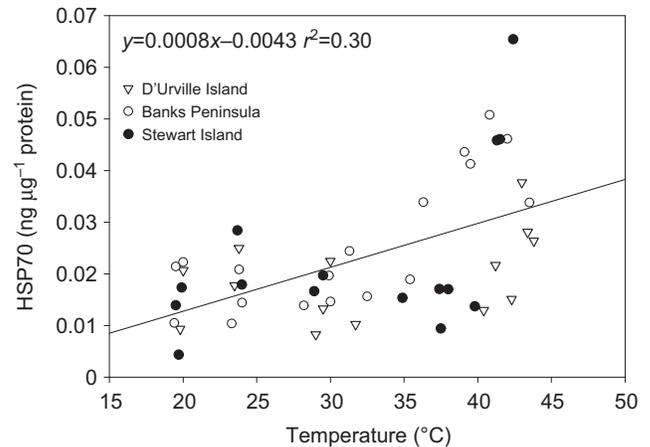


Fig. 6. Relationship between levels of inducible HSP70 in larvae of *P. canaliculus* from three populations in southern New Zealand that were exposed to an acute thermal shock for 3 h and allowed to recover for 18 h at 20°C. Note, regression of HSP70 versus temperature (solid line) is shown for all sites pooled.

control conditions (7 day old veligers at 20°C; N.L.C.R., unpublished data). Larval metabolism is also likely to increase rapidly with temperature; for example, oyster veliger growth rates increased according to a Q_{10} coefficient of approximately 3.4 between 17 and 32°C (Rico-Villa et al., 2009). If larval metabolism follows a similar trajectory, oxygen consumption rates could exceed 20 ng O₂ individual^{–1} h^{–1} at the survival tipping-point temperatures measured here. The oxygen content of air-equilibrated seawater also decreases with rising temperature, at 20°C holding ~7.5 ng O₂ ml^{–1}, falling to ~6.1 ng O₂ ml^{–1} at 32°C (Benson and Krause, 1984). It therefore seems inevitable that some degree of hypoxia was present in all experimental vials, becoming exacerbated at higher challenge temperatures. The monitoring of survival following thermal shock underwater should therefore be considered to be an assessment of the integrated effects of elevated temperature and all corresponding physical covariates within the water body.

With regards to HSP70 induction, the temperature at which larval *P. canaliculus* initiated HSP70 expression (known as T_{on}) was not population specific and appeared to occur above 35°C. In summer-acclimated Pacific oysters (*Crassostrea gigas*), induction of HSP69 was not apparent until experimental thermal challenge temperatures reached 40°C, yet T_{on} was lower (37°C) in winter-acclimated oysters (Hamdoun et al., 2003). Given that our experiments were performed in the austral summer months, it may be expected that the winter T_{on} for *P. canaliculus* will be somewhat lower.

It is thus apparent that the initiation of HSP70 production is influenced by a complex suite of temperature cues. Previous authors have noted the role ambient temperature fluctuations have in the temperature at which HSP70 production is induced (Dong et al., 2008; Tomanek and Somero, 1999). Organisms inhabiting moderately variable thermal environments (i.e. subtidal habitats) rarely display induced HSP70 production and only do so at temperatures well above those normally experienced in their natural environment (Tomanek, 2008). In our results, the T_{on} for HSP70 expression approaches the 100% lethal temperature for *P. canaliculus* larvae, implying there is little protection provided by the HSR for larvae of this species. However, intrinsic (e.g. primary sequence) and extrinsic factors (e.g. compatible osmolytes) are known to also stabilise proteins and potentially increase the thermal

range of an organism (Tomanek, 2008). What role these play in stabilising proteins of *P. canaliculus* is currently unknown and deserves further investigation.

Whether (as in the case of thermotolerance) an ontogenetic increase in HSP70 expression occurs to protect *P. canaliculus* larvae once they are settled in the eurythermal intertidal environment is unknown. Adult and veliger larvae of the native California oyster (*Ostreola conchaphila*) showed increased expression of HSP70 following a heat shock of 33°C or greater, yet a similar response was not seen in the early embryonic stages, i.e. eight cell and blastula, of this species (Brown et al., 2004). Initiating the expression of HSPs often results in reduced synthesis of other proteins and may be metabolically expensive (Hamdoun et al., 2003; Parsell and Lindquist, 1994). Thus for *P. canaliculus*, a trade-off may exist between balancing the demands imposed by larval development and the negative effects incurred by initiating HSP70 expression.

The apparent lack of thermotolerance variability between *P. canaliculus* populations is intriguing given that collection sites were separated by nearly 1000 km, a distance over which populations can be reasonably expected to show adaptive differentiation to their local environment (Sanford and Kelly, 2011). Metapopulation dynamics, temporal variation in abiotic factors and phenotypic plasticity can all prevent local adaptation occurring in marine invertebrates (Sanford and Kelly, 2011). However, our use of F2 larvae obviates the first two masking agents; thus, it is evident that the larval phenotype of *P. canaliculus* is remarkably robust when faced with acute thermal stress. Hamdoun and colleagues (Hamdoun et al., 2003) assert that the HSR of mussels and oysters exhibits a high level of phenotypic plasticity. Whilst it is tempting to see confirmation of this within our work, experiments to indicate whether developmental or reversible plasticity is present are now needed to confirm or disprove this for *P. canaliculus*. Nonetheless, whether the lack of site differences in thermotolerance is maintained by high gene flow is unknown as the larval dispersal 'neighbourhoods' of *P. canaliculus* are still to be determined, although much progress is beginning to be made in this regard (Dunphy et al., 2011). The use of full-sib F1 families in the present trial means that only four original parents are represented in each F2 genotype, raising concern that individual effects could overshadow geographic/population-level effects. However, a small pool of founding parents would be expected to increase the likelihood of phenotypic differences between families. The conservative thermotolerance results observed are therefore all the more surprising and likely to be a general limitation in this species. Given the difficulty in obtaining F2 individuals, we were only able to utilise larvae from South Island sites in our experimental work, and were thus unable to provide estimates of the thermotolerance of this species throughout its entire range (i.e. including North Island of New Zealand sites). Nevertheless, single-strand conformation polymorphism (SSCP) and random amplified polymorphic DNA (RAPD) analyses of *P. canaliculus* phylogeography recognise three clades of this species around New Zealand, with the D'Urville Island, Banks Peninsula and Stewart Island sites being located within the Northern, Southern and Stewart Island clades, respectively (Apte and Gardner, 2002; Star et al., 2003). Thus, we were able to compare some of the broad scale genetic patterns that exist in *P. canaliculus* around New Zealand. The possibility of introgression of cultured mussels into local natural populations is an issue of concern for New Zealand (Apte et al., 2003). For many years, the mussel aquaculture industry has actively transferred larval *P. canaliculus* from the far north of New Zealand to southern on-growing sites. Definitive methods to identify where introgression has occurred

remain to be developed; thus, we have been accordingly circumspect when drawing our conclusions.

In conclusion, the present study provides the first account of HSP70 induction and thermotolerance in *P. canaliculus*. It appears that this species possesses great acclimatory capacity (as opposed to fixed genotypic differences along a stress gradient) as there was little evidence of local adaptation among F2 individuals sourced from three sites separated by 900 km. Whilst our results are encouraging insofar as near-future ocean surface temperatures are unlikely to approach the acute LT₅₀ of *P. canaliculus* veligers, mussel populations are predicted to experience significant alterations to predator-prey dynamics under predicted climate change (Harley, 2011), and it may well be that these constitute a larger driver of localised extinctions of mussel populations than temperature effects *per se*. Future effort now needs to be focused on describing the thermotolerance profile of this species along its entire distribution using northern F2 larvae. Additionally, how thermotolerance or HSP70 expression varies with ontogeny must be assessed if a robust estimate of complete life-cycle resilience is to be achieved. Lastly, in order to refine our understanding of anthropogenic influences upon *P. canaliculus* in general (including climate change and aquaculture), a characterisation of larval dispersal neighbourhoods of this species is needed, including definitive evidence for or against introgression by cultured populations.

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