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1   Latitudinal comparison of thermotolerance and HSP70 production in F2 larvae of the

2   Greenshell mussel (*Perna canaliculus*).

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22   **Short title:** Thermotolerance of F2 mussel larvae

23

24   **Summary**

25   We report the first measures of thermotolerance (recorded as percent mortality and induced

26   HSP70 production) for pelagic larvae of three populations of the New Zealand Greenshell

27   (green-lipped) mussel *Perna canaliculus*. Our goal was to determine whether distinct

28   populations of *P. canaliculus* were more susceptible to predicted climate change than others,

29   and whether such patterns of susceptibility were either genetically controlled (local

30   adaptation of populations) or simply reflect the acclimatory capacity of this species. F2 larvae

31   from three *P. canaliculus* populations (D'Urville Island, Banks Peninsula and Stewart Island)

32   were subjected to an acute thermal challenge (3 h exposure to a fixed temperature in the

33   range 20 - 42°C). No latitudinal patterns in either % mortality or HSP70 protein production

34   were apparent. For all populations LT_{50} was between 32.9 and 33.9 °C, with significant

35   amounts of HSP70 induction only occurring in those individuals that experienced

36   temperatures of 40°C or greater. The data presented therefore do not support the hypothesis

37   that genetic adaptation of *P. canaliculus* to distinct thermal environments will be reflected by

38   a corresponding difference in acute heat tolerance. In fact, the apparently vulnerable veligers

39   show a surprisingly wide thermal safety margin. To develop a comprehensive understanding

40   of ocean warming upon this species, subsequent studies should consider the impacts of sub-

41   lethal stress upon fitness in addition to chronic thermal challenge and, critically, the response

42   of sedentary juvenile and adult stages.

43   **Keywords** thermotolerance, % mortality, HSP70, F2 larvae, *Perna canaliculus*, local

44   adaptation,

45

46   **Introduction**

47   Global sea surface temperatures (SSTs) and the frequency and magnitude of 'extreme' events

48   are predicted to increase with climate change (Bindoff et al., 2007; Rahmstorf and Coumou,

49   2011). Such a situation will threaten the current distribution of many marine taxa as the

50   range of a species often reflects its physiological ability to withstand maximal environmental

51   temperatures (Hofmann and Todgham, 2010). For intertidal environments, traditional models

52   have assumed a continuous latitudinal gradient of temperature stress in which those

53   populations residing at the edges of a species distributions are more at risk of localised

54   extinction than those located at the centre (Sagarin and Somero, 2006). However, recent work

55 has shown the situation to be much more complex with a mosaic of thermal stress acting on
56 both local and regional scales (Helmuth, 2009). Furthermore, counter-intuitive latitudinal
57 patterns of vulnerability to increased temperatures have also been reported; i.e. cold-adapted
58 high latitude populations being more heat tolerant than low latitude warm-adapted
59 populations (Kuo and Sanford, 2009). This may be due to warm-adapted populations often
60 living closer to their absolute tolerance limits and accordingly, have less acclimation potential
61 (Somero, 2011). Such intriguing findings have sparked great interest in mapping the effects
62 of climate change on intertidal organisms, particularly identifying those species that will be
63 'winners' under predicted climate change scenarios (Somero, 2010).

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

78 The use of whole system bioassays, such as assessing mortality in response to acute thermal
79 challenges, has also proven a robust comparative tool and provided excellent insights into
80 inter- and intra-specific responses of invertebrates to heat stress (Kuo and Sanford, 2009;
81 Pandolfo et al., 2010). By pairing these whole organism assays with the HSR, an integrative
82 understanding of the impact of thermal stress upon the organism is afforded. Selection of
83 study organisms is critical however, and preference is given to those that exhibit a wide
84 geographic distribution, and variation in physiological responses to abiotic factors (Mykles et
85 al., 2010). In this regard, marine mussels are an excellent study organism as they are often a
86 dominant component of near-shore and intertidal ecosystems with explicit responses to
87 increasing temperature, for example contractions of biogeographic range and extreme

88   temperature-induced mortality events (Jones et al., 2010; Somero, 2012; Sorte et al., 2011).

89   Furthermore, pronounced sub-lethal effects are evident in mussels exposed to temperature

90   stress, with both northern (*M. edulis*) and southern hemisphere (*P. canaliculus*) mussel

91   species exhibiting reduced growth and reproductive output in response to acute temperature

92   challenges (Petes et al., 2007, Petes et al., 2008). Additionally, short term responses are also

93   evident with freshwater mussel species increasing heart beat frequency when experiencing

94   thermal stress (Pandolfo et al., 2010).

95   A key limitation to any studies of thermotolerance in marine invertebrates is an inability to

96   resolve whether the patterns observed are genetically based, i.e. local adaptation to specific

97   environmental conditions of a site, or a reflection of the phenotypic plasticity of species

98   (Sanford and Kelly, 2011). The performance of wild-sourced stock will inevitably be

99   influenced by life history and acclimation, factors which may also influence the F1 offspring

100 due to differing maternal investment in egg production (Phillips, 2007). Unbiased assessment

101 therefore requires the use of F2 larvae of F1 parents that have been raised under the same

102 conditions. Such experiments are rare due to the logistics involved in maintaining such

103 organisms (Kuo and Sanford, 2009). However, a potential source of F2 larvae, hitherto

104 untapped, is that of aquaculture hatcheries where extensive breeding programs are often

105 undertaken and where the parentage and site of collection of broodstock individuals can be

106 assured.

107 In the present study we tested the thermotolerance of F2 larvae of the Greenshell mussel

108 (*Perna canaliculus* Gmelin 1791) that had been sourced from three populations from the

109 South Island of New Zealand. This species is widely distributed throughout New Zealand,

110 from the Kermadec Islands (30°S) to its southern limit at Stewart Island (46°S), where it

111 often forms dense beds within the subtidal and intertidal zones. Not only ecologically

112 important, this species is also New Zealand's largest source of aquaculture export revenue

113 thus there is considerable commercial interest in selective breeding programmes (Fitzpatrick

114 et al., *in press*). Larval development of *P. canaliculus* takes up to 6 weeks and during this

115 time it is thought that larvae may be dispersed up to several hundred kilometres (Hayden,

116 1995; Jeffs et al., 1999). Whilst such a distance could reduce the likelihood of local

117 adaptation of populations occurring, recent evidence suggests that dispersal of marine

118 invertebrate populations may not be as 'open' as once thought (Becker et al., 2007). Thus far

119 genetic analyses have been able to identify large-scale haplotype structure for *P. canaliculus*

120 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 (LT_{50}). The hypothesis was larval *P. canaliculus* derived from parents sourced from warmer, northern latitudes would show greater resilience (i.e. lower % 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 discriminate whether any observed patterns in thermotolerance were a result of local adaptation or indicative of phenotypic plasticity for this species.

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, sea surface temperature (SST) readings were obtained from the SST50 dataset available from NOAA's Comprehensive Large Array Stewardship System (CLASS, http://www.nsof.class.noaa.gov/). This dataset provides twice weekly SST (°C) measurements 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 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°C ± 0.6 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

151 from different 'site pure' families crossed to create a single F2 half-sib family from each of

152 the 3 geographic populations.

153 Once fertilised, embryogenic and trochophore stages were maintained for 48 h in static 18°C

154 water containing EDTA. Once larvae had reached the veliger stage, they were transferred to

155 20°C flow-through systems for the remainder of larval rearing. Here, larvae were fed a

156 mixture of *Isochrysis galbana* (T-Iso clone) and *Chaetoceros calcitrans* (forma *pumilum*) at a

157 cell ratio of 1:2 and concentration of 40 cells μL^{-1}. Measurements of shell length were

158 obtained by capturing digital images of larvae and using Image J software as set out in

159 Fitzpatrick et al. (2010). To ensure that only live larvae were used in experiments, larvae

160 were collected from the top of the rearing tanks; i.e. still swimming, thus ensuring that

161 measures of mortality were not inflated.

162 *Acute heat shock treatment*

163 To map the impact of an acute thermal shock on *P. canaliculus* larvae, samples of seven day

164 old larvae (~120 µm shell length) from each of the three sites were exposed to a thermal

165 challenge for three hours. To achieve this, a sand bath was set up which consisted of an

166 insulated polystyrene box ($1000 \times 600 \times 200$ mm) filled with fine, dry sand. Heat exchange

167 coils were buried at opposing ends of the bath, one receiving thermostatically-controlled

168 water at 15°C, the other 60°C; thus providing a temperature gradient ranging between 20 and

169 43°C (see Table 1 for temperature measurements of each section). Larvae to be monitored for

170 survival were stocked at 5 larvae mL^{-1} in 10 mL of seawater within 18 mL acid washed glass

171 liquid scintillation vials. For each population, a total of 18 vials were used to assess survival

172 of larvae (see Fig. 2 for schematic of experimental design). Three replicate vials from each

173 population were randomly distributed along each isothermic row; a total of 6 rows provided

174 exposure to a representative range of temperatures across the gradient. Water within each vial

175 equilibrated to surrounding sand temperature within 5 min of insertion. Individual vial

176 temperatures were measured after 2 h using a thermocouple thermometer (Sper Scientific,

177 model 800008) to allow a precise thermal response curve to be constructed.

178 Larvae were exposed to an acute thermal shock by being held in the temperature bath for

179 three hours before being allowed to recover for 18 h at 20°C. Following the recovery period,

180 live and dead larvae were quantified by the addition of 0.7 ml of 85 % ETOH to each vial as

181 a sedative, causing larvae to cease swimming and drop to the bottom of the vessel for

182 enumeration. Dead larvae were deemed to be those showing two or more of the following:

183 uncontrolled gaping, extrusion of gut through gaping valves and/or a loss of internal
184 structures.

Heat shock protein (HSP70) quantitation

186 In order to map the production of the inducible form of HSP70 in *P. canaliculus* following
187 acute heat exposure, a second experiment was run. Protocols followed those described above
188 except that a volume of 15 ml of water was used in vials and a density of 75 larvae mL^{-1} to
189 ensure sufficient protein was obtained for analysis.

190 The inducible form of HSP70 was quantified using an HSP70 ELISA kit (EKS-700B Enzo
191 Life Sciences, New York, USA). Replicates of larvae were homogenised using polypropylene
192 pestles (Raylab, Auckland, NZ). Extraction buffer used for homogenisation consisted of the 5
193 X extraction buffer supplied with the HSP70 ELISA kit to which a broad spectrum protease
194 inhibitor (SIGMAFAST ^{TM}, Sigma-Aldrich) was added to prevent protein catabolism.

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

205 Levels of HSP70 were expressed as ng/μg total protein, with protein levels in the mussel
206 tissue extracts quantified via the Bicinchoninic Acid Assay (Pierce BCA kit, 23225,
207 Rockford, Illinois, USA). Bovine serum albumin (BSA) was used as a protein standard.

Statistical analyses

209 Comparisons of seasonal SST data among sites were made using a 2-Way ANOVA with
210 interaction; *site* and *season* as the main factors. Normality and homogeneity of variance were
211 tested with Shapiro–Wilk's and Levene's tests respectively. Methods to estimate the acute
212 lethal temperature at which 50% of the mussel larvae died (LT$_{50}$) followed the methods of

213 Kuo and Sanford (2009). Namely, a logistic binomial regression was fitted to the number of

214 live and dead larvae at each temperature, and LT_{50} then estimated using the inverse prediction

215 function (based on maximum likelihood estimates) in JMP (version 9.0, SAS Institute,

216 Carolina, USA). Logistic regression analyses were run with, and without, an interaction term

217 between *temperature* and *site.* Due to a lower AIC value for the 'no interaction model'

218 (840.07 interaction model vs. 842.91 no interaction model) we did not include an interaction

219 term in our logistic regression (Agresti, 2007). Using the model parameters *site, temperature*

220 and *site × temperature*, the effect of temperature on the level of HSP70 production among

221 sites was tested using least squares multiple regression (Zar, 2010).

Results

223 Thermal regimes among sites differed in an expected pattern with average monthly

224 temperatures decreasing with increasing latitude; i.e. D'Urville Island recording the highest

225 SST for any season, followed by Banks Peninsula and Stewart Island respectively (see Fig.

226 3). Strong seasonal differences were found within and between sites, with the exception of

227 Banks Peninsula and Stewart Island during the winter, where no significant temperature

228 difference was detected. This result is reflected in the significant *site × season* interaction

229 effect evident in Table 2. Additionally, differences in amplitude of thermal regime were

230 observed among sites with the difference between winter and summer temperatures being 6

231 °C for both Banks Peninsula and D'Urville Island compared to 3 °C for Stewart Island (Fig.

232 3)

233 Population differences in size were evident after one week of larval culture; D'Urville and

234 Stewart Island showed similar mean shell lengths (158.1 ± 1.3 µm S.E.M. and 158.8 ± 1.3

235 µm S.E.M. respectively). Larvae sourced from Banks Peninsula broodstock showed a small,

236 but significant increase in size (163.1 ±1.64 µm S.E.M.; One-way ANOVA, d.f. 2,170; F =

237 3.89, p<0.05).

238 Mortality of larvae from the three sites following a 3 h temperature challenge and an 18 h

239 recovery period are shown in Fig. 4A,B. With regards to percent mortality, the temperature

240 band of 32 - 37°C represents a key response region for temperature related mortality in this

241 species, with a rise in mortality from <10% to >90% over these temperatures (see Fig. 4A).

242 An inverse prediction model showed that for a given temperature, individuals sourced from

243 Stewart Island exhibited slightly higher levels of mortality compared to individuals sourced

244 from either D'Urville Island or Banks Peninsula (Fig. 4B). However, estimates of LT_{50} were

245   not significant among the populations tested with 33.6 °C ±0.8 c.i., 34.0 °C ±0.6 c.i., and

246   33.0°C ±0.7 c.i. recorded for D'Urville Island, Banks Peninsula and Stewart Island

247   respectively (see Fig. 5 and Table 3).

248   Levels of HSP70 production in *P. canaliculus* larvae did not correlate with site of origin (see

249   Table 4 and Fig. 6), with only temperature having a significant effect on the production of

250   HSP70 in this species. However, the relationship between temperature and HSP70 production

251   was not strong e.g. r^2 of 0.30, with low levels of HSP70 in larvae held at or below 35 °C

252   followed by an abrupt, albeit variable, increase in heat shock protein production in larvae

253   held above 40 °C.

254   **Discussion**

255   Measures of acute thermotolerance and HSP70 production in larval *P. canaliculus* veligers

256   are reported here for the first time. A key aim of the study was to characterise intraspecific

257   thermotolerance patterns for F2 larvae of parents gathered from differing sites in southern

258   New Zealand. It was hypothesised that *P. canaliculus* larvae derived from parents sourced

259   from warmer, northern latitudes would show greater resilience (i.e. lower % mortality and

260   HSP70 production) to acute thermal stress than stock from colder southern sites.

261   However, when challenged with temperatures ranging from 20 - 42°C, F2 *P. canaliculus*

262   larvae from the 3 populations demonstrated very similar mortality and HSP70 responses. This

263   is despite the SST profiles of the sites from which the original broodstock were collected

264   conforming to an expected latitudinal gradient of warmer SSTs in the north vs. colder SSTs

265   in the south. Interestingly, winter SSTs at two sites (Banks Peninsula and Stewart Island)

266   were similar, probably due to the dominance of the Southland Current pushing up the east

267   coast of the South Island (Sutton, 2003). Further complicating the hydrodynamic situation in

268   this area are alternating influxes of either cold sub-antarctic or warmer sub-tropical surface

269   waters that occur near Banks Peninsula (Chiswell, 1994; Shaw and Vennell, 2000). This

270   situation of alternating hydrodynamic features may account for the similarity of winter SSTs

271   between these two sites and the dissimilarity of summer SSTs.

272   Despite being raised under identical husbandry conditions there were differences in size

273   among the larvae used in our experiments. Banks Peninsula larvae exhibited higher rates of

274   growth compared to their conspecifics from D'Urville and Stewart Island sites. This

275   presumably reflects the genetically distinct nature of the larvae used, however such size

276 differences had little impact on thermotolerance capability among these populations. Lee and

277 Boulding (2010) reported similar results for thermotolerance trials using the intertidal

278 gastropod *Littorina keenae.* In this northern hemisphere species, body size exhibited a

279 latitudinal cline with larger individuals found at colder, northern sites yet no distinct

280 latitudinal cline in thermotolerance to temperatures 30°C and above was observed (Lee and

281 Boulding, 2010).

282 For *P. canaliculus,* increases in larval mortality were only observed once water temperatures

283 exceeded 29°C with 100% mortality of larvae that experienced water temperatures of 42°C.

284 Such findings reflect those observed for F1 larvae of the freshwater mussel species'

285 *Lampsilis siliquoidea*, *Potamilus alatus*, and *Ligumia recta* all of which exhibited 100%

286 mortality when acutely exposed to temperatures of 42°C (Pandolfo et al., 2010). To the best

287 of our knowledge Kuo and Sanford (2009) is the only other study to utilise F2 marine

288 invertebrate larvae in thermotolerance trials. They found distinct regional differences in LT_{50}

289 for *Nucella canaliculata* larvae sourced from cool and warm water sites (Oregon vs.

290 California), indicating a strong level of local adaptation in this species. Despite using similar

291 scales of latitudinal separation, we did not observe differences in larval *P. canaliculus* LT_{50}

292 among sites in the present study. A key explanation may lie in the differing larval dispersal

293 strategies of our respective study organisms. Direct developing organisms (such as *N.*

294 *canaliculata*) are thought to have greater potential for local adaptation than planktonic larvae

295 (e.g. *P. canaliculus*) which can disperse over large distances (Parsons, 1998).

296 Using expected climate change scenarios, increases in SST for the New Zealand EEZ are

297 expected up to a maximum of 4 °C over the next 100 years (Boyd and Law, 2011). For the

298 most northern population we tested (D'Urville Island) this would equate to average summer

299 temperatures peaking at 22 °C, well within the acute LT_{50} temperature of 33.5 °C calculated

300 here for *P. canaliculus*, suggesting a wide thermal safety margin. However, before *P.*

301 *canaliculus* can be classified as a 'winner' under predicted climate change scenarios the

302 ontogeny of thermal susceptibility of this species needs to be characterised for all life stages.

303 Thermotolerance trials using adult *P. canaliculus* observed 100% mortality 24 h after a 3 h

304 exposure to 35°C (Dunphy and Ragg unpublished data). Furthermore, during 2005 wild adult

305 populations of *P. canaliculus* at Banks Peninsula suffered high rates of mortality during a 3

306 day heat wave event where air temperatures reached 36°C (Petes et al., 2007). Such thermally

307 induced mortality isn't restricted to elevated temperatures, with sudden drops in water and air

308 temperature producing significant mortality in adults and juveniles of the Green mussel,

309   *P.viridis* (Firth et al., 2011; Urian et al., 2011). Temperature variations are predicted to

310   increase in magnitude and frequency with the progression of climate change (Rahmstorf and

311   Coumou, 2011) and further highlight the complex interplay between biotic (e.g. reproduction,

312   competition) and abiotic (e.g. temperature, desiccation) factors that determine the

313   physiological susceptibility of a species (Tomanek and Helmuth, 2002). Thus it may be that

314   the eurythermal intertidal life stages (juvenile and adult) of *P. canaliculus* are actually more

315   thermally sensitive than the larval stages developing in stenothermal subtidal environments.

316   Recent studies with a terrestrial ectotherm (the mealworm *Tenebrio molitar*) demonstrate that

317   adults were less thermally resilient than larvae (Belén Arias et al., 2011). Such observations

318   broadly agree with a general hypothesis advanced by Pörtner and Farrell (2008), who suggest

319   that the thermotolerance window is expected to increase through the gamete and larval stages

320   to maximum values in juveniles, declining again as sexual maturity is reached.

321   Furthermore, chronic sub-lethal thermal stress may be of greater relevance to the pelagic

322   larvae of mussel species. For example, Buchanan (1998) observed high mortality in *P.*

323   *canaliculus* larvae reared at 24°C, compared to apparent optima at 16 - 19°C. Ragg et al.

324   (2010) also note that the effect of subtle stress may not be immediately detectable in *P.*

325   *canaliculus* larval survival and growth, but become manifest as reduced pediveliger

326   competency to metamorphose and settle. Thus for marine ectotherms, quantifying

327   thermotolerance may be necessary for all life stages particularly for those species where

328   larval and adult forms exist in very different environments i.e. stenothermal subtidal larvae

329   vs. intertidal eurythermal adults.

330   It should be noted that the 3 h immersed thermal shock administered in the current trials does

331   not represent a simple challenge. Young veligers of the green mussel, *Perna perna*, were

332   found to consume ~2 ng O_2 individual^{-1} h^{-1} at 24°C (Lemos et al., 2003). Veligers of *P.*

333   *canaliculus* may maintain an even higher metabolic rate, with preliminary measurements

334   suggesting an oxygen consumption rate of ~5 ng O_2 individual^{-1} h^{-1} under our control

335   conditions (7 day old veligers at 20°C; N. L. C. Ragg, unpublished data). Larval metabolism

336   is also likely to increase rapidly with temperature; for example oyster veliger growth rates

337   increased according to a Q_{10} co-efficient of approximately 3.4 between 17 and 32°C (Rico-

338   Villa et al., 2009). If larval metabolism follows a similar trajectory, oxygen consumption

339   rates could exceed 20 ng O_2 individual^{-1} h^{-1} at the survival tipping-point temperatures

340   measured here. The oxygen content of air-equilibrated seawater also decreases with rising

341   temperature, at 20°C holding ~7.5 ng O_2 mL^{-1}, falling to ~6.1 ng O_2 mL^{-1} at 32°C (Benson

342   and Krause, 1984). It therefore seems inevitable that some degree of hypoxia was present in

343   all experimental vials, becoming exacerbated at higher challenge temperatures. The

344   monitoring of survival following thermal shock under water should therefore be considered to

345   be an assessment of the integrated effects of elevated temperature and all corresponding

346   physical covariates within the water body.

347   With regards to HSP70 induction, the temperature at which larval *P. canaliculus* initiated

348   HSP70 expression (known as T_{on}) was not population specific and appeared to reside above

349   35 °C. In summer-acclimated Pacific oysters (*Crassostrea gigas*) induction of HSP 69 was

350   not apparent until experimental thermal challenge temperatures reached 40°C, yet T_{on} was

351   lower (37°C) in winter-acclimated oysters (Hamdoun et al., 2003). Given that our

352   experiments were performed in the austral summer months it may be expected that the winter

353   T_{on} for *P. canaliculus* will be somewhat lower.

354   It is thus apparent that the initiation of HSP70 production is influenced by a complex suite of

355   temperature cues. Previous authors have noted the role ambient temperature fluctuations have

356   on the temperature at which HSP70 production is induced (Dong et al., 2008; Tomanek and

357   Somero, 1999). Organisms inhabiting moderately variable thermal environments (i.e.

358   subtidal habitats) rarely display induced HSP70 production and only do so at temperatures

359   well above those normally experienced in their natural environment (Tomanek, 2008). In our

360   results, the T_{on} for HSP70 expression approaches the 100% lethal temperature for *P.*

361   *canaliculus* larvae, implying there is little protection provided by the heat shock response for

362   larvae of this species. However, intrinsic (e.g. primary sequence) and extrinsic factors (e.g.

363   compatible osmolytes) are known to also stabilise proteins and potentially increase the

364   thermal range of an organism (Tomanek, 2008). What role these play in stabilising proteins

365   of *P. canaliculus* is currently unknown and deserves further investigation.

366   Whether (as in the case of thermotolerance) an ontogenetic increase in HSP70 expression

367   occurs to protect *P. canaliculus* larvae once settled in the eurythermal intertidal environment

368   is unknown. Adult and veliger larvae of the native California oyster (*Ostreola conchaphila*)

369   showed increased expression of HSP70 following a heat shock of 33°C or greater; yet a

370   similar response was not seen in the early embryonic stages i.e. 8 cell and blastula, of this

371   species (Brown et al., 2004). Initiating the expression of heat shock proteins often results in

372   reduced synthesis of other proteins and may be metabolically expensive (Hamdoun et al.,

373   2003; Parsell and Lindquist, 1994). Thus for *P. canaliculus* a trade-off may exist between

374 balancing the demands imposed by larval development and the negative effects incurred by

375 initiating HSP70 expression.

376 The apparent lack of thermotolerance variability between *P. canaliculus* populations is

377 intriguing given that collection sites were separated by nearly 1000 km, a distance over which

378 populations can be reasonably expected to show adaptive differentiation to their local

379 environment (Sanford and Kelly, 2011). Metapopulation dynamics, temporal variation in

380 abiotic factors and phenotypic plasticity can all prevent local adaptation occurring in marine

381 invertebrates (Sanford and Kelly, 2011). However, our use of F2 larvae obviates the first two

382 masking agents, thus it is evident that the larval phenotype of *P. canaliculus* is remarkably

383 robust when faced with acute thermal stress. Hamdoun et al. (2003) assert that the heat shock

384 response of mussels and oysters exhibits a high level of phenotypic plasticity. Whilst it is

385 tempting to see confirmation of this within our work, experiments to indicate whether

386 developmental or reversible plasticity is present are now needed to confirm or disprove this

387 for *P. canaliculus*. Nonetheless, whether the lack of site differences in thermotolerance is

388 maintained by high gene flow is unknown as the larval dispersal 'neighbourhoods' of *P.*

389 *canaliculus* are still to be determined, although much progress is being to be made in this

390 regard (Dunphy et al., 2011). The use of full-sib F1 families in the present trial means that

391 only 4 original parents are represented in each F2 genotype, raising concern that individual

392 effects could overshadow geographic/population-level effects. However, a small pool of

393 founding parents would be expected to increase the likelihood of phenotypic differences

394 between families. The conservative thermotolerance results observed are therefore all the

395 more surprising and likely to be a general limitation in this species.

396 Given the difficulty in obtaining F2 individuals we were only able to utilise larvae from

397 South Island sites in our experimental work, and were thus are unable to provide estimates of

398 the thermotolerance of this species throughout its entire range (i.e. including North Island of

399 New Zealand sites). Nevertheless, SSCP and RAPD analyses of *P. canaliculus*

400 phylogeography recognise three clades of this species around New Zealand, with the

401 D'Urville Island, Banks Peninsula and Stewart Island sites being located within the Northern,

402 Southern and Stewart Island clades respectively (Apte and Gardner, 2002; Star et al., 2003).

403 Thus, we were able to compare some of the broad scale genetic patterns that exist in *P.*

404 *canaliculus* around New Zealand. The possibility of introgression of cultured mussels into

405 local natural populations is an issue of concern for New Zealand (Apte et al., 2003). For

406 many years, the mussel aquaculture industry has actively transferred larval *P. canaliculus*

from the far north of New Zealand to southern ongrowing 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 LT_{50} 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 focussed 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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590 **Tables**

591 Table 1: Water temperatures of experimental vials recorded at each of the six divisions within

592 a sandbath used in the acute heat shock challenge of *P. canaliculus* larvae.

593 Table 2: Results of 2-way ANOVA comparing seasonal thermal regimes among D'Urville

594 Island, Banks Peninsula and Stewart Island sites for the years 2007-2011 (Data from NOAA).

595 Table 3: Results from binomial logistic regression describing the relationship between

596 temperature and probability of mortality of *P. canaliculus* larvae from three sites after

597 experiencing an acute thermal challenge.

598 Table 4: Results of multiple regression comparing levels of HSP70 production of *P.*

599 *canaliculus* veligers and temperature among sites.

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602 Table 1

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

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606 Table 2

Source of variation	DF	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 × Site	6	29.7	5.0	6.2	<0.001
Residual	168	133.1	0.8		
Total	179				

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608 Table 3

Whole model test				
Model	-Loglikelihood	DF	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	Std error	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
Temp	0.48	0.02	521.41	<0.0001

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612   Table 4

ANOVA					
Source	DF	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	Std error	t ratio	Prob>t	
Intercept	0.0039594	0.006394	0.62	0.5391	
Site	-0.002987	0.001702	-1.75	0.0866	
Temp	0.0007485	0.00017	4.41	<.0001*	
Site × Temp	-0.000323	0.000211	-1.53	0.1344	

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616   **Figures**

617   Fig. 1: Field sites for collection of adult *Perna canaliculus* used to generate F1 and F2

618   offspring for this study

619   Fig. 2: Schematic of sand bath used to quantify mortality level and HSP70 expression in

620   larval *P. canaliculus*. Three replicate vials for each geographic family were randomly

621   distributed across each isothermic row. Heat exchange from the water baths was carried out

622   via coils of 7 mmØ polyethylene tubing buried in the sand.

623   Fig. 3: Monthly SST (°C) of nearshore environment of source population sites averaged for

624   the Years 2007-2011. Austral seasons are superimposed above their respective months (Data

625   from NOAA).

626   Fig. 4: Mortality of larval *P. canaliculus* from three southern New Zealand populations which

627   were exposed to an acute thermal shock for 3 h and allowed to recover for 18 h at 20°C. A)

628   Percent mortality of thermally challenged *P. canaliculus* larvae; B) Mortality response curves

629   fitted using logistic regression and inverse prediction which describe the probability of 'x'
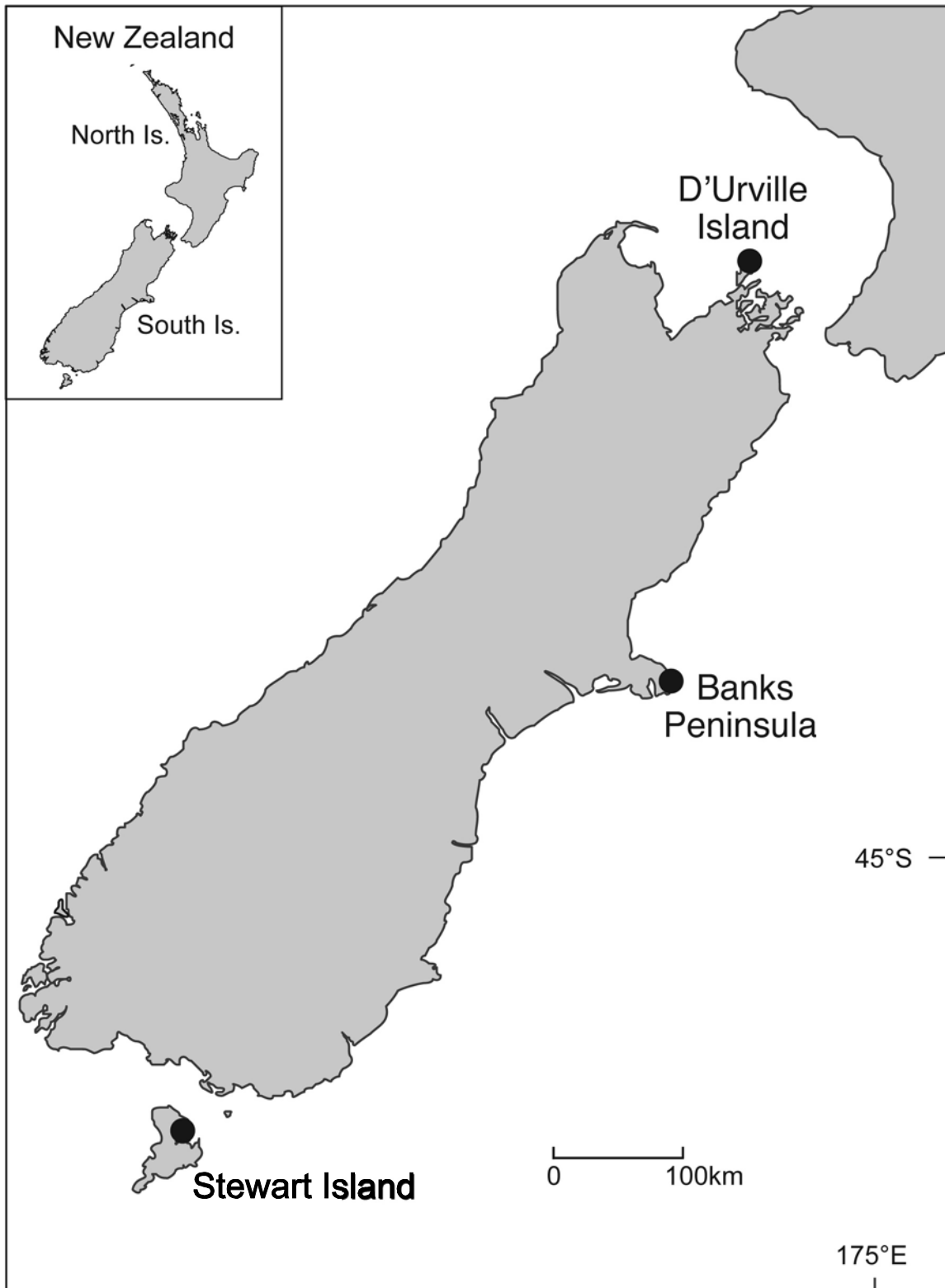
630   mortality for a given temperature.

631   Fig. 5: Estimates of LT_{50} ($\pm$ 95% c.i.) for larval *P. canaliculus* from three sites in southern

632   New Zealand which were exposed to an acute thermal shock for 3 h and allowed to recover

633   for 18 h at 20°C.

634   Fig. 6: Relationship between levels of inducible HSP70 in larvae of *P. canaliculus* from three

635   populations in southern New Zealand which were exposed to an acute thermal shock for 3 h

636   and allowed to recover for 18 h at 20°C. Note regression of HSP70 vs. temperature shown for

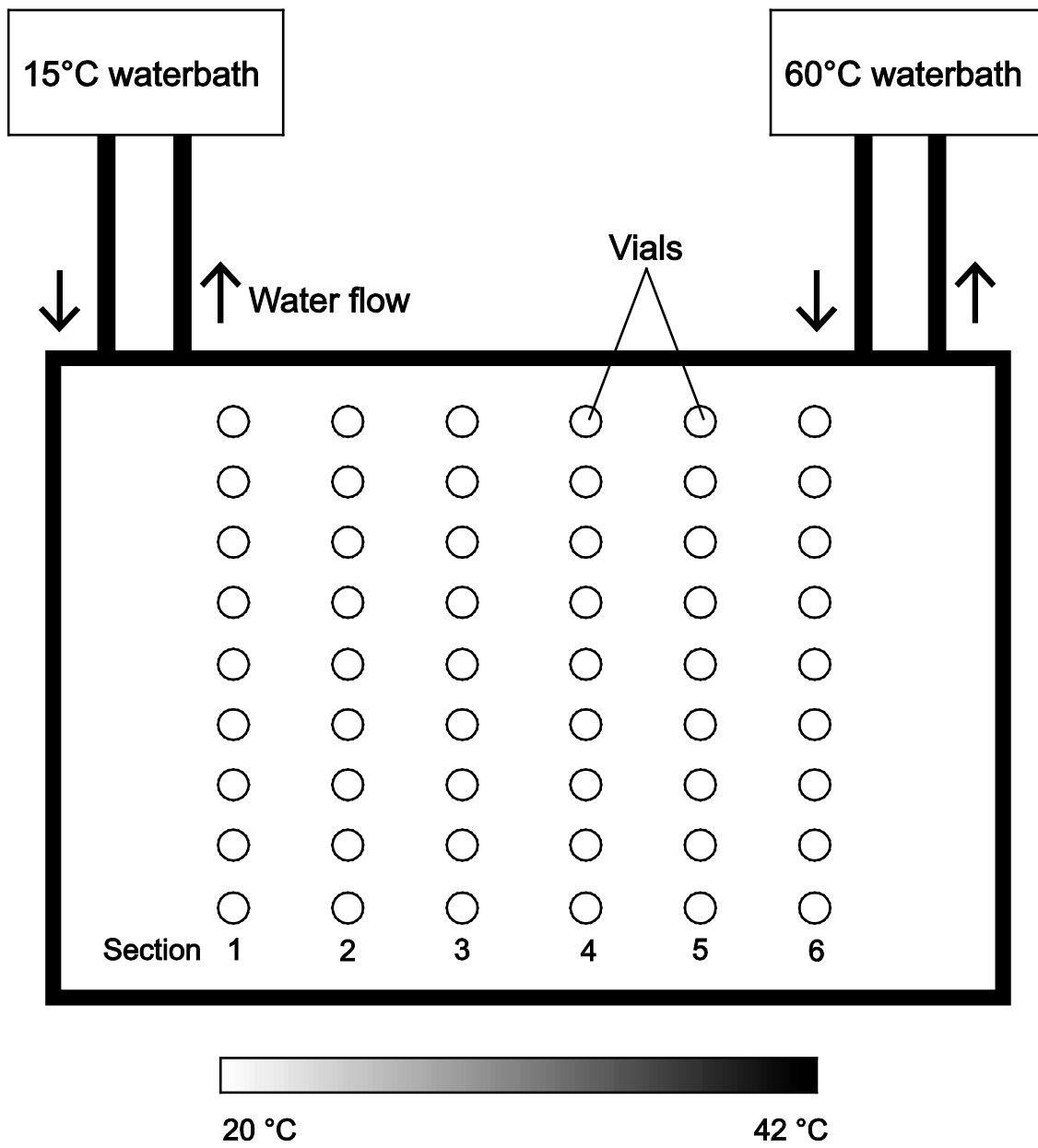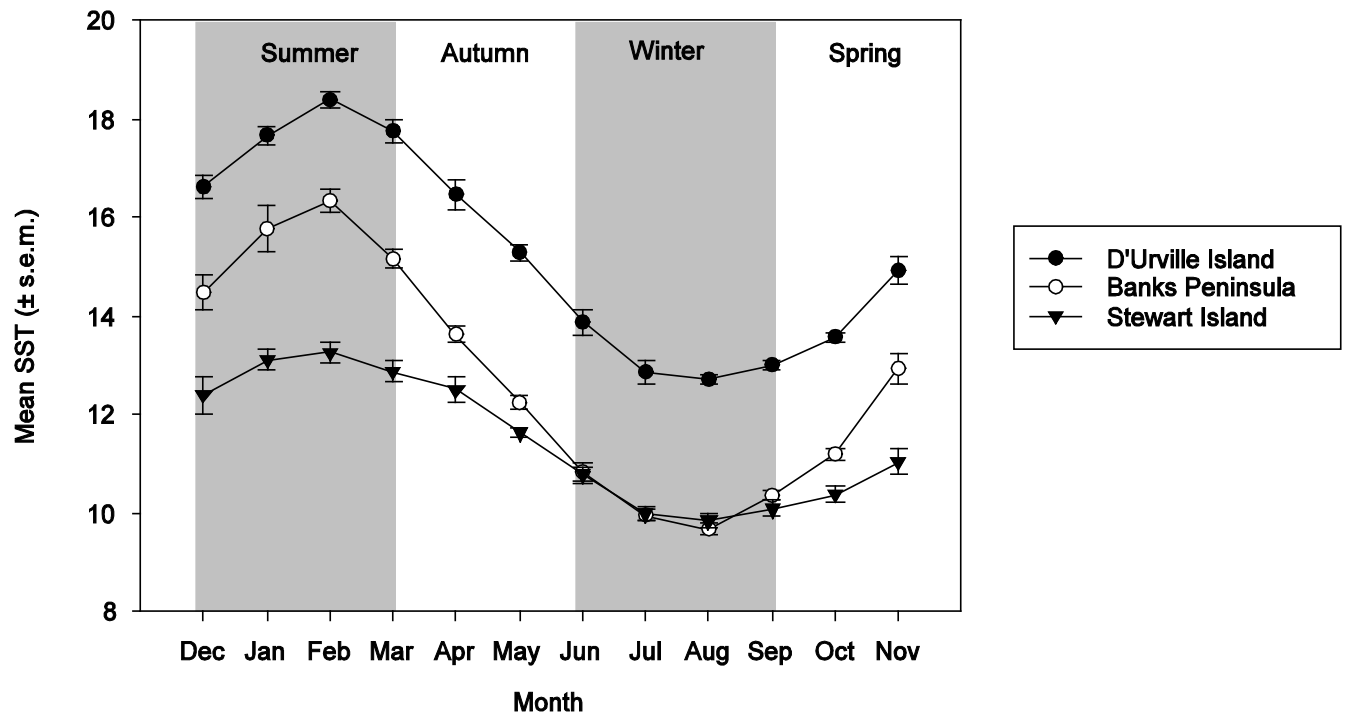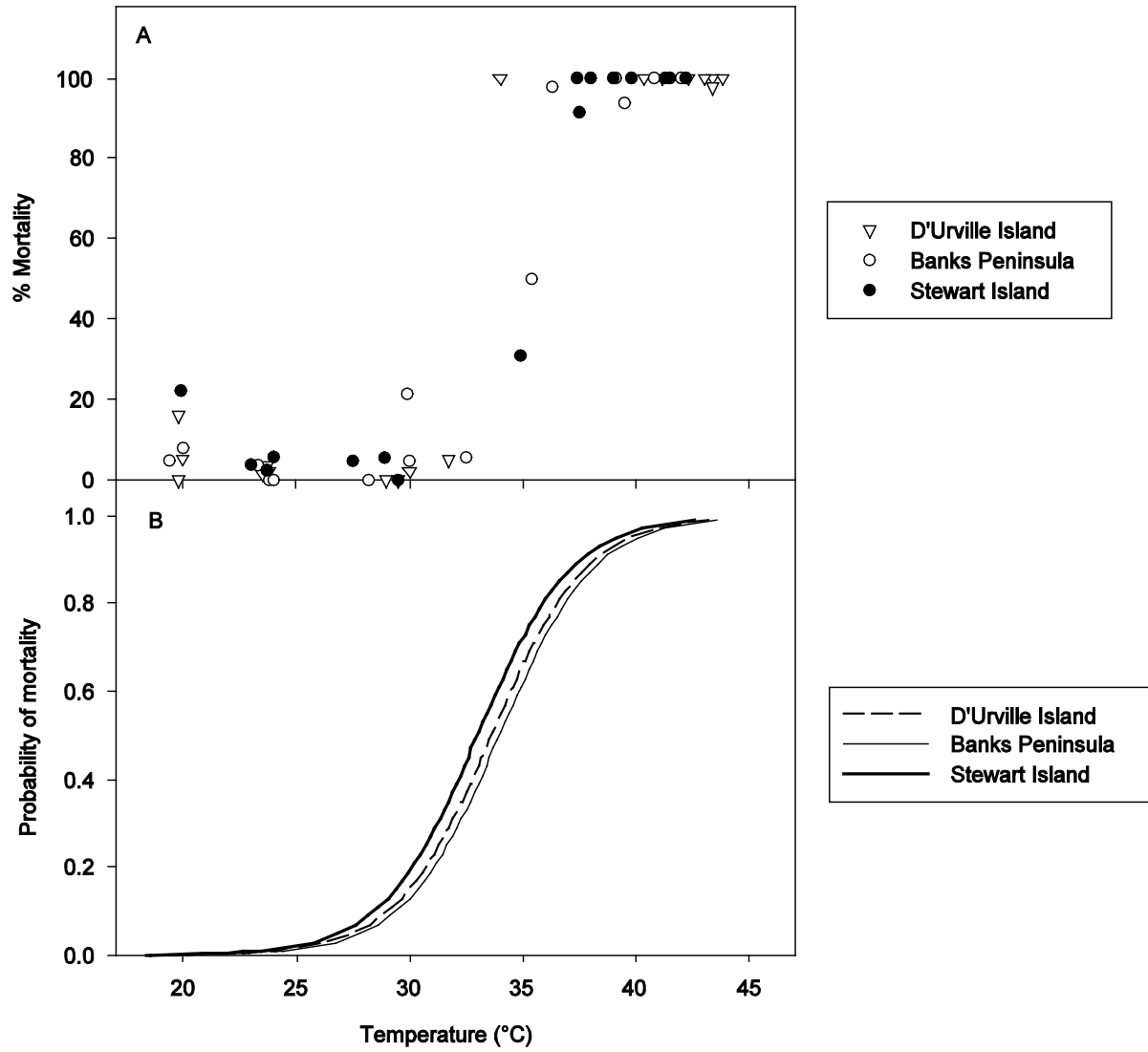637   all sites pooled.

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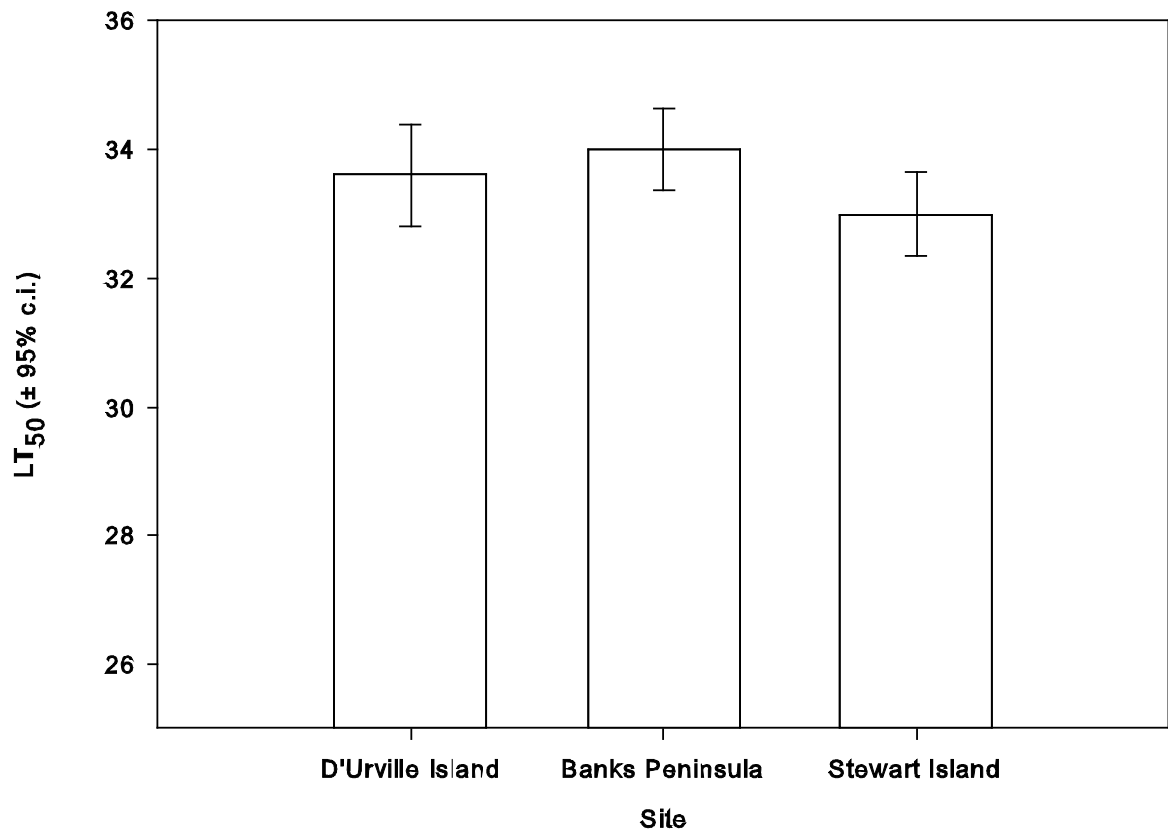New Zealand

North Is.

South Is.

D'Urville
Island

Banks
Peninsula

45°S

Stewart Island

0   100km

175°E

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