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Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*)

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Summary

The three species of blue mussels, *Mytilus trossulus* Gould 1850, *M. edulis* Linnaeus 1758 and *M. galloprovincialis* Lamarck 1819, have distinct global distribution patterns that are hypothesized to reflect differences in their tolerances of temperature and salinity. We examined effects on heart rate (beats min^{-1}) of acute exposure and acclimation to different combinations of temperature and salinity to test this hypothesis and, in the context of the invasive success of *M. galloprovincialis*, to gain insights into the factors that may explain the replacement of the temperate Pacific native, *M. trossulus*, by this Mediterranean Sea invader along much of the California coast. Heart rate of *M. trossulus* was significantly higher than that of *M. galloprovincialis*, consistent with evolutionary adaptation to a lower habitat temperature (temperature compensation) in the former species. Heart rates of *M. trossulus*/*M. galloprovincialis* hybrids were intermediate between those of the parental species. Following acclimation to 14°C and 21°C, heart rates of all species exhibited partial compensation to temperature.

Heart rate increased with rising temperature until a high temperature was reached at which point activity fell sharply, the high critical temperature (H_{crit}). H_{crit} increased with increasing acclimation temperature and differed among species in a pattern that reflected their probable evolutionary adaptation temperatures: *M. galloprovincialis* is more heat tolerant than the other two congeners. Ability to sustain heart function in the cold also reflected evolutionary history: *M. trossulus* is more cold tolerant than *M. galloprovincialis*.

Heart rates for all three congeners decreased gradually in response to acute reductions in salinity until a low salinity (S_{crit}) was reached at which heart rate dropped precipitously. S_{crit} decreased with decreasing salinity of acclimation and was generally lowest for *M. galloprovincialis*. Mortality during acclimation under common garden conditions was greatest in *M. trossulus* and was highest at high acclimation temperatures and salinities. These intrinsic differences in basal heart rate, thermal and salinity responses, acclimatory capacity, and survivorship are discussed in the contexts of the species' biogeographic patterning and, for the invasive species *M. galloprovincialis*, the potential for further range expansion along the Pacific coast of North America.

Key words: acclimation, biogeography, invasive species, *Mytilus trossulus*, *Mytilus edulis*, *Mytilus galloprovincialis*, salinity, temperature.

Introduction

Determining the roles played by physiological adaptations in establishing biogeographic patterning is an important goal of ecological and evolutionary physiology (Hochachka and Somero, 2002). Identifying the physiological determinants of latitudinal and vertical distribution patterns can not only explain past and present biogeographic patterning, but also assist in formulating predictions about the effects of climate change on biological communities (Parmesan et al., 1999; Roemmich and McGowan, 1995), especially in the case of range expansions and contractions. Similarly, physiological analysis may assist in explaining and predicting the success of introduced species that have entered a new environment due to human activities that have allowed traditional biogeographic barriers to be bypassed, as species are transported beyond their natural ranges (Carlton and Geller, 1993). There are many cases in which these introduced species persist and become invasive in the new habitat, warranting an analysis of the mechanism(s) accounting for successful range expansion and invasion. One hypothesis is that invasive species are physiologically poised to adjust to new habitats by being particularly tolerant of environmental stressors. Here, we

explore the effects of acute and acclimatory changes in temperature and salinity on *Mytilus* blue mussel congeners, including native and invasive species with distinct distribution patterns along the coast of California.

There are three *Mytilus* blue mussel congeners worldwide: *Mytilus trossulus*, *M. edulis* and *M. galloprovincialis* (McDonald and Koehn, 1988; McDonald et al., 1991). *M. trossulus* is native to the North Pacific and is thought to have given rise to the latter two species, from the North Atlantic and the North Atlantic/Mediterranean, respectively (Seed, 1992). The blue mussel species hybridize in all known regions of overlap but only *M. galloprovincialis* has a demonstrated ability to invade novel locations, including South Africa, Japan and California (Seed, 1992). The congeners differ in their expected tolerance to habitat temperature and salinity, based on their speciation pattern and on their current geographic distribution. *M. trossulus* is thought to be the most tolerant of cold and low salinity conditions as a species that originated in the North Pacific, while *M. galloprovincialis* is thought to be the most tolerant of warm, high salinity conditions as a species that originated in the Mediterranean (Seed, 1992). The third blue mussel congener, *M. edulis*, is thought to have evolved from ancestral *M. trossulus* (*via* a trans-Arctic migration) and given rise to the Mediterranean population of blue mussels, which evolved into *M. galloprovincialis* (Barsotti and Meluzzi, 1968; Vermeij, 1991). *M. edulis* shares latitudinal overlap to the north with *M. trossulus* (both occur at latitudes >66°N) and with both species to the south (28–35°N) (Hilbish et al., 2000). Because *M. galloprovincialis* is limited in its northern distribution (current known limit is Great Britain, ~55°N), it is thought that this species may be limited by cold conditions.

The California hybrid zone between *M. trossulus* and *M. galloprovincialis* was only recently described (McDonald and Koehn, 1988; McDonald et al., 1991) and thus has been the subject of relatively few studies. This zone of secondary contact was created by the introduction of *M. galloprovincialis* to Southern California, presumably by shipping, in the first half of the twentieth century (Geller, 1999; McDonald and Koehn, 1988). *M. galloprovincialis* has replaced *M. trossulus* over a large fraction of the California coast, from the Mexican border to the approximate latitude of Monterey (37°N). In the Monterey and San Francisco Bays, the native and invader co-exist and are found in a mosaic hybrid zone that comprises habitats differing widely in temperature and salinity (Braby and Somero, 2006). Although *Mytilus* blue mussels occur commonly on the open coast of both the North Atlantic and parts of the North Pacific (in addition to other geographic regions), they are rare in open coast habitats in California, and abundant only in protected, often estuarine, sites. In these protected sites, the mosaic hybrid zone pattern of *Mytilus* species is hypothesized to be evidence of physiological adaptation to local abiotic environmental conditions (Harrison and Rand, 1989; Sarver and Foltz, 1993; Rawson et al., 1999). In support of this conjecture there is evidence that adult distribution correlates with temperature and salinity gradients (Sarver and Foltz, 1993; Suchanek et al., 1997; Rawson et al., 1999; Braby, 2004; Braby and Somero, 2006). It is suggested that *M. trossulus* is less tolerant of high temperature and more tolerant of both low salinity and low temperature, consistent with the evolutionary history of the species. It is known that *Mytilus* congeners have different tolerances of high temperature and that thermal tolerance changes with acclimation/acclimatization history (Hofmann and Somero, 1996; Roberts et al., 1997; Buckley et al., 2001). However, these limited data on thermal physiology do not provide an adequate basis for explaining the distribution patterns of the blue mussels or for predicting the future course of the invasion by *M. galloprovincialis*.

Even less is known about the importance of salinity effects on the distribution of these species. There is some evidence that *M. trossulus* may be more euryhaline than the other blue mussel congeners, although results to date are equivocal. Thus, although laboratory experiments comparing growth rates and feeding in adult *M. trossulus* and *M. edulis* across salinity treatments found no difference between the congeners' performance (Gardner and Thompson, 2001), differences in larval survival rates of the two species suggest that *M. trossulus* is more euryhaline (Qiu et al., 2002). However, no physiological studies have addressed the question of whether *M. trossulus* and *M. galloprovincialis* respond differently to changes in salinity.

To elucidate the roles of temperature and salinity in establishing the distribution patterns of blue mussels, we investigated the effects of acute and acclimatory changes in temperature and salinity on heart function in intact, immersed animals. Heart rate has been used successfully as a proxy for whole-animal stress in Mytilids in response to toxins (Davenport, 1977; Grace and Gainey, 1987; Depledge et al., 1996; Wedderburn et al., 2000), temperature (Pickens, 1965; Helm and Trueman, 1967; Coleman and Trueman, 1971), salinity (Stickle and Sabourin, 1979; Nicholson, 2002) and predation (Rovero et al., 1999). Here, we demonstrate that significant differences in the responses of the three blue mussel congeners to variations in temperature and salinity may account, at least in part, for their different distribution patterns and may help to explain and predict the invasive success of *M. galloprovincialis*.

Materials and methods

Animal collection and care

We collected blue mussels from protected, subtidal habitats (floating docks in harbors) whose temperatures and salinities could be readily measured (Braby and Somero, 2006). Locally collected specimens (Monterey Bay and San Francisco Bay mosaic hybrid zone) were brought to the Hopkins Marine Station in coolers within 24 h of collection, and then transferred to closed-circulation, aerated, and filtered 75 l seawater aquaria. Because the congeners and their hybrids can only be distinguished by genetic methods (see below), we also

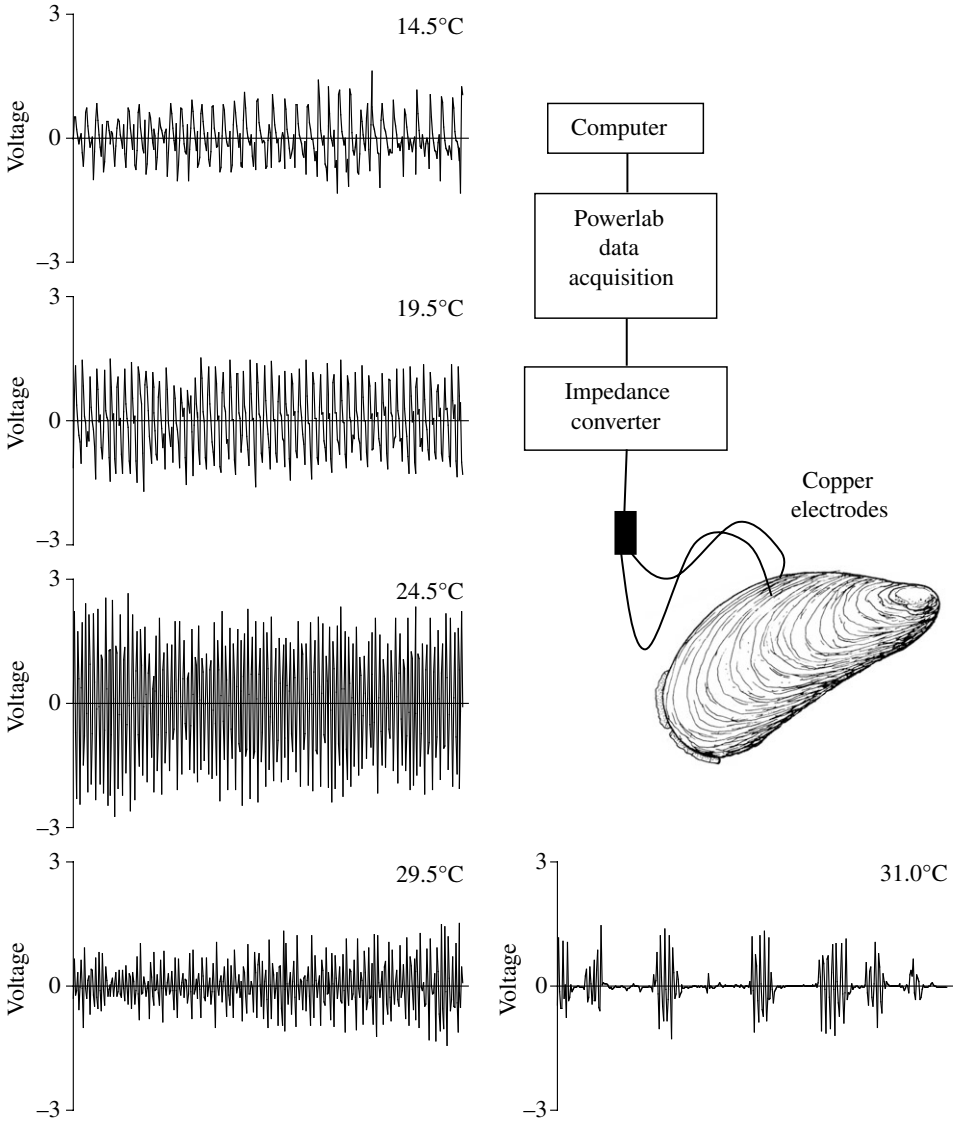

Fig. 1. Heart rate response to temperature and methods for impedance pneumography. Heart activity patterns from a single individual over a range of temperatures from 14.5–31°C are shown. Each heart rate trace spans 3 min. Inset shows the schematic of the heart rate monitoring apparatus, and the placement of electrodes. Mussel drawing adapted from (Brusca and Brusca, 1990).

collected animals from blue mussel populations outside of the California hybrid zone in order to ensure adequate and equal numbers of each species in our studies. We received animals within 36 h of collection in moist (but not immersed) conditions from four locations: Coos Bay, Oregon (43°N; *Mytilus trossulus* Gould); Morro Bay (35°N) and Santa Barbara, California (34°N; *Mytilus galloprovincialis* Lamarck); and, for experiments with the Atlantic congener, Narragansett Bay, RI (41°N; *Mytilus edulis* Linnaeus). After size sorting animals (retaining those within the 30–60 mm range) and cleaning encrusting organisms from the shells, we marked the shells with nail lacquer, so that all species could be acclimated in common-garden tanks. Each week, we fed animals 2–3 times with commercially available phytoplankton mix (Reed Mariculture, Shellfish Diet – 1800 Formula, Campbell, CA, USA) and also exchanged half the tank volume with filtered seawater each week, cleaning out accumulated pseudofeces. With the exception of acclimation treatments (described below), we held animals at 13–14°C and 28 p.p.t. salinity (obtained by diluting filtered Monterey Bay seawater with deionized water).

Heart rate measurement and environmental control

We monitored mussel heart rates (beats min^{-1}) using impedance pneumography (Fig. 1). After drilling a small hole (<1 mm diameter) in each valve immediately exterior to the pericardial space at the posterior end of the hinge region, we inserted fine copper electrodes (40-gauge magnet wire, with 2 mm of tip exposed) into the pericardial space and held them in place with quick-drying surgical glue (Super Glue™ – Gel Formula). This method produces an analog impedance signal, which is converted to a voltage signal (UFI, Impedance Converter model 2991) and digitally recorded using a data acquisition system (ADInstruments, PowerLab/16SP, Colorado Springs, CO, USA), sampling at 100 Hz. We immobilized and immersed mussels in an aerated, temperature-controlled experimental chamber, in which temperature was maintained or changed by a computer-controlled water bath

(Lauda Brinkmann, RC 6 CS, Westbury, NY, USA). The water bath circulated fluid through a stainless steel heat-exchanger coil in the experimental chamber, which allowed rapid changes in chamber water temperature. The chamber temperature (ADInstruments, T-type thermocouple probe), conductivity (YSI, 3417 conductivity probe, Yellow Springs, OH, USA) and the heart rates of up to six mussels could be recorded simultaneously and continuously by the data acquisition system during a given experiment. Each experiment was begun early in the morning (one run each day) and only one environmental factor was tested on each individual (either temperature or salinity, but not both).

Temperature stress and heart rate

To test the response of heart rate to simulated diel water temperature fluctuations, we used increasing temperature ramps at a rate of change of 6°C h^{-1}, somewhat faster than the thermal changes seen in blue mussel habitat during the summer months (maximum rate +1.6°C h^{-1}) (Braby, 2004). Each experiment consisted of a 1 h recovery period at the acclimation temperature (to allow the animals to recover from the electrode implantation procedure), followed by an increase in temperature (1°C per 10 min interval), while salinity was held constant at the acclimation level. Temperature increases continued until all individuals showed a significant drop in heart rate (the critical temperature, H_{crit}) (Fig. 2A), at which point we stopped the temperature ramp, and decreased the temperature back to the acclimation (initial) value. All animals that exhibited a stable basal heart rate and a characteristic heat ramp response recovered from the heat stress. A small subset of individuals (5 of 89 specimens) was not included in the analysis because the heart activity of these individuals appeared erratic from the beginning of the experiment.

To test the response of hearts to low temperature events, as might occur during emersion in winter, we used decreasing temperature ramps that simulated cooling rates to be expected in habitats to north of the hybrid zone (Oregon, Washington, and Alaska) (Fig. 2B). Each experiment consisted of a 1 h post-surgical recovery period followed by a decreasing ramp (–1°C per 15 min interval) from the acclimation temperature to approximately 0°C (±1°C). Salinity was held constant at the acclimation level. Animals were held near 0°C for 1 h and then returned to the acclimation temperature to observe short-term recovery.

Salinity stress and heart rate

We used decreasing salinity ramps to test the response of heart rate to salinity fluctuations, as seen in subtidal habitats during periods of spring tides and heavy winter rain run-off (Fig. 2C) (Braby and Somero, 2006). Each experiment consisted of a 1 h post-surgical recovery period at the acclimation temperature, followed by a decrease in salinity (2–3 p.p.t. h^{-1}), while temperature was held constant at the acclimation level. We decreased chamber salinity by pumping in low salinity water through a peristaltic pump, while an overflow valve kept the volume of the experimental chamber

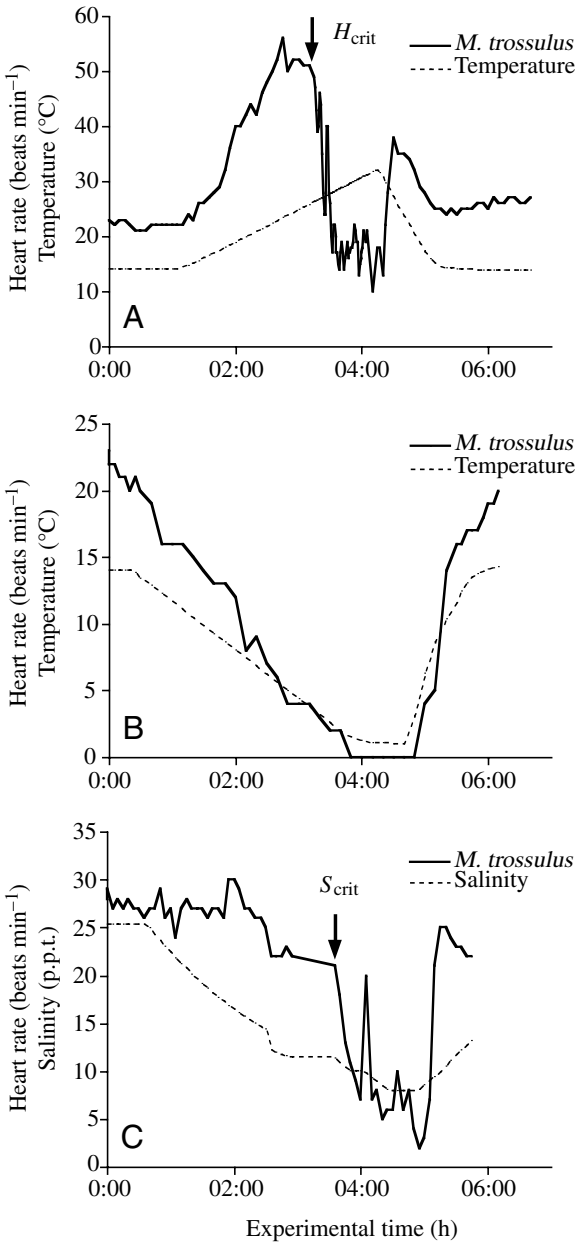

Fig. 2. Environmental exposure and heart rate response to stress in *M. trossulus*. (A) Heat stress; as temperature increases (broken line; beginning at 01:00 h, experimental time), heart rate increases until the animal reaches a temperature at which heart rate falls rapidly (H_{crit}). With continued heat stress, heart rate remains depressed, but as soon as temperature drops, heart rate returns to a normal level. (B) Cold stress; as temperature decreases from acclimation level, heart rate decreases to zero. (C) Low salinity stress; as salinity decreases from acclimation level, heart rate decreases very gradually until the animal reaches a salinity at which there is a rapid fall in heart rate (S_{crit}). Heart rate remains depressed during continued low salinity treatment but recovers immediately when salinity begins to increase.

constant. Each experiment continued until all individuals showed a significant drop in heart rate (critical salinity, S_{crit}), at which point we stopped the salinity ramp. All animals that

exhibited a stable heart rate at the beginning of the experiment survived the salinity stress.

Effects of thermal and salinity acclimation on heart rate

During winters of 2003 and 2004, we conducted multi-factor acclimations with six acclimation tanks in each year: three salinities (22 p.p.t., 28 p.p.t. and 34 p.p.t. in both years) and two temperatures (14°C and 21°C in 2003; 7°C and 14°C in 2004). These temperatures and salinities are conditions that the mussels regularly encounter in the field in the California hybrid zone (Braby and Somero, 2006). Two large temperature-controlled aquaria (550 l each) served as water baths and held the three smaller acclimation tanks (75 l) with the salinity treatments. Each acclimation tank had its own filtration/aeration unit and we exchanged half the tank volume each week with temperature-equilibrated seawater of the appropriate salinity (from a mixture of filtered seawater and deionized water or InstantOcean® supersaturated seawater). The acclimation protocol was as follows: (1) animals were initially held at 14°C and at their collection salinity for 2 days; (2) acclimation tank salinity was changed by 1 p.p.t. day^{-1} until it reached the target acclimation salinity; (3) temperature was changed by 2°C day^{-1} until it reached the target acclimation temperature. Using temperature data loggers (Dallas Semiconductor, iButtons with ±0.5°C accuracy, Dallas, TX, USA) and a salinity refractometer (Reichert, temperature compensated, Depew, NY, USA), we monitored the temperature and salinity every 2 days and adjusted these as needed. Acclimation conditions stayed within ±1°C and ±1 p.p.t. of the targeted temperatures and salinities throughout the acclimation period. Every 2 days during the acclimation period, we fed animals commercially available phytoplankton mix (Reed Mariculture, Shellfish Diet – 1800 Formula). During the experimental period, we fed animals on a daily basis to eliminate any potential experimental differences due to nutritional state. We began each acclimation period with 30 individuals of each species (a total of 90 individuals per tank in 2002–3 and a total of 60 animals per tank in 2003–4) and we acclimated them for 3 weeks before beginning experiments. On each experiment day, we removed six animals from one acclimation tank for experimental purposes, as well as any sick (gaping) or dead animals. No effect of holding (acclimation) time on heart rates or critical temperatures or salinities was observed.

Data analysis

Because of the high variability of mussel heart beat amplitude over the course of an experiment (Fig. 1), we were unable to use the PowerLab software directly to calculate heart rate throughout the experiments. Thus, setting an arbitrary threshold for amplitude failed to capture the effects of treatments on beats min^{-1}. Instead, we extracted heart rate manually by selecting the time period of interest and visually counting beats for the selected period. We estimated beats min^{-1} for 1 min time intervals, selecting once every 5 min for heating ramps, once every 10 min for cooling ramps and once every 20 min for salinity ramps. When the animal approached its temperature or salinity limit, we calculated beats min^{-1} for every 1 min. For heat and salinity ramps, we mathematically derived critical values by finding the common solution of the best-fit lines before and after the distinct shift in heart rate (Stillman and Somero, 1996). When challenging animals with low temperature stress, we calculated the lowest heart rate (LHR) during the 1 h holding period near 0°C.

We evaluated the effects of treatments on these three response variables (H_{crit}, S_{crit} and LHR) by applying a multiplicative three-factor ANOVA model with species, acclimation temperature, and acclimation salinity as the three factors. We followed the ANOVAs with *post-hoc* multiple comparisons (Student–Newman–Keuls, P=0.05), which compared species at each of the temperature/salinity combinations.

Genetic identification

Congeners in the *Mytilus* blue mussel complex are difficult to distinguish because of their morphological similarity, so we genotyped all the animals used in these experiments. We dissected animals after each experiment, and during the 2003 experiments made qualitative notes of the reproductive status of each individual (*M. trossulus* and *M. galloprovincialis* were developed and *M. edulis* was not). We used multiple DNA isolation protocols throughout the course of these experiments, including membrane spin columns (Macherey-Nagel, Nucleospin® DNA extraction kit, Easton, PA, USA), guanidinium salt and silica bead extraction (Hoss and Paabo, 1993), and Proteinase K tissue digestion [10 mmol l^{-1} Tris-HCl pH 8, 1 mmol l^{-1} EDTA, 0.3% Tween, 0.3% nonylphenyl-polyethylenglycol (Sigma, IGEPAL CA-630), 0.03 units μl^{-1} Proteinase K; 55°C for 12 h, 98°C for 10 min]. We used previously described polymerase chain reaction (PCR)-based methods to amplify two nuclear loci: the byssal thread protein locus (*Glu-5′*) (Rawson et al., 1996) and the ribosomal internal transcribed spacer region (*ITS-1*) (Heath et al., 1995).

The *Glu-5′* locus amplifies a different sized fragment for *M. galloprovincialis* (300 bp), *M. edulis* (350 bp) and *M. trossulus* (240 bp), because of an insertion in the *M. galloprovincialis* gene and a double insertion in the *M. edulis* gene. An F1 *M. trossulus*/*M. galloprovincialis* hybrid (T/G hybrid) amplifies both sized fragments (240 bp and 300 bp). We used the published primers (F-GTAGGAACAAAGCATGAACCA; R-GGGGGGATAAGTTTTCTTAGG), slightly modified PCR chemistry (15 μl reaction volume with 1 μl DNA template, 0.1 Units Taq polymerase, 1× Taq Buffer [50 mmol l^{-1} KCl, 30 mmol l^{-1} Tricine, pH 8.6], 200 nmol l^{-1} dNTPs, 2 mmol l^{-1} $MgCl_2$, 300 nmol l^{-1} of each primer) and slightly modified cycling conditions (initial denaturation of 1.5 min at 94°C; 35 cycles of 94°C for 20 s, 53°C for 30 s and 72°C for 45 s; final extension of 2 min at 72°C).

The *ITS-1* locus amplifies a similar sized fragment in all three species (~950 bp), but *M. trossulus* can be distinguished from *M. galloprovincialis*/*M. edulis* by the number of recognition sites for the *Hha*I restriction enzyme. After a

restriction digest of the PCR products, there are several fragments but two unique fragment sizes (*M. trossulus*= 280 bp; *M. galloprovincialis*/*M. edulis*=450 bp). We used the published primers (F-GTTTCCGTAGGTGAACCTG; R-CTCGTCTGATCTGAGGTCG), slightly modified PCR chemistry (same recipe as for *Glu-5′*, described above) and slightly modified cycling conditions (initial denaturation of 1.5 min at 94°C; 30 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 1 min; final extension of 2 min at 72°C). To digest the PCR product, we then added 0.2 μl *Hha*I enzyme (New England Biolabs, Ipswich, MA, USA), 1.5 μl 10× buffer (New England Biolabs – Buffer 4) and 0.15 μl bovine serum albumin (100 μg ml^{-1}) to each PCR reaction and digested for 4 h at 37°C.

We visualized PCR products for both loci using gel electrophoresis on a 2% agarose gel, stained with ethidium bromide. To arrive at a final genotype designation, we scored individuals at each locus separately and then scored across both loci. These final designations are: *M. galloprovincialis*, *M. trossulus*, T/G hybrid (potentially includes backcrossed genotypes), and *M. edulis*.

Results

Interspecies differences in heart rate

Heart rates of the three species differed significantly under common measurement and acclimation conditions (Fig. 3A; ANOVA, P<0.0001, see Tables 1, 2). The highest heart rate was exhibited by *M. trossulus*, the species adapted to the lowest temperatures. At a common temperature and salinity of

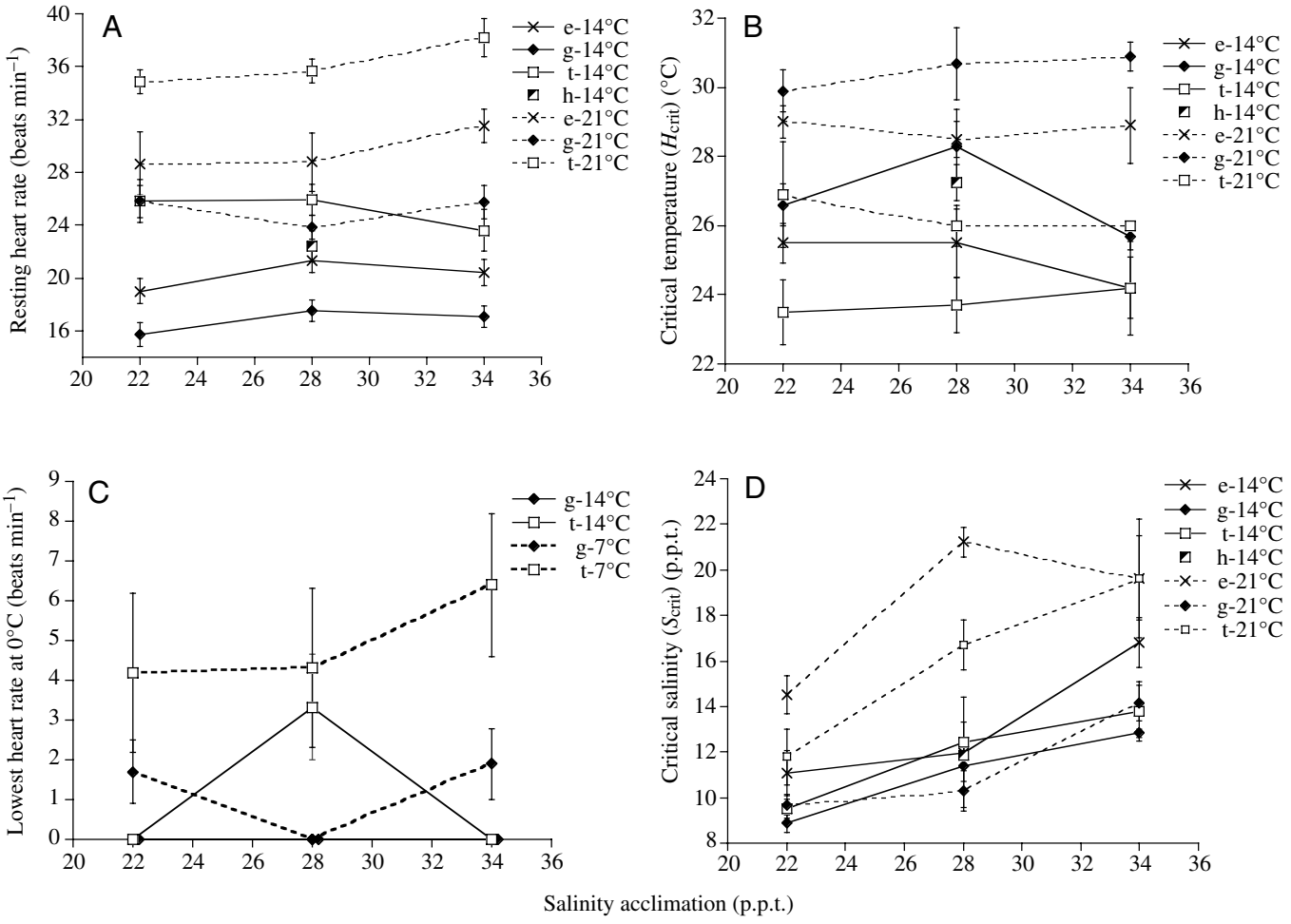

Fig. 3. Heart rate response to temperature and salinity in differently acclimated mussels. Species are referred to by the first letter of the species' name: *M. trossulus* (t), *M. galloprovincialis* (g), *M. edulis* (e), and *M. trossulus*/*M. galloprovincialis* hybrids (h). Symbols connected by solid lines are 14°C-acclimated; symbols connected by dotted lines are 21°C-acclimated. Values are means ± s.e.m. For N and P values, see Table 2. (A) Resting heart rate measured at the acclimation temperatures (14°C or 21°C). (B) Response to heat stress (H_{crit}); all species show an increase in H_{crit} with increased acclimation temperature. While there are some differences in H_{crit} among salinity treatments, salinity acclimation does not contribute statistically to the observed pattern. (C) Response to cold stress (lowest heart rate at 0°C); at comparable acclimations, *M. trossulus* has an equal or higher heart rate than *M. galloprovincialis*. Note that to make overlapping symbols visible, we offset the g-14°C data by +0.2°C along the x-axis. (D) Response to low salinity stress (S_{crit}); there is a clear increase in S_{crit} with increasing salinity acclimation in all species.

Table 1. *The effects of species (M. trossulus, M. edulis or M. galloprovincialis), acclimation temperature and salinity acclimation, as well as interactions between all three*

Response	ANOVA	R^2	Species	Temperature (T)	Salinity (S)
RHR (log)	**<0.0001**	0.7533	**<0.0001**	**<0.0001**	0.3324
H_{crit}	**<0.0001**	0.6004	**<0.0001**	**<0.0001**	0.7791
LHR (log)	**0.0001**	0.4534	**0.0012**	**<0.0001**	0.8231
S_{crit} (log)	**<0.0001**	0.7337	**<0.0001**	**<0.0001**	**<0.0001**

Response	Sp×T	Sp×S	T×S	Sp×T×S
RHR (log)	0.8190	0.7740	0.0658	0.4491
H_{crit}	0.6201	0.8110	0.5681	0.8625
LHR (log)	0.2173	**0.0411**	**0.0230**	0.4998
S_{crit} (log)	**0.0091**	0.7577	0.6493	0.0701

Acclimation temperatures (T=7, 14 or 21°C); salinity acclimation (S=22, 28 or 34 p.p.t.).

P-values are from three-factor ANOVA. Significant results are in bold type. Species (Sp) and temperature acclimation (T) contribute greatly to the variation in all four heart rate parameters (in both years), while salinity (S) only contributes greatly to the variation in response to salinity stress. Because there are significant interaction terms, *post-hoc* comparisons were done only within a single temperature and salinity combination.

Heart activity parameters: RHR, resting heart rate at acclimation temperature (beats min^{-1}); H_{crit}, critical temperature during heat stress (°C); LHR, lowest heart rate at 0°C (beats min^{-1}); S_{crit}, critical salinity during salinity stress (p.p.t.).

measurement, heart rates for *M. trossulus* were approximately 1.5 times those of the more warm-adapted *M. galloprovincialis*. Heart rates of the third species, *M. edulis*, were intermediate. Hybrids between *M. galloprovincialis* and *M. trossulus* were measured only at 14°C and 28 p.p.t. The heart rates of hybrids (22±1.17, mean ± s.e.m.) were intermediate between and significantly different from those of the parent species (Fig. 3A) (ANOVA, P<0.0001). There was no evidence of a change in basal heart rate over the acclimation period nor was there evidence of a correlation between size and basal heart rate (data not shown).

Heat stress and heart rate

In response to acute increases in measurement temperature, heart rate initially increased and, then, abruptly decreased when the critical high temperature (H_{crit}) was reached (Fig. 2A). During continued exposure to temperatures >H_{crit}, heart rate remained depressed but quickly returned to the initial rate when the water bath was returned to the initial experimental temperature (=acclimation temperature; see Fig. 2A). This indicates that the pronounced fall in heart rate that occurs when H_{crit} is reached is not lethal, but rather is a temporary response to an elevated environmental temperature and is likely coincident with valve closure (see Discussion).

Significant differences were observed in comparisons of the different species and, within a species, of differently acclimated individuals. All species exhibited increases in H_{crit} as acclimation temperature was increased (Fig. 3B) (ANOVA, P<0.0001, Tables 1, 2). The combination of acclimation temperature and species explained a significant portion of the variation seen in H_{crit} (ANOVA, P<0.0001, Tables 1, 2), but salinity acclimation was not a significant contributor to the observed pattern (P=0.7791).

Although not all interspecific comparisons were statistically significant at each temperature/salinity combination, there are consistent trends in H_{crit} among species that suggest that the congeners respond differently to heat stress (Fig. 3B). *Mytilus trossulus* had the lowest H_{crit} and so appears to be the most sensitive to heat stress, followed by *M. edulis*, and *M. galloprovincialis*. These relationships among the three species do not vary as a function of temperature acclimation. At the one acclimation treatment so studied (14°C, 28 p.p.t.), *M. trossulus*/*M. galloprovincialis* hybrids had H_{crit} values intermediate to those of the parental species. Depending on acclimation salinity, *M. trossulus* increased its H_{crit} by 1.8–3.4°C during acclimation to 21°C. *Mytilus galloprovincialis* and *M. edulis* showed slightly greater acclimatory plasticity as they increased H_{crit} by 2.4–5.2°C and 2.0–4.7°C, respectively, during acclimation at 21°C.

Cold stress and heart rate

Heart rate fell steadily with decreasing measurement temperature, but no sharp breaks in cardiac activity such as those seen during heating were observed (Fig. 2A,B). After cold stress and a return to the acclimation temperature, the heart rates of both species tested (*M. trossulus* and *M. galloprovincialis*) quickly recovered to values seen at the start of the experiment (acclimation temperature). Comparing the lowest heart rates measured near 0°C (LHR) (Fig. 3C), it is clear that *M. trossulus* was able to maintain heart function at significantly lower temperatures than *M. galloprovincialis* (ANOVA, P<0.0001, Tables 1, 2) under most acclimation conditions. Both species showed significant acclimation effects. The heart rate of 14°C-acclimated animals was severely depressed by cold stress, with a measurable heart rate only in *M. trossulus* and only at a single salinity (28 p.p.t.). Following acclimation to 7°C, an increase in heart rate of up to 6 beats min^{-1} was observed in *M. trossulus*, but for *M. galloprovincialis* heart rate increased by only 2 beats min^{-1}. At common winter temperatures, therefore, the heart rate of *M. trossulus* would exceed that of *M. galloprovincialis* by at least two- to threefold, depending on salinity.

Salinity stress and heart rate

Heart rate decreased gradually as the salinity of the medium was reduced and exhibited a characteristically sharp drop when salinity reached a certain low value, the critical low salinity (S_{crit}) (Fig. 2C). S_{crit} was positively correlated with acclimation salinity in all species (Fig. 3D; Tables 1, 2). In addition, S_{crit} was positively correlated with acclimation temperature in some treatments. Acclimation to a higher temperature increased S_{crit} of *M. trossulus* and *M. edulis* (Fig. 3D, ANOVA, P<0.0001,

Table 2. *Resting heart rate and heart rate response to stress – average responses, by species*

	M. edulis	*M. galloprovincialis*	*M. trossulus*	*N* e	*N* g	*N* t
RHR						
14°C, 22 p.p.t.	18.99±0.95	15.70±0.89	25.86±1.63	10	9	9
14°C, 28 p.p.t.	21.30±0.87	17.53±0.85	25.91±1.17	10	10	9
14°C, 34 p.p.t.	20.45±0.98	17.06±0.79	23.62±1.55	10	10	10
21°C, 22 p.p.t.	28.65±2.39	25.80±1.21	34.88±0.88	7	7	8
21°C, 28 p.p.t.	28.77±2.20	23.88±0.91	35.70±0.91	7	8	9
21°C, 34 p.p.t.	31.50±1.26	25.72±1.28	38.24±1.44	8	9	7
H_{crit}						
14°C, 22 p.p.t.	25.50±0.57	26.60±0.60	23.50±0.93	4	6	6
14°C, 28 p.p.t.	25.50±0.99	28.30±1.08	23.70±0.80	4	7	7
14°C, 34 p.p.t.	24.20±0.89	25.70±0.41	24.20±1.36	3	7	6
21°C, 22 p.p.t.	29.00±0.47	29.90±0.60	26.90±1.53	4	3	4
21°C, 28 p.p.t.	28.50±0.51	30.70±1.04	26.00±0.59	4	4	4
21°C, 34 p.p.t.	28.90±1.09	30.90±0.41	26.00	4	6	1
LHR						
7°C, 22 p.p.t.	–	2.28±0.94	3.80±2.42	–	7	5
7°C, 28 p.p.t.	–	0	4.33±1.96	–	5	6
7°C, 34 p.p.t.	–	1.86±0.94	6.40±1.81	–	7	5
14°C, 22 p.p.t.	–	0	0	–	6	6
14°C, 28 p.p.t.	–	0	3.33±1.33	–	5	6
14°C, 34 p.p.t.	–	0	0	–	5	5
S_{crit}						
14°C, 22 p.p.t.	11.09±0.99	8.89±0.39	9.53±0.42	3	4	4
14°C, 28 p.p.t.	11.99±2.40	11.39±0.70	12.41±0.92	4	4	4
14°C, 34 p.p.t.	16.81±1.10	12.84±0.18	13.79±1.31	4	4	4
21°C, 22 p.p.t.	14.52±0.83	9.68±0.44	11.80±1.23	3	4	4
21°C, 28 p.p.t.	21.23±0.66	10.28±0.89	16.73±1.10	3	4	5
21°C, 34 p.p.t.	19.64±2.61	14.15±0.77	19.65±1.85	4	3	5

Results of heart rate experiments for all physiological indices measured. Each index (mean ± s.e.m.) is listed by species (e, *M. edulis*; g, *M. galloprovincialis*; t, *M. trossulus*), and the number of individuals tested.

Heart activity parameters: RHR, resting heart rate at acclimation temperature (beats min^{-1}); H_{crit}, critical temperature during heat stress (°C); LHR, lowest heart rate at 0°C (beats min^{-1}); S_{crit}, critical salinity during salinity stress (p.p.t.).

Tables 1, 2). Of all the species, *M. edulis* was most affected by the interaction of warm temperature acclimation and the response of heart rate to salinity, raising its S_{crit} by 4–9 p.p.t., depending on the salinity of acclimation. In contrast, *M. galloprovincialis* had a similar S_{crit}, regardless of the acclimation temperature. Among the congeners, heart rate of *M. galloprovincialis* is sustained at the lowest ambient salinities, that of *M. edulis* ceases at the highest S_{crit}, and *M. trossulus* is intermediate. Pilot studies showed that *M. trossulus/M. galloprovincialis* hybrids were not different in their salinity tolerance in comparison to the three congeners at moderate acclimation levels (14°C and 28 p.p.t.) (S_{crit}≈11.8 p.p.t.).

Q_{10} and temperature compensation of heart rate during thermal acclimation

To determine whether temperature compensation of heart rate occurred during thermal acclimation (14°C and 21°C), we calculated the Q_{10} of heart rate of 14°C-acclimated individuals (each species), using data from the initial hour of heat ramps. The Q_{10} values differed only slightly among species (*M. galloprovincialis*=2.38, *M. trossulus*=2.03, *M. edulis*=2.08). We then used these Q_{10} values to predict heart rate at the higher acclimation temperature (21°C), in the absence of temperature compensation. We calculated that we should see a 60–70% increase in heart rate during acclimation from 14°C to 21°C, if there were no temperature compensation. In fact, we observed partial temperature compensation due to acclimation, with rate increases of only 35–64% in heart rate (Fig. 4), depending on acclimation treatment.

Mortality during temperature and salinity acclimation

There was differential survival among species during the acclimation treatments (Fig. 5). Only 3 of 348 *M. galloprovincialis* failed to survive and fatalities were all in the 21°C acclimation group in 2003. Significantly higher mortality occurred for *M. edulis* and *M. trossulus* (the highest was 66% in *M. edulis* at 21°C and 28 p.p.t.) and there was a positive

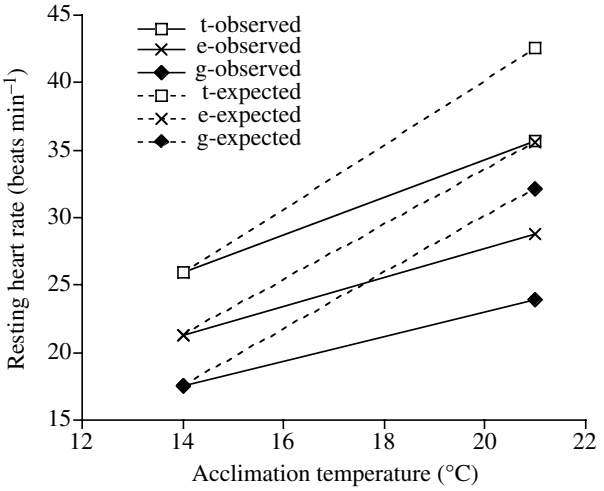

Fig. 4. Temperature compensation of resting heart rate (RHR; beats min^{-1}). t, *M. trossulus*; g, *M. galloprovincialis*; e, *M. edulis*. Symbols connected by solid lines are observed RHR values averaged across salinity treatments. Symbols connected by broken lines are observed RHRs at 14°C and expected RHRs at 21°C, using the species-specific Q_{10} estimate and assuming no physiological compensation. If there was complete temperature compensation, there would be no difference between the observed values for 14°C- and 21°C-acclimated animals (symbols connected by a horizontal line; not shown). However, the observed RHR at 21°C is lower than expected for all species, indicating partial compensation with acclimation temperature.

correlation between acclimation temperature and mortality for these two congeners. The overall mortality in *M. trossulus* was comparable in both acclimation years, but the pattern of mortality in response to salinity treatment was different between years. In 2003, mortality in the 21°C-acclimated specimens was positively correlated with salinity acclimation; this relationship was not observed at 14°C, however. In 2004, mortality in the 14°C-acclimated specimens was positively correlated with salinity, but this trend was not found in the 7°C-acclimated animals.

Discussion

Limits to biogeographic patterns are clearly the result of many ecological, historical, and physiological factors. However, among these factors, we perhaps know the least about the roles of physiological limits in governing distribution ranges. In this manuscript, we present data that enable us to address a number of questions about the roles played by fixed genetic differences in temperature and salinity tolerance and by varying capacities for acclimation to these two variables in setting distribution patterns of closely related blue mussel congeners. The evolutionary history of the blue mussel complex suggests that differences in tolerance of temperature and salinity and in the capacities to acclimatize to changes in both variables should exist among species. The congeners are separated by approximately 3.5–5 million years of evolutionary divergence, yet the two most diverged congeners in the blue mussel complex (*M. galloprovincialis* is the newest species and *M. trossulus* the oldest) (Barsotti and Meluzzi, 1968; Seed, 1992; Vermeij, 1991), remain capable of hybridizing in all known regions of contact. However, the habitats in which these two species arose differ in temperature and salinity characteristics, despite sympatric extant populations. The Mediterranean Sea is a warm, enclosed sea with a relatively high and stable salinity, compared to the North Pacific Ocean, which is relatively cool and may have coastal regions of low salinity during periods of run-off from winter storms. From these native habitat differences, one would predict *M. trossulus* to be successful in habitats with fluctuating salinity and cooler temperatures and *M. galloprovincialis* to be more successful in habitats with stable and higher salinities and higher temperatures. However, the current global distribution and invasive success of *M. galloprovincialis* suggest that it may be the most physiologically adaptable of the *Mytilus* congeners and that it may have fewer physiological limitations to range expansion than its congeners.

Thermal physiology

Habitat temperature is often cited as a limiting factor determining where ectothermic species can live (Hochachka

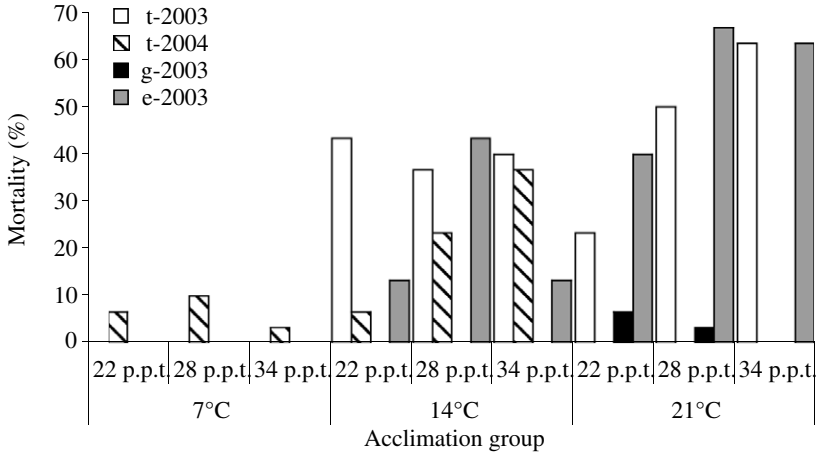

Fig. 5. Mortality in temperature and salinity acclimation treatment groups. There was nearly equal mortality of *M. edulis* (e) and *M. trossulus* (t) in 2003, regardless of acclimation treatment, and both species had greater mortality at the warmer acclimation temperature. In the following year, *M. trossulus* had equivalent mortality at the 14°C acclimation and decreased mortality under 7°C acclimation, reinforcing the positive relationship between acclimation temperature and mortality. Only three *M. galloprovincialis* (g) individuals died in both acclimation years (out of 348 total), and all three were from the 21°C acclimation in 2003 (*M. galloprovincialis* 2004 is not included in the graph because there was no observed mortality).

and Somero, 2002). Because water temperature and blue mussel species composition were highly variable among the sites we examined (Braby, 2004; Braby and Somero, 2006), any observed differences between *M. trossulus* and *M. galloprovincialis* in their responses to temperature could provide a mechanistic explanation for the distribution of these species within the mosaic hybrid zone present in San Francisco and Monterey Bays. Previous work (Sarver and Foltz, 1993) on the distributions of these two congeners in this hybrid zone suggested that *M. trossulus* was better adapted to conditions of low temperature and low salinity than *M. galloprovincialis*. However, this earlier study did not examine physiological traits, but focused strictly on the distributions of the two species.

Our study provides evidence for genetically fixed differences in the thermal responses of heart function among the three blue mussel congeners, differences that may account, in part, for their current biogeographic patterning and, in the context of predicting the further invasive success of *M. galloprovincialis*, for the ability of this invader to extend its range northward. *Mytilus trossulus* is more cold tolerant than the invasive *M. galloprovincialis*, as evidenced by its capacity for sustaining cardiac function at low temperatures (Fig. 3C). Under winter conditions, cardiac activity would be substantially lower in the invasive species than in *M. trossulus*, a physiological difference that could favor the native species at higher latitudes, where winter temperatures during emersion may reach 0°C. Likewise, our discovery of a significantly higher intrinsic rate of cardiac activity in *M. trossulus* relative to *M. galloprovincialis* (Fig. 3A) suggests that the native species is more cold-adapted (cold-compensated) than the Mediterranean invader. Our data on heart rates provide an interesting complement to an earlier study of blue mussels (Pickens, 1965), which was conducted before it was known that the blue mussels found along the Pacific Coast of North America were not *M. edulis*, but instead comprised *M. trossulus*, *M. galloprovincialis* and their hybrids. Pickens reported that *'M. edulis*' populations from Alaska (putatively, *M. trossulus*) had significantly higher heart rates than *'M. edulis*' populations from Southern California (putatively, *M. galloprovincialis*). We conjecture, therefore, that replacement of *M. trossulus* by *M. galloprovincialis* at higher latitudes may be impeded by the native species' better ability to sustain cardiac function at colder temperatures.

Other studies support the view that *M. trossulus* has a more cold-adapted physiology than *M. galloprovincialis*, and, therefore, would be at a competitive advantage at low, but not high, temperatures. A recent study (Fields et al., 2006) compared the thermal sensitivities of cytosolic malate dehydrogenase (cMDH) of *M. trossulus* and *M. galloprovincialis* and showed that the cMDH of *M. trossulus* exhibited kinetic properties consistent with adaptation to lower temperatures relative to the ortholog of *M. galloprovincialis*. Hofmann and Somero provide additional evidence that *M. trossulus* is the more cold-adapted species (Hofmann and Somero, 1996), through showing that *M. galloprovincialis* induces synthesis of heat-shock proteins at a higher temperature than *M. trossulus* (in 13°C-acclimated animals, induction temperatures were 25°C and 23°C, respectively). The greater tolerance of high temperatures by *M. galloprovincialis*, e.g. the higher values for H_{crit} (Fig. 3B), and the much lower mortality found at the highest acclimation temperature (21°C) (Fig. 5) indicate that this invasive species is likely to have a competitive advantage over the native under conditions of high temperature. These differences could underlie the success of the invader in replacing *M. trossulus* along much of the coastline of southern and central California.

Although *M. trossulus* appears better adapted, physiologically and biochemically, than *M. galloprovincialis* to function at low temperatures and thus seems poised to outcompete the invader at higher latitudes, the occurrence of higher mortality of *M. trossulus* under common garden conditions at both low and high temperatures (Fig. 5) raises a caveat about the relative competitive abilities of these two species. Higher mortalities for northern populations of '*M. edulis*' (likely, *M. trossulus*) than for southern populations (likely, *M. galloprovincialis*) have also been reported (Pickens, 1965). Pickens' data and ours suggest that some currently unknown difference in robustness exists between these two species, and these differences in adult survivorship could potentially balance or even outweigh the competitive differences due to differential adaptations to temperature at the physiological and biochemical level.

Another caveat about the significance of physiological adaptations also merits consideration. The sharp decreases in cardiac activity seen at extremes of temperature may be a consequence of a complex behavioral response to stress, rather than merely the direct effects of temperature on the heart itself. Pickens compared the effects of temperature on hearts *in situ* and on hearts isolated from the mussels (Pickens, 1965). Hearts under both experimental conditions exhibited a similar Q_{10} effect as measurement temperature was varied. However, the isolated hearts sustained function at higher and lower temperatures than hearts *in situ*. The differences in the thermal responses between these two experimental preparations may arise from valve closure. A few studies have quantified valve closure response to toxins (Depledge et al., 1996) and to salinity stress (Nicholson, 2002) in Mytilid mussels. We qualitatively demonstrated that temperatures near H_{crit} elicited valve closure (Braby, 2004); however, this was done after the experiments reported in this paper were completed. Thus, the sharp fall in heart rate seen at temperature extremes in intact animals could be a consequence of a behavioral response to stress, valve closure, which in turn elicits physiological adjustments. Closing of the valves would restrict gas exchange, thus limiting aerobic metabolism. Under conditions of limiting oxygen, reductions (or cessations) of cardiac activity might be necessary. In the context of thermal effects, valve closure responses could be especially advantageous during periods of emersion, when the threat of desiccation at high temperatures may be significant. Future research should quantify valve closure in response to temperature (and salinity) stress and to

determine which comes first, valve closure or heart rate depression.

Salinity physiology

Valve closure, which we assume is coupled with a reduction in cardiac activity under all circumstances, could also be important for immersed animals facing sharp reductions in salinity, as might occur during winter rainstorms in the northeastern Pacific. For osmoconformers like *Mytilus*, reductions in ambient salinity will lead to dilution of the internal fluids and perturbation of the ionic compositions of the cells. In *Mytilus*, the physiological response to chronic hypo-osmotic conditions is to actively change the cytosolic concentration of various organic osmolytes, including free amino acids (Yancey et al., 1982), which may require 2 or more days to achieve (de Vooys, 1991; Gosling, 1991; Hawkins and Bayne, 1991). However, in response to acute hypo-osmotic stress, conservation of the composition of the body fluids would be favored by closing the valves and, thereby, reducing exchange of water and solutes with the ambient seawater. In the case of acute hypo-osmotic stress, therefore, it may be advantageous to close the valves (and, consequently, reduce cardiac activity) at relatively high salinities. Thus, a high S_{crit} might be advantageous in coping with hypo-osmotic stress, for this would allow a mussel to isolate its body fluids from falling salinity at an earlier stage of stress exposure. Earlier studies have, in fact, shown that acute fluctuations in salinity trigger valve closure in mussels (de Vooys, 1991; de Zwaan and Mathieu, 1991; Seed and Suchanek, 1991).

Viewed from this perspective, the higher S_{crit} found for *M. trossulus* relative to *M. galloprovincialis* might represent an adaptation for coping with hypo-osmotic stress, which is apt to be more common in coastal Pacific habitats than in the Mediterranean Sea. If this is the case, then another difference between the native and invasive species appears to be important in determining their biogeographic patterns and in determining the further invasive movement of *M. galloprovincialis*. The greater ability of *M. trossulus* to isolate its internal fluids from hypo-osmotic stress could help it to out-compete *M. galloprovincialis* in habitats where such stress is prevalent. Especially during winter storms, when the combination of low temperatures and reduced salinity exists in coastal regions, *M. trossulus* might be significantly better able to function than its invasive competitor. We conjecture that the reduction in feeding time and the depression in metabolic activity that are likely to result from valve closure are more than balanced by the reduction in physiological stress associated with volume regulation due to hypo-osmotic conditions.

S_{crit} varied directly with acclimation salinity, such that animals held at the high salinity reduced cardiac activity at higher salinities than specimens acclimated to lower salinities (Fig. 3D). Acclimation temperature also influenced S_{crit}. For *M. trossulus*, S_{crit} is lower in 14°C-acclimated specimens relative to 21°C-acclimated specimens (Fig. 3D). This finding suggests that winter-acclimatized individuals may be better able to cope with reduced salinities than summer-acclimatized individuals. There was relatively little change in S_{crit} during thermal acclimation in *M. galloprovincialis*. *M. edulis* exhibited the largest changes in S_{crit} as functions of acclimation conditions. Our experiments did not monitor the time course of the change in S_{crit} during acclimation, but a recent study shows that cyclical hypo-osmotic stress confers greater salinity tolerance with each cycle (quantified by valve closure) (Sukhotin et al., 2003) and that this increased tolerance occurs over the course of a few hours.

Hybrid physiology

Hybrids between *M. trossulus* and *M. galloprovincialis* exhibited intermediate values for resting heart rate (Fig. 3A) and H_{crit} (Fig. 3B). S_{crit} values fell within the range of the parental species (Fig. 3D). These limited data provide no evidence for any distinct advantage or disadvantage on the part of adult hybrids. However, there is some indication from reproductive studies that larval success of hybrid crosses of these two species is lower than for parental species (Matson et al., 2003).

What is the most critical physiological factor in setting distribution patterns of blue mussels?

Our studies of adult blue mussels reveal a number of physiological differences that correlate with the species' distribution patterns. *M. galloprovincialis* is better able to cope with higher temperatures than either *M. trossulus* or *M. edulis*. Assuming that a high value for S_{crit}, which is likely paired with valve closure, denotes an adaptive response to hypo-osmotic stress, then *M. trossulus* appears better adapted than its invasive competitor to tolerate reduced salinity. These differences between *M. trossulus* and *M. galloprovincialis* in tolerance of thermal and salinity extremes are consistent with the evolutionary histories of the two species. However, despite this agreement between physiological response and evolutionary history, two important questions about the role of these physiological differences in setting distribution patterns remain. First, which environmental variable is more important in setting distributions, temperature or salinity? Second, do the physiological data obtained in this study reflect the distribution patterns of the native and invasive species within the mosaic hybrid zone found in the San Francisco and Monterey Bays?

Based on our study of the distributions of the native and invasive species within this mosaic hybrid zone (Braby and Somero, 2006), it appears that salinity is the more important variable in the context of determining the distribution of species. Thus, *M. trossulus* was found to be dominant in the warmest habitats studied, which are shallow estuarine regions with the highest variation in salinity.

Adult distribution patterns may also reflect differences in larval recruitment over space and time and different sensitivities of congeneric larvae to environmental factors. Other studies of *Mytilus* have shown that the larvae of *Mytilus* congeners have differences in mortality due to salinity and

temperature treatments (Matson et al., 2003; Qiu et al., 2002). In the case of the two Pacific coast species used in this study, there is only limited knowledge of where larvae spend most of their planktonic life. In the open ocean, there is likely to be little variation in temperature and salinity. The degree to which larvae are subject to coastal variability in both temperature and salinity is highly dependent on their retention in bays and estuaries. If high retention occurs, then larval physiology may play a significant role in determining adult distribution patterns. In fact, there is some evidence that *Mytilus* may be recruiting more locally than previously thought (Gilg and Hilbish, 2003). Nonetheless, the differences in adults' responses to temperature and salinity reported here suggest that genetically fixed differences in physiological traits – differences that are likely present in larval stages as well – make a strong contribution to distribution patterns, including those of invasive species.

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