The Onset Temperature of the Heat-Shock Response and Whole-Organism Thermal Tolerance Are Tightly Correlated in both Laboratory-Acclimated and Field-Acclimatized Tidepool Sculpins (*Oligocottus maculosus*)

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ABSTRACT

We examined the relationship between thermal tolerance, measured as critical thermal maximum (CT_{max}), and aspects of the heat-shock response in tidepool sculpins (*Oligocottus maculosus*) acclimated to constant laboratory temperatures or acclimatized to field conditions. The CT_{max} of fish laboratory acclimated to 6°, 13°, and 20°C were 27.6° ± 0.1°C, 29.5° ± 0.1°C, and 30.8° ± 0.1°C, respectively, increasing linearly by 0.2°C for each 1°C increase in acclimation temperature. The CT_{max} of field-acclimatized fish from the low intertidal (29.9° ± 0.1°C) was significantly lower than that of fish from the mid- (30.5° ± 0.1°C) and high (30.4 ± 0.1°C) intertidal. CT_{max} and the onset temperature of *hsp70* induction in gill (T_{on}) were highly correlated in both laboratory-acclimated and field-acclimatized sculpins, with T_{on} occurring at 2°C below CT_{max} in all cases. However, there was no consistent relationship between CT_{max} and the maximum levels of gill *hsp70* mRNA. Predicted "acclimation" temperature (15.9° ± 0.5°C) and mean habitat temperature (15.9° ± 1.6°C) were similar for sculpins from low intertidal pools, but this relationship was not apparent in mid- and high intertidal fish. Mark-recapture experiments indicated that approximately 80% of fish from low intertidal pools were residents of that pool, but residency rates were less than 50% in mid- and high intertidal pools, which may explain the lack of correlation between CT_{max} and habitat variables in these groups. These data indicate that gill *hsp70* T_{on} and CT_{max} are highly correlated indicators of the thermal performance of tidepool sculpins in both laboratory and field settings.

Introduction

Because of its profound effects on biochemical and physiological processes, temperature is thought to be an important factor that limits the biogeographic distribution of ectotherms (see, e.g., Fry 1947; Hochachka and Somero 2002). Consistent with this idea, species from colder habitats are often less tolerant of high temperatures than those from warmer habitats (e.g., Fields et al. 1987; Krebs and Loeschcke 1995; Garbuz et al. 2003; Fangue et al. 2006). However, the precise aspects of thermal physiology that are most important in establishing this relationship are not well understood.

One potentially important component of the response to high temperatures at the cellular level is the ability to mount a heat-shock response (HSR). The HSR involves the rapid transcription and translation of a family of proteins known as the heat-shock proteins (Hsps), which act as molecular chaperones to combat the protein denaturation and aggregation associated with exposure to thermal stress (Lindquist and Craig 1988). Thus, it has been suggested that changes in the HSR may play a role in thermal adaptation (for a review, see Feder and Hofmann 1999) and may be involved in specifying the biogeographic distributions of organisms (Tomanek 2008). However, the extent to which the HSR and measures of whole-organism thermal tolerance are reflections of similar underlying phenomena at the molecular level is far from clear. For example, most common measures of whole-organism thermal tolerance (such as knockdown temperature or critical thermal maximum [CT_{max}]) are most likely to be integrated measures of neural function, which may be most responsive to the effects of temperature on molecular characteristics such as membrane fluidity, while the HSR is presumably most closely associated with the effects of temperature on protein three-dimensional structure.

Remarkably few studies have attempted to directly relate changes in Hsp expression to changes in whole-organism thermal tolerance in natural populations, and these studies have yielded conflicting results (see, e.g., Fangue et al. 2006; Dahlhoff and Rank 2007; Jensen et al. 2009; Bahrndorff et al. 2009, 2010).

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However, studies on the HSR and thermal tolerance of genetically engineered strains of *Drosophila melanogaster* that carry different numbers of copies of the gene for *hsp70* strongly suggest that there may be a mechanistic link between these two phenomena (Feder and Krebs 1998; Gong and Golic 2006; Bettencourt et al. 2008). These inconsistent data sets have led to a call for additional studies into the relationship between the HSR and whole-organism thermal tolerance (see, e.g., Bahrndorff et al. 2009, 2010).

Here we use the tidepool sculpin (*Oligocottus maculosus*), a species that is found throughout the intertidal zone along the west coast of North America from the Gulf of Alaska to central California (Morris 1960), to investigate the relationship between the HSR and whole-organism thermal tolerance. Over the course of the tidal cycle, tidepools undergo pronounced changes in a variety of abiotic factors, including temperature, salinity, and oxygen (Truchot and Duhamel-Jouve 1980; Morris and Taylor 1983). The extent of these fluctuations typically differs among tidepools, with pools in the low intertidal experiencing much less variation than those in the high intertidal zone. Tidepool sculpins exhibit strong site fidelity to their home tidepool, returning to the same pool across multiple tidal cycles and returning to the home tidepool following experimental translocation (Green 1971; Khoo 1974; Craik 1981; Yoshiyama et al. 1992). There is little genetic differentiation among tidepool sculpins across regional geographic scales, however (Altman and Taylor 2003), opening the possibility that thermal history could vary substantially among geographically proximate and genetically similar sculpins from tidepools at different intertidal heights. Both the HSR (e.g., Dietz and Somero 1992; Roberts et al. 1997; Tomanek and Somero 1999; Buckley et al. 2001; Barua and Heckathorn 2004) and whole-organism thermal tolerance (reviewed in Beitinger et al. 2000) can be altered by an organism's thermal history. Thus, tidepool sculpins may present an interesting study system in which to ask whether these two traits are correlated and change in parallel in response to varying thermal history in both the laboratory and the field.

The goals of this study were (1) to determine the degree of plasticity in whole-organism thermal tolerance and the HSR in *O. maculosus*, (2) to ask whether these two traits are correlated across a range of thermal environments in both the laboratory and the field, and (3) to examine which aspects of the natural thermal environment affect thermal tolerance and the HSR in field-acclimatized fish from tidepools at different heights within the intertidal zone. By using both laboratory-acclimated and field-acclimatized fish from different environments, we address whether plasticity in the HSR response and whole-organism thermal tolerance have a direct relationship and whether this relationship is consistent in both the simple, highly controlled environment of the laboratory and the complex, multivariate natural environment.

Material and Methods

Fish Collection and Husbandry

Tidepool sculpins for physiological experiments were collected in the area around Bamfield, British Columbia, in July and August 2003. Fish for the laboratory acclimation studies were collected via beach seine from Ross Islets (48°52′12″N, 125°09′42″W), and field-acclimatized fish were collected using dip nets from low, middle, and high intertidal tidepools at Wizard Rocks (48°51′30″N, 125°09′45″W). All fish were transported to Bamfield Marine Sciences Centre in 32 parts per thousand (ppt) seawater at their collection temperature.

Field-acclimatized tidepool sculpins from each tidepool were held in outdoor tanks at 32 ppt, under natural photoperiod, with ambient ocean temperature (~12°C) for a maximum of 2 h until the determination of CT_{max}. Fish for laboratory acclimation experiments were held in glass aquaria with recirculating seawater at 32 ppt and natural photoperiod. Tank temperature was initially maintained at the ambient ocean temperature for each group but was then increased or decreased by 0.5°C/d until acclimation temperatures of 6.3° ± 0.39°C, 13.1° ± 0.38°C, and 20.4° ± 0.55°C (mean ± SEM) were achieved. Fish were acclimated to each of these temperatures for 25, 20, and 26 d, respectively, before experimentation and were fed fresh mussels ad lib. daily. Feeding was stopped 24 h before experimentation. All animal husbandry and experiments were performed according to an approved University of British Columbia and Bamfield Marine Sciences Centre animal care protocol (A01-0180).

Thermal Tolerance Methodology

The CT_{max} of both laboratory-acclimated and field-acclimatized sculpins was determined as an index of whole-organism acute thermal tolerance ($n = 10$ fish per group). CT_{max} trials were performed essentially as in the study by Fangue et al. (2006), using loss of equilibrium as an experimental endpoint. Briefly, water temperature was increased at a rate of 0.3°C/min until the fish were unable to maintain their position in the water column. The water in the test chambers was vigorously aerated throughout to prevent thermal stratification and to maintain dissolved oxygen levels. We conducted a preliminary experiment to determine the effect of temperature ramping rate on CT_{max} values using a separate group of field-acclimatized tidepool sculpins collected from Ross Islets and held at ambient ocean conditions (~12°C) for 2 d before testing. We found a small but significant difference in CT_{max} determined using a ramping rate of 0.3°C/min (29.6° ± 0.1°C) versus all slower rates (30.0° ± 0.1°C; tested at 0.025°C/min, 0.05°C/min, and 0.1°C/min). We selected the more rapid rate because it has been broadly used in studies of CT_{max} in fishes (Beitinger et al. 2000), allowing ready comparison of our data to those collected for other species, and to ensure that there was no opportunity for field-acclimatized fish to acclimate to the laboratory conditions. However, this does open the possibility that our CT_{max} data represent slight underestimates of the actual maximal thermal tolerance of these animals. Most CT_{max} trials required approximately 60 min to complete (with a range from 35 min for 20°C acclimated fish to 72 min for 6°C acclimated fish). Immediately following loss of equilibrium, fish were removed from the test chamber, and weights and standard lengths were de-

termined. Fish were then returned to tanks at their acclimation temperature for recovery. No mortality occurred as a result of the CT_{max} procedure.

Heat-Shock Experiments

In order to assess the effects of prior thermal history on the HSR, both laboratory-acclimated and field-acclimatized sculpins were exposed to an experimental heat-shock treatment that paralleled the CT_{max} trials described above. As a pre-CT_{max} control, fish were sampled directly from their holding tanks ($n = 6$ per field-acclimatized and laboratory-acclimated group). The remaining fish were introduced into the CT_{max} apparatus at their acclimation temperature or under ambient ocean conditions (approximately 12°C) for the field-acclimatized groups. A subset of fish was then transferred to a recovery tank for 1 h and sampled ($n = 6$ per group) as a handled control. The temperature in the CT_{max} apparatus was then increased at a rate of 0.3°C/min, and groups of six fish were removed from the apparatus at temperatures equivalent to 6°, 4°, 2°, and 1°C below their respective CT_{max} and at their CT_{max}. Following this heat shock, the fish were returned to their acclimation/acclimatization temperature for 1 h, after which they were killed by lethal anesthesia (MS-222, 2 g/L) and gill tissue was sampled and immediately placed into liquid nitrogen. Tissue samples were stored at −80°C until analysis.

RNA Isolation and Real-Time Polymerase Chain Reaction Analysis of hsp70, hsc70, *and* hsp90 *Expression*

We chose to monitor the HSR at the RNA level rather than at the protein level because the constitutive and inducible members of the hsp70 family from tidepool sculpins are difficult to separate on one-dimensional protein gels. This technical issue and the large amounts of the constitutive heat-shock cognate hsc70 protein present under control conditions make accurate detection of the T_{on} of hsp70 induction challenging at the protein level in this species (see, e.g., Todgham et al. 2006).

RNA was extracted from tissues using the guanidinium isothiocyanate method (Chomczynski and Sacchi 1987) using Trizol Reagent (Invitrogen Life Technologies, Burlington, Ontario). Isolated RNA was quantified spectrophotometrically, and RNA integrity was assessed using agarose-formaldehyde gel electrophoresis (1% w/v agarose, 16% formaldehyde). Only samples that had an A260/280 ratio of at least 1.8 and that showed two bright rRNA bands of equal intensity were used for cDNA synthesis. First-strand cDNA was synthesized from 5 μg total RNA using oligo(dT_{18}) primer and RevertAid H Minus M-MuLV reverse transcriptase in 20 μl total volume following the manufacturer's instructions (MBI Fermentas, Burlington, Ontario), with incubation at 42°C for 60 min. The resulting cDNA was stored at −80°C until use.

Gene expression was determined with quantitative real-time polymerase chain reaction (qRT-PCR) on an ABI Prism 7000 sequence analysis system (Applied Biosciences, Foster City, CA), using gene-specific primers for *hsp70*, *hsc70*, *hsp90β*, and elongation factor 1 α (*EF-1α*; Todgham et al. 2005). All primers were synthesized and desalted by Integrated DNA Technologies. Previous studies (Todgham et al. 2005, 2006) and our data indicate that *EF-1α*, *hsc70*, and *hsp90β* are constitutively expressed genes whose expression does not usually change substantially with thermal stress.

Real-time PCR reactions were performed using 1 μL cDNA and 4 pmol forward and reverse primer with SYBR Green Master Mix (Applied Biosciences) as follows: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. All samples were analyzed in duplicate, and coefficients of variation between duplicates were never greater than 10% (and were usually below 5% for *hsc70*, *hsp90β*, *EF-1α*, and in the induced samples for *hsp70*). Melt curve analysis was performed to confirm presence of a single amplicon, and representative reactions were sequenced to ensure the identity of the amplicons. Non-reverse-transcribed controls were performed for all samples to assess levels of genomic DNA contamination. Contamination was never greater than 0.01% for any primer set or sample, except for control samples for *hsp70*, in which expression was very close to the minimum level of detection of the technique.

One highly induced sample from a high intertidal field-acclimatized fish (exposed to a temperature 1°C below its CT_{max}; approximately 29.5°C) was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set (with five fivefold serial dilutions of the input cDNA). Standard curves always had r^2 values greater than 0.980 and efficiencies between 90% and 110%. A standard curve was run on each plate, and expression levels for each sample were determined on the basis of comparison to its standard curve in order to account for any possible variation in PCR efficiency from day to day. Because of this standard curve approach, our data are expressed relative to the level of expression in the randomly chosen individual. Results for *hsp70* were then normalized using the geometric mean of the relative mRNA levels of *EF-1α*, *hsc70*, and *hsp90β*. The purpose of this normalization is to control for variation in reverse transcription efficiency, which is apparent among batches (Fig. A1 in the online edition of *Physiological and Biochemical Zoology*). This variation did not represent a consistent difference between laboratory-acclimated or field-acclimatized fish, and levels of *EF-1α*, *hsc70*, and *hsp90β* were highly correlated with each other, which is typical for variation among samples due to differences in the efficiency of the reverse transcription reaction among batches. The use of the geometric mean of the expression of multiple internal control genes has been recommended as the most appropriate method for the analysis of real-time PCR data sets (Vandesompele et al. 2002). Although there were no substantial changes in *hsc70* or *hsp90β* mRNA with heat shock in most groups, we did observe modest (less than twofold) induction of *hsc70* and *hsp90β* genes in heat-shocked 20°C laboratory-acclimated fish at 2° and 4°C below the CT_{max} (Figs. A2, A3 in the online edition of *Physiological and Biochemical Zoology*). Despite this induction, we chose to include both *hsc70* and *hsp90β* in our normalization control to focus on regulation that is specific to

the inducible *hsp70*. However, because this approach will tend to bias downward our estimate of the levels of *hsp70* in these groups, we also present the results for *hsp70* normalized only to *EF-1α* for comparison (Fig. A4 in the online edition of *Physiological and Biochemical Zoology*). These two different normalization methods generated exactly the same patterns, and thus the choice of normalization approach does not affect the validity of our conclusions.

Determination of Tidepool Temperatures

In order to obtain an estimate of the thermal history of the field-acclimatized sculpins, we deployed Smartbutton temperature data loggers (ACR Systems) in three high, two mid-, and two low intertidal tidepools at Wizard Rocks, from which fish were subsequently collected for field acclimatization experiments. The loggers were deployed in July of 2003, several weeks before collection of fish, which allowed us to determine the thermal profiles of these habitats. Preliminary experiments to determine the variability in thermal microhabitats within each tidepool revealed limited thermal stratification within the pools (with temperatures varying by less than 1°C across each pool). Because of this limited stratification and the level of accuracy of the loggers, we chose to deploy only a single temperature logger in each pool, which should have provided a reasonable estimate of conditions throughout the pool and hence sculpin body temperatures. Each logger was secured to a large rock at the bottom of the tidepool. In the mid- and high tidepools, water temperatures were recorded every 20 min for 16–18 consecutive days before the collection of field-acclimatized fish. The original loggers were lost from both low tidepools, so data are available only for the 9 d subsequent to fish collection from replacement loggers deployed in these tidepools.

Low tidepool 1 was located at 48°51′51″N, 125°9′58″W and had dimensions of 2.7 m × 1.08 m × 2.31 m (maximum length × width × depth), with depth measured to the top of the barnacle zone. Low tidepool 2 was located at 48°51′46″N, 125°9′64″W and had dimensions of 2.7 m × 1.08 m × 2.31 m. Mid-tidepool 1 was located at 48°51′48″N, 125°9′63″W and had dimensions of 2.5 m × 1.06 m × 1.05 m. Mid-tidepool 2 was located at 48°51′48″N, 125°9′65″W and had dimensions of 2.4 m × 0.9 m × 1.44 m. High tidepool 1 was located at 48°51′46″N, 125°9′60″W and had dimensions of 4.4 m × 0.72 m × 1.35 m. High tidepool 2 was located at 48°51′47″N, 125°9′58″W and had dimensions of 2.38 m × 0.5 m × 1.95 m.

Mark-Recapture Experiments

Although tidepool sculpins are known to exhibit strong site fidelity and homing behavior (Green 1971; Khoo 1974; Craik 1981; Yoshiyama et al. 1992), most previous studies on site fidelity have been conducted at a single tidal height (approximately in the lower part of the mid-intertidal). Thus, it is unknown whether sculpins also home to tidepools at other tidal heights. This information is critical in order to determine whether the temperatures measured using data loggers in tidepools are a reasonable approximation of the thermal history of sculpins collected in the field. (Note that sculpins are small fish and thus cannot be effectively implanted with temperature data loggers at an individual level). We thus conducted mark-recapture experiments in June and July of 2005. The first two experiments assessed site fidelity at different temporal scales. In all mark-recapture experiments, fish were between 2.7 and 7.3 cm in length, with approximately equal numbers of males and females.

In experiment 1, which was initiated on June 14, 2005, we captured all of the fish in a single tidepool in the low intertidal zone of Wizard Rock (48°51′30″N, 125°09′45″W) near Bamfield, British Columbia. Tidepool sculpins were lightly anesthetized with MS-222 (~100 mg/L in seawater) and were then marked using a visible implant elastomer tag (Northwest Marine Technology, Shaw Island, WA), which was subcutaneously injected into the area above the right pectoral fin. To validate this methodology, we tagged 30 tidepool sculpins and housed them in the laboratory for 2 mo. All sculpins survived the tagging, and no tags were lost during this time. We also captured some of the tagged sculpins the subsequent summer at Wizard Rocks, suggesting that this tagging protocol had few negative effects on the fish. The standard length of the fish was measured, and the marked fish were returned to their pool of origin. All of the fish in this pool were captured 1, 2, and 3 wk following marking, and the number of marked and unmarked tidepool sculpins in the pool was recorded.

In experiment 2, three adjacent tidepools that spanned from the low to the high intertidal areas of Wizard Rock were used. All fish were captured from these three pools on June 22, 2005, and marked and measured as described above, except that the color and the location of the tag varied and were used to indicate the tidepool of capture. The fish were then returned to their pool of capture. All fish in these pools were recaptured 1, 2, 4, 6, and 13 d following marking, and the number of unmarked and marked tidepool sculpins of each type was recorded.

Statistics

All data were analyzed using SPSS (IBM) or Graphpad Prism 5 (Graphpad Software). Preliminary investigation of the data indicated that sex was not a significant covariate for any of the tested variables, and this factor was not included in subsequent analyses. Data from mark-recapture studies were analyzed by ANCOVA on arcsine-transformed data, with tidepool of capture as the fixed factor and date of recapture as the covariate. Tidepool temperature data were analyzed using a nonparametric Kruskal-Wallis ANOVA followed by a Dunn's multiple comparison test. Whole-organism thermal tolerance data (CT_{max}) were tested for normality and homogeneity of variance before one-way ANOVA, and all data met the assumptions of the test. Simple linear regression was used to examine the relationship between CT_{max} and acclimation temperature, and these data were then used to estimate the predicted acclimation

temperature of the field-acclimatized fish (as in Otto and Gerking 1973; Heath et al. 1993; Beitinger and Bennett 2000; Fangue and Bennett 2003). The 95% confidence limits of the regression equation were used to determine the error of the estimate of the predicted acclimation temperatures of field-acclimatized fish.

Real-time PCR data for heat-shock gene expression was first log transformed and then analyzed with two-way ANOVA, using acclimation or acclimatization group and heat-shock temperature as factors. We used a nonparametric Dunn's post test to perform preplanned multiple comparison tests of all groups against their respective controls, because some pairwise comparisons failed the assumptions for parametric tests.

Results

Whole-Organism Thermal Tolerance

Figure 1 presents the CT_{max} of laboratory-acclimated and field-acclimatized sculpins as an estimate of their whole-organism thermal tolerance. In laboratory-acclimated fish, there was a significant difference in the CT_{max} of the fish at each acclimation temperature (6°, 13°, and 20°C; $P < 0.001$). Because there were no significant differences in CT_{max} in field-acclimatized sculpins from replicate pools at the same tidal height, data from the replicate pools were combined before analysis. In field-acclimatized fish, there was a significant difference only between the CT_{max} of fish from the low intertidal pools and those of fish from the middle and upper intertidal pools. The CT_{max} of fish from the middle and high intertidal pools were similar to that of 20°C laboratory-acclimated fish.

Linear regression of CT_{max} versus laboratory acclimation temperature was highly significant (simple linear regression, $P < 0.001$, $y = 0.2249x + 26.316$, $r^2 = 0.9835$); CT_{max} increased by approximately 0.2°C for every 1°C increase in acclimation temperature. This relationship was then used to estimate the predicted acclimation temperature of field-acclimatized fish, given their observed CT_{max}. This analysis indicated that only the low tidepool fish had a significantly lower predicted acclimation temperature (15.9° ± 0.52°C) than did fish in the other tidepool locations (18.8° ± 0.65°C and 18.1° ± 0.61°C for mid- and high tidepool fish, respectively).

Heat-Shock Gene Expression

The effect of heat shock on the relative levels of gill *hsp70* mRNA is shown in Figure 2. Two-way ANOVA of relative *hsp70* mRNA levels in laboratory-acclimated fish (Fig. 2*A*) revealed significant effects of acclimation temperature ($P < 0.0001$) and heat-shock treatment ($P < 0.0001$) and a significant interaction between the two variables ($P < 0.0001$). Similarly, in field-acclimatized fish (Fig. 2*B*), two-way ANOVA of *hsp70* mRNA revealed a significant effect of tidepool height ($P = 0.0099$) and heat-shock temperature ($P < 0.0001$) and a significant interaction between the two variables ($P = 0.0355$). Relative levels of *hsp70* mRNA were not significantly higher in handled controls compared with unhandled controls in any group but increased significantly relative to the unhandled controls 2°C below the respective CT_{max} of each group. Thus, the T_{on} for *hsp70* mRNA induction was 2°C below the CT_{max} in both laboratory-acclimated and field-acclimatized fish, and the actual temperature of induction varied among groups in parallel with the changes in CT_{max}. This result was robust to different approaches to data normalization, since T_{on} was also 2°C below the CT_{max} for all groups when the data were normalized to only *EF-1α* (Fig. A4).

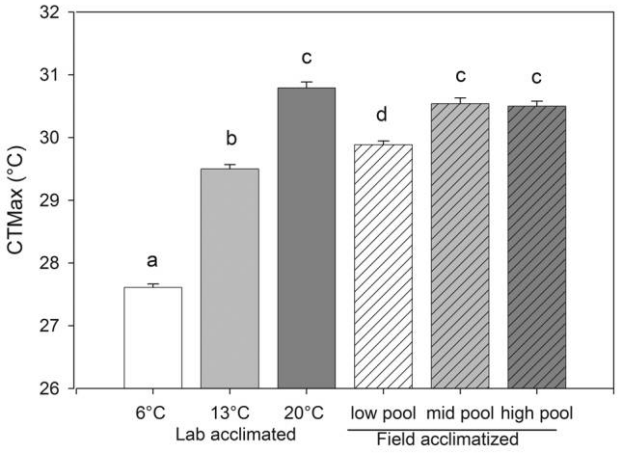

Figure 1. Critical thermal maximum (CT_{max}) as an estimate of whole-organism thermal tolerance in laboratory-acclimated and field-acclimatized tidepool sculpins (mean ± SEM). Different letters indicate groups having significantly different CT_{max} ($P < 0.001$).

In contrast to the close relationship between CT_{max} and the temperature of onset of the HSR, there was no consistent relationship between the extent of induction of *hsp70* mRNA and CT_{max}. Groups with similar CT_{max} (e.g., 20°C laboratory acclimated fish and mid- and high intertidal fish; Fig. 1) had rather different maximal levels of *hsp70* mRNA (Fig. 2; this observation was not altered by normalization method; Fig. A4). Alternatively, laboratory-acclimated sculpins with different CT_{max} had quite similar maximum levels of *hsp70* mRNA following heat shock (Fig. 2; regardless of normalization method; Fig. A4). A similar conclusion is generated by examining the data in terms of fold increase relative to control values (Fig. A5 in the online edition of *Physiological and Biochemical Zoology*).

Tidepool Temperatures

Figure 3 presents the temperature profiles of the tidepools at different intertidal heights. As expected, pools in the high intertidal underwent larger temperature fluctuations and reached higher maximal temperatures than did those in the middle and low intertidal, whereas low intertidal pools showed the least variation in temperature. Mean temperature (Table 1) differed significantly between low, mid-, and high intertidal pools ($P < 0.0001$). There were no significant differences in mean temperature between the replicate low pools or high pools, but the

two mid-intertidal pools had slightly different mean temperatures ($P < 0.001$). Average daily maximum temperature was also generally greatest in the high intertidal pools and lowest in the low intertidal, with intermediate temperatures in the mid-intertidal pools, but this measure did not distinguish the different tidal heights as clearly as did mean temperature (Table 1).

Mark-Recapture Experiments

Our first mark-recapture experiment was designed to assess site fidelity across several weeks using fish from a single low intertidal tidepool that were all marked and returned to their pool of origin (Fig. 4). A total of 25 fish were initially marked (mean size 4.7 ± 0.14 cm); 68% of these fish were recaptured in the same tidepool in week 1, 80% were recaptured in the tidepool in week 2, and 68% of these fish were recaptured in the tidepool in week 3 following marking. A number of unmarked fish entered the pool in the weeks following marking, such that at any given sampling time, an average of 52.9% of the fish in this tidepool were marked, with the remaining fish being unmarked (Fig. 4). No marked fish were ever found in nearby tidepools at any tidal height. These data suggest that many sculpins within a low intertidal tidepool remain there through multiple tidal cycles but that sculpins also leave and enter the pool—by moving back and forth into the subtidal area—in appreciable numbers.

Table 2 presents the results of our second mark-recapture experiment, which was designed to assess site fidelity in the low, mid-, and high intertidal areas and the degree of interchange between these areas. We present the data in two different ways: (1) as the percent of fish captured that were "resident" in the pool and (2) as the percent of marked fish that were recaptured. The first index is the most relevant to this study, since our goal in this experiment was to determine whether a group of tidepool sculpins captured from a single tidepool are likely to have similar thermal histories. We provide the second index in order to directly compare our results with those of previous studies (Green 1971; Khoo 1974; Craik 1981; Yoshiyama et al. 1992).

There was a statistically significant difference in percentage of resident fish between tidepools at different tidal heights ($P < 0.0001$), and day of recapture was not a significant covariate (Table 2). Site-fidelity was greatest in the low intertidal (81.1% resident), whereas sculpins in the high intertidal were less likely to remain in their pools of origin (42%–49.5% resident). In particular, recapture rate was very low in the high intertidal pool on the day immediately following marking, when only 25% of the marked animals (seven of 28) were recovered. The number of resident fish increased over time in the high intertidal, reaching slightly above 50% of the marked animals (15 of 28) at 13 d following marking.

A similar pattern was evident when the data are presented as recapture rates (Table 2). There was a statistically significant difference in recapture rates between tidepools at different tidal heights ($P = 0.0002$), and day of recapture was not a significant covariate. Percent recapture was greatest in the low intertidal (78.6% recapture) and lowest in the high intertidal (39.3% recapture). As with percent resident fish, percent recapture increased over time in the high intertidal, from 25% of marked fish on the day following marking up to 53.6% of marked fish 2 wk following marking.

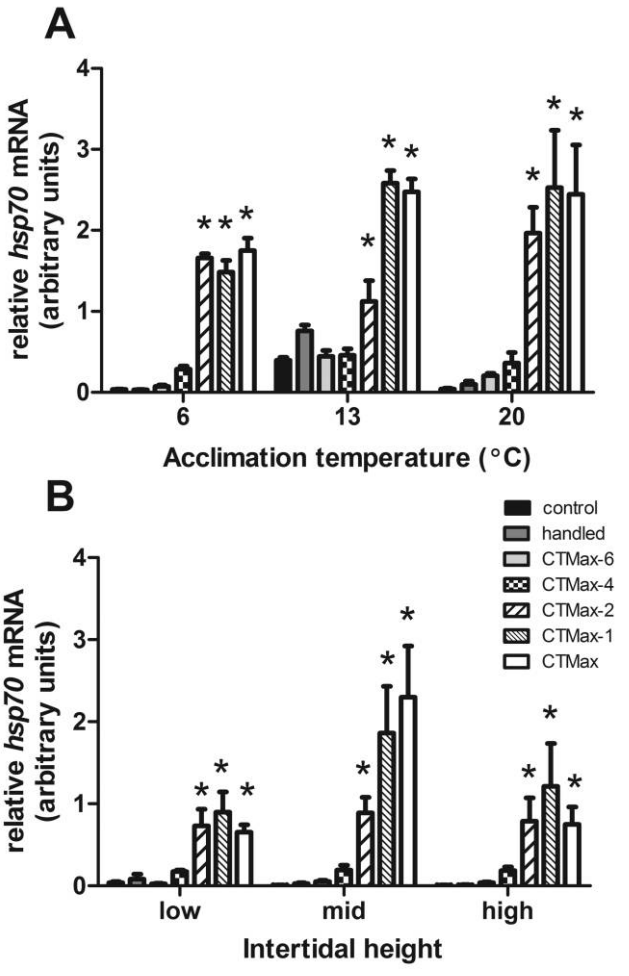

Figure 2. Relative levels of heat-shock protein 70 (*hsp70*) mRNA following heat shock in laboratory-acclimated (*A*) and field-acclimatized (*B*) tidepool sculpins exposed to the critical thermal maximum (CT_{max}) protocol and sampled at various temperatures at and below their respective CT_{max} (e.g., CT_{max}-1 is 1°C below CT_{max}, CT_{max}-2 is 2°C below CT_{max}) during exposure to a temperature-ramped CT_{max} trial. *Hsp70* mRNA data (mean ± SEM) are expressed relative to levels in a randomly chosen individual from the high intertidal that had been exposed to CT_{max}-1. To control for differences in reverse transcription efficiency, these data are also normalized to the geometric mean of the levels of the constitutively expressed genes *hsc70* and *hsp90β* and elongation factor 1a (*EF-1α*). Asterisks indicate groups that are statistically significantly different from their respective control value ($P < 0.01$).

Few fish moved from a pool at one tidal height to one of the nearby pools at the other tidal heights, and there was no movement of marked fish from the mid- or high intertidal pools into the low intertidal pool (Table 2). Substantial numbers

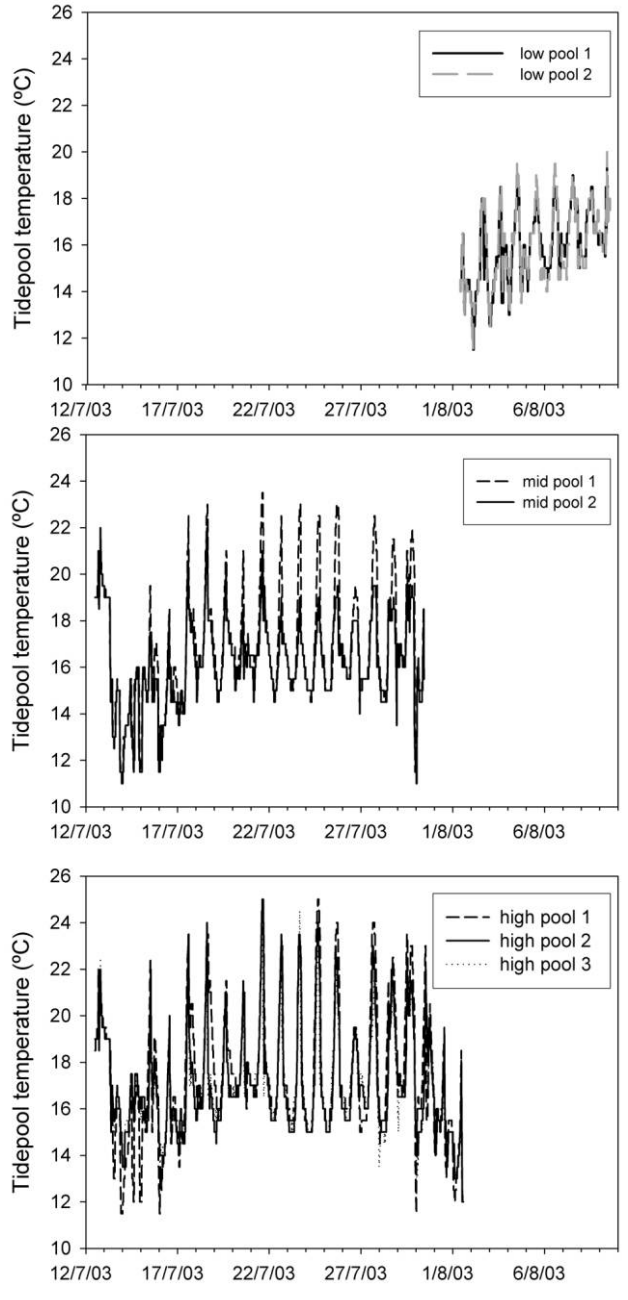

Figure 3. Tidepool temperatures in replicated low, middle, and high intertidal pools near Bamfield, British Columbia.

of unmarked fish entered the mid- and high intertidal pools, but this was much less evident in the low intertidal pool.

Discussion

Effects of Thermal History on Whole-Organism Thermal Tolerance

Consistent with a large body of work that suggests that CT_{max} is a plastic trait in many fish species, CT_{max} changed with thermal acclimation in tidepool sculpins (Fig. 1). However, the slope of the relationship between acclimation temperature and CT_{max} was very shallow compared with that of other fish species. Beitinger et al. (2000) reviewed the available data on the plasticity of CT_{max} for 20 species of fish across nine distinct families and found a mean slope of 0.41 ± 0.7. With a slope of approximately 0.2, tidepool sculpins are among the least plastic of the fish species surveyed, with only the desert pupfish (*Cyprinodon nevadensis*) being less plastic (with a slope of 0.13). This relative lack of acclimatory plasticity in CT_{max} could reflect the large daily thermal variation that is a dominant feature of the habitats of both pupfish and sculpins. Theory suggests that this type of habitat, with large variation across short temporal scales, might select for the maintenance of high resistance at all times rather than selecting for the ability to adjust CT_{max}. Similar patterns of high resistance and low plasticity of thermal tolerance have been observed in other high-intertidal species (e.g., Stillman 2002; Tomanek and Somero 2002).

In all groups but the 6°C laboratory-acclimated fish, we estimate the CT_{max} of sculpins to be between 29° and 31°C. This estimate is similar to the upper lethal temperature of this species (30°C in fish acclimated to 11°C, assessed as temperatures that were lethal within 10 min of direct transfer; Nakano and Iwama 2002). We never observed temperatures approaching CT_{max} in the natural habitat during our measurement period (Table 1), but sculpins have previously been observed in water temperatures up to 32.5°C in tidepools near Bamfield, British Columbia (Nakano and Iwama 2002), which exceeds both their measured CT_{max} and their upper lethal temperature. These observations suggest that (similar to other high intertidal organisms; see, e.g., Stillman 2002) these fish are living on the edge of their tolerance window.

Effects of Thermal History on the HSR

We found that *hsp70* mRNA exhibited a classic pattern of induction in response to heat shock and that the T_{on} of induction for *hsp70* was altered by thermal acclimation in the laboratory (Fig. 2*A*), which is consistent with the results of studies in many other organisms (reviewed in Feder and Hofmann 1999; Barua and Heckathorn 2004; for examples in fishes, see Dietz and Somero 1992; Dietz 1994; Lund et al. 2006). In contrast, laboratory thermal acclimation had no consistent effect on the maximum amount of *hsp70* mRNA produced during heat shock, with all laboratory-acclimated groups achieving similar maximum levels of *hsp70* mRNA in the gill.

We observed a T_{on} for *hsp70* mRNA induction of 27.0°–28.4°C in field-acclimatized animals. Tidepool temperatures did not reach these levels during our measurement period (Table 1). Thus, it is possible that the HSR is of limited relevance in these organisms in the field. However, Todgham et al. (2006) monitored *hsp70* mRNA levels in fish in situ in mid-intertidal tidepools during the course of a low tide period and found significant elevations of *hsp70* mRNA at midday and in the evening under conditions in which the tidepool temperatures never exceeded 22°C. The differences between these two ob-

Table 1: Summary of tidepool temperatures

Tidepool	Mean (°C)	Median (°C)	Modal (°C)	Minimum (°C)	Maximum (°C)	Average Daily Maximum (°C)	Maximum Thermal Range (°C)
Low:							
1	15.9 ± 1.6^{A}	16	15.5	11.5	20	18.4 ± .9^{A}	8.5
2	15.9 ± 1.7^{A}	15.5	15	11.5	23	19.1 ± 1.7^{AB}	11.5
Middle:							
1	16.8 ± 2.4^{C}	16.5	15	11	23.5	21.1 ± 2.0^{BC}	12.5
2	16.2 ± 1.9^{B}	16	15.5	11	22	19.3 ± 1.5^{AB}	11
High:							
1	17.4 ± 2.8^{D}	17	16	11.5	25	22.0 ± 2.2^{C}	13.5
2	17.2 ± 2.3^{D}	16.5	16.5	13.5	25	21.6 ± 2.3^{C}	11.5
3	17.1 ± 2.1^{D}	17	17	13	24.5	21.2 ± 2.1^{BC}	11.5

Note. Temperatures sharing the same letter do not differ significantly (mean ± SD).

servations could be accounted for by differences in the rate of temperature increase, differences in the time spent above a critical temperature threshold, differences in environmental stressors other than temperature, or differences in the time course of the HSR. In this study, we measured mRNA levels 1 h following the heat shock, whereas Todgham et al. (2006) measured these levels at the peak of the temperature increase. Given that mRNA responses can be very rapid (Gracey et al. 2008; Levy et al. 2011), it is possible that the protocol we utilized caused us to miss transient changes in heat-shock protein mRNA levels. There are relatively few studies of mRNA levels in intertidal organisms that provide detailed information on the time course of the response to thermal stress (although see Buckley et al. 2006; Healy et al. 2010), and this may be an important consideration for the general conclusions that can be drawn from these data. It is also possible that these timing issues are an underlying cause of the lack of relationship between thermal history and the maximal levels of *hsp70* mRNA induction that we observed.

Relationship between the HSR and Whole-Organism Thermal Tolerance

One of the most striking observations from our experiments is that the T_{on} of the HSR and CT_{max} are very closely related, regardless of acclimation or acclimatization condition, with T_{on} being approximately 2°C below the CT_{max} in all cases. This suggests that despite the possibility that these two phenomena are associated with rather different processes at the molecular level, there is some underlying relationship. Despite these similarities, however, there are also some differences in the HSRs of sculpins that have very similar thermal tolerance at the whole-animal level (as assessed using CT_{max}). Sculpins acclimated to 20°C in the laboratory had much higher resting and induced levels of *hsp70* than did fish acclimatized to the high intertidal, despite the fact that there was no difference in CT_{max} between these groups (Figs. 1, 2). Fold induction of *hsp70* mRNA also differed between these groups, but in this case, the field-acclimatized fish had a much greater fold induction of *hsp70* mRNA following heat shock than did the laboratory-acclimated animals, largely because field-acclimatized fish had very low resting levels of *hsp70* mRNA (Fig. A5). These data clearly demonstrate that there can be substantial alteration in some aspects of the HSR without alteration of whole-organism thermal tolerance.

The Thermal History of Field-Collected Sculpins

Previous studies have demonstrated that tidepool sculpins show substantial site fidelity and tend to stay within their home tidepool or within a small group of neighboring pools across multiple tidal cycles (Green 1971; Khoo 1974; Craik 1981; Yoshiyama et al. 1992). Our data are consistent with these

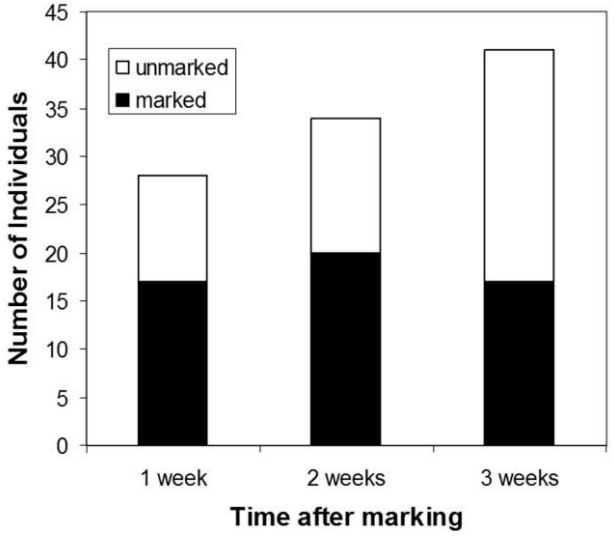

Figure 4. Mark-recapture experiment 1: number of marked and unmarked fish in a low intertidal tidepool following marking. A total of 25 fish (all of the fish in the pool at that time) were initially marked.

Table 2: Mark-recapture experiment 2: site fidelity of tidepool sculpins in the low, middle, and high intertidal zone

	Low (14)	Middle (41)			High (28)							
Days following Marking	Marked	Unmarked	Resident (%)	Recapture (%)	Marked	Unmarked	Resident (%)	Recapture (%)	Marked	Unmarked	Resident (%)	Recapture (%)
1	12 L	2	85.7	85.7	16 M, 1 L	16	48.4	39.0	7 H, 3 M	8	55.5	25.0
2	11 L	2	84.6	78.5	19 M, 1 H	14	55.9	46.3	11 H, 3 M	18	34.4	39.3
4	11 L	3	78.6	78.5	12 M, 1 H	16	41.3	29.3	9 H, 5 M	10	37.5	32.1
6	12 L	4	75	85.7	22 M, 1 H	21	50	53.7	13 H, 2 M	16	41.9	46.4
13	9 L	2	81.8	64.3	26 M, 1 H	23	52	63.4	15 H, 2 M	20	40.5	53.6
Mean			81.1	78.6			49.5	46.3			42.0	39.3

Note. Numbers in parentheses indicate the total number of fish originally marked from each pool. Fish lengths (mean ± SD): 5.36 ± 0.48 cm (low intertidal), 5.07 ± 0.99 cm (middle intertidal), and 4.96 ± 1.19 cm (high intertidal). Marked indicates the number of marked fish from a particular pool caught in low (L), middle (M), and high (H) pools at each sampling point. Unmarked indicates the number of unmarked fish captured in a particular pool at each sampling point. Resident (%) is calculated as the observed number of fish bearing the home pool mark divided by the total number of fish captured in the pool. Recapture (%) is calculated as the observed number of fish bearing the home pool mark divided by the total number of fish originally marked.

observations, falling well within the range of previous reports (see Table 2; Fig. 4; see also Table A1 in the online edition of Physiological and Biochemical Zoology). Thus, our experiments support the observations of previous studies. However, our experiments are novel in that they allow us to examine the percentage of resident fish at various intertidal heights, which is critical in order to estimate the thermal history of sculpins collected from various tidepools in nature.

Overall, few fish moved from pools at one tidal height to another (Table 2), and no marked fish from the mid or high intertidal pools colonized the low intertidal pool. However, substantial numbers of unmarked fish entered both the mid and high intertidal pools, with fewer entering the low intertidal pool (Table 2; Fig. 4). Taken together, our data suggest that a sample of fish from either the mid or high intertidal is likely to be of mixed thermal history (being composed of a roughly equal mix of resident and nonresident fish), whereas a sample of fish from the low intertidal is likely to have more uniform thermal histories, and that their thermal histories may be distinct from that of fish from other tidal heights.

Interestingly, the CT_{max} of fish from the mid- and high intertidal were more variable than the CT_{max} of most laboratory-acclimated fish or fish from the low intertidal. The standard deviations of the CT_{max} of mid- and high intertidal fish were 0.39° and 0.37°C, respectively, whereas the standard deviation of the CT_{max} of low intertidal fish was 0.19°C, which was similar to those of fish acclimated to lower temperatures in the laboratory (standard deviations of 0.17° and 0.20°C for fish acclimated to 6° and 13°C, respectively). This is potentially consistent with the more mixed thermal histories that likely characterize samples of fish taken from the mid- and high intertidal, on the basis of their low percentage residency in mark-recapture studies. Fish acclimated to 20°C in the laboratory also had relatively variable CT_{max} (this group had a standard deviation of 0.30°C for CT_{max}). A similar pattern of high variability can be observed in the induced levels of *hsp70* mRNA for 20°C laboratory-acclimated fish and for mid- and high intertidal field-acclimatized fish (Fig. 2). Thus, it is also possible that the high variability in high-temperature laboratory-acclimated and mid- and high intertidal field-acclimatized fish might be associated with the more stressful nature of their environments. In support of this hypothesis, we observed modest induction of the constitutively expressed *hsc70* and *hsp90β* in 20°C laboratory-acclimated fish (Figs. A2, A3), and we have had limited success acclimating sculpins to temperatures higher than 20°C in the laboratory (N. A. Fangue and A. E. Todgham, personal observations), which suggests that these conditions are stressful for sculpins. In addition, we have previously shown that heat-shock proteins are regularly induced in fish in mid- and high intertidal pools in nature (Todgham et al. 2006).

Despite the relatively modest plasticity in CT_{max} exhibited by laboratory-acclimated tidepool sculpins, we did observe a significant difference in the CT_{max} of field-acclimatized sculpins from the low intertidal compared with field-acclimatized sculpins from the mid- and high intertidal. The thermal profiles of low intertidal pools are substantially less variable than the thermal profiles of pools in the mid- and high intertidal. Coupled with our observation of the high percentage of resident fish within low intertidal compared with mid- and high intertidal pools, these data suggest that differences in thermal history between sculpins in the low intertidal and those in the mid- and upper intertidal may cause the observed differences in CT_{max} between fish from these different habitats.

The predicted acclimation temperature (calculated based on the relationship between acclimation temperature and CT_{max} in the laboratory; as in Otto and Gerking 1973; Heath et al. 1993; Beitinger and Bennett 2000; Fangue and Bennett 2003) of field-acclimatized tidepool sculpins from the low intertidal was extremely similar to the mean temperature of their habitat (predicted acclimation temperature 15.9° ± 0.52°C versus a mean habitat temperature of 15.9° ± 1.6°C). This similarity suggests that in the low intertidal zone, sculpins may acclimate to the mean temperature of their habitat rather than the maximum temperature or even the average daily maximum temperature. The similarity between the predicted acclimation temperature and mean habitat temperature was rather unexpected, given the fact that even in the low intertidal, temperature fluctuates on a daily basis (Fig. 3), and many other environmental pa-

rameters (e.g., salinity, oxygen, and pH) are also likely to vary, which is in marked contrast to the constant conditions of laboratory acclimation. Possibly low intertidal pools simply do not undergo environmental fluctuations that are sufficiently extreme as to induce physiological adjustments in addition to the generalized "acclimation" effect. In support of this possibility, we did not observe such a striking similarity between the predicted acclimation temperature and CT_{max} for sculpins collected from the mid- and high intertidal. The predicted acclimation temperature of mid-intertidal sculpins was 18.8° ± 0.65°C compared with a mean habitat temperature of approximately 16°C (Table 1) and 18.1° ± 0.61°C compared with a mean habitat temperature of 17.4° ± 2.8°C for fish from the high intertidal. In both cases, the predicted acclimation temperature of tidepool sculpins was higher than the mean temperature of the habitat. A deviation in this direction cannot be accounted for by the potentially mixed thermal history of fish in the mid- and high intertidal pools (which contain both residents and recent immigrants from habitats that are presumably lower in temperature). Instead, the higher CT_{max} of the fish from these tidepools is most likely to be accounted for by the effect of brief high-temperature exposure in the high intertidal (i.e., heat hardening in response to exposure to maximum daily temperatures). This observation suggests that mean habitat temperature is not the only factor affecting CT_{max} and the HSR in these more extreme habitats. These potential differences in the factors influencing the thermal tolerance of fish from the low and mid-/high intertidal zones emphasizes the challenges of making the link between whole-organism thermal tolerance, the HSR, and factors influencing the biogeographic ranges of organisms in natural environments.

Conclusions and Perspectives

The clear relationship between the T_{on} of the HSR (as measured using *hsp70* mRNA levels in the gill) and whole-organism thermal tolerance (as measured using CT_{max}) is a strong indicator that both of these traits have some relationship at the molecular level, either direct or indirect. The observation that this correlation is maintained in both the simple, controlled laboratory environment and the complex, highly variable field environment also supports the possibility of a functional basis of this relationship. These data are in strong support of previous suggestions (e.g., Tomanek 2008) that the T_{on} of the HSR may be an important indicator of the upper limits of an organism's optimal thermal habitat. However, the observed differences between the extent of HSRs of laboratory-acclimated and field-acclimatized tidepool sculpins with similar whole-organism thermal tolerance emphasize the need for caution in extrapolating the results of laboratory studies to their likely effects under natural conditions, particularly when T_{on} is not measured. Considering the plasticity of both whole-organism thermal tolerance and the HSR will be particularly critical when attempts are made to connect descriptions of the HSR based on laboratory studies to ideas about the factors specifying the biogeographic ranges of organisms or the likely effects of global climate change on species distributions.

Acknowledgments

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