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The Adaptive Cline at LDH (Lactate Dehydrogenase) in Killifish *Fundulus heteroclitus* Remains Stationary After 40 Years of Warming Estuaries

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Abstract

Since the 1970s, water temperatures along the Atlantic seaboard of the United States have risen by an average of 0.5 °C in summer months and 2.2 °C in winter months. In response, the distribution and abundance of several nearshore species have changed dramatically, but no study has attempted to document whether estuarine populations have evolved greater thermal tolerance. Here, we re-examine the classic latitudinal cline at lactate dehydrogenase (LDH) in the killifish *Fundulus heteroclitus* that was originally described by Dennis Powers and associates from samples collected between 1970 and 1972. Laboratory and field evidences indicated that northern and southern isozymes at muscle LDH are locally adapted to cold and warm temperatures, respectively. Despite the potential for evolutionary response at this adaptive locus, we detected no significant shift of the LDH cline from 20 to 30 *F. heteroclitus* collected at each of 13 locations between the early 1970s and 2010. We conclude that the microevolution of LDH-mediated thermal tolerance has not occurred, that shifts in alleles are too incremental to be distinguished from random processes, or that *F. heteroclitus* uses phenotypic and genetic mechanisms besides LDH to respond to warmer waters.

Subject areas: *Molecular adaptation and selection; Population structure and phylogeography*

Key words: *fish, genetic adaptation, marine population, microevolutionary response to climate change*

Poleward expansions or range contractions as a result of warming temperatures are common to terrestrial and marine habitats worldwide (Hughes et al. 2003; Hays et al. 2005; Perry et al. 2005; Hickling et al. 2006; Parmesan 2006; Jones et al. 2010). Such dramatic responses to warming arise when species live near the limits of their thermal tolerance (Hoegh-Guldberg 1999; Tomanek and Somero 1999; Deutsch et al. 2008; Somero 2010). Other species show more subtle or no demographic response to rapidly changing environments (Hoffmann and Willi 2008; Booth et al. 2011), and instead rely upon phenotypic response or genetic adaptation (Helmuth et al. 2006; Hoffmann and Sgro 2011). Forecasting ecosystem and community responses to a future warming environment is limited in part by our current inability to predict demographic and microevolutionary responses of most species (Helmuth et al. 2006).

Because of the fundamental effects of temperature upon physiology and biotic interactions, rapidly warming temperatures should represent a type of environmental change that results in adaptive response. Such evolutionary responses are rarely detected, however; whether this is because populations rarely adapt or because of the greater difficulty to detect changes in genetic composition remains an open question (Hoffmann and Sgro 2011). Time series of allele frequencies at loci that are under thermal selection, or strongly linked to those loci, offer an opportunity to detect microevolutionary responses, but identifying these loci and alleles is nontrivial (Strand et al. 2012). As an example, alleles at alcohol dehydrogenase in *Drosophila melanogaster* (Kreitman and Hudson 1991) vary with latitudinal changes in environmental temperature across multiple continents and have recently shifted with recent climate change (Oakeshott et al. 1982; Umina et al. 2005).

A similar clinal co-variation has been observed in *Fundulus heteroclitus*, the Atlantic Killifish, a shallow-water estuarine fish along the northwestern Atlantic coastline. This fish inhabits

subtidal estuarine creeks and has limited dispersal during its adult or juvenile life (Duvernell et al. 2008; Able et al. 2012). Estuarine habitat along the Atlantic coast of the United States exhibits a dramatic transition in mean sea surface temperature from about 10 °C in the north to about 23°C in the south. *Fundulus heteroclitus* inhabits this transitional zone, and its ability to adapt to these temperature fluctuations has been suggested to be in part the result of variation in the metabolic enzyme lactate dehydrogenase (LDH) expressed principally in muscle tissue. LDH in *Fundulus* has been one of the most well-studied latitudinal clines in evolutionary biology and has been cited to have temperature-dependent kinetic characteristics that associate with differences in swimming performance, metabolism, developmental rates, as well as survivorship at high temperatures (Powers et al. 1991).

The gene that codes for LDH has 2 allozyme alleles distinguished by a single nucleotide polymorphism (SNP, Powell et al. 1992). These allozyme alleles vary in frequency in a clinal fashion along the coast, where populations of *F. heteroclitus* in Maine are fixed for 1 allele, whereas those in Georgia are fixed for the other allele (Powers and Place 1978). A field survey of LDH alleles in Long Island indicated higher southern allele frequencies in populations near a warm water outflow from a nuclear power plant (Mitton 1973). Although these studies point to selection as a major driver of the LDH cline in *Fundulus* (Brown and Chapman 1991; Powers et al. 1991; Crawford and Powers 1992), it remains a possibility that neutral fixation of LDH and historical population differentiation played a role in the observed clinal pattern (Hilbish 1996; Strand et al. 2012).

Powers and Place (1978) originally described the LDH cline in *F. heteroclitus* from samples collected in 1970–1972. Between 1970–1972 and 2008–2009, estuarine water temperatures have risen an average of 0.6 °C and 2.2 °C during summer and winter months, respectively (Figure 1; see also Maul et al. 2001; Nixon et al. 2004; Allen et al. 2008), and the distribution and abundance of several nearshore species have shifted northward (e.g., Jones et al. 2009, 2010; Nye et al. 2009). Given the strong evidence that LDH plays a role in thermal tolerance, we re-examined the LDH cline to test for microevolutionary response to warming estuaries. Although the original 1970–1972 survey utilized allozyme technology, we estimated allele frequencies of 2010 fishes at a SNP that determines electrophoretic differences at LDH (see Powell et al. 1992). We predicted that a range-wide response to increasing estuarine temperatures would shift the midpoint of the historical cline toward northern latitudes.

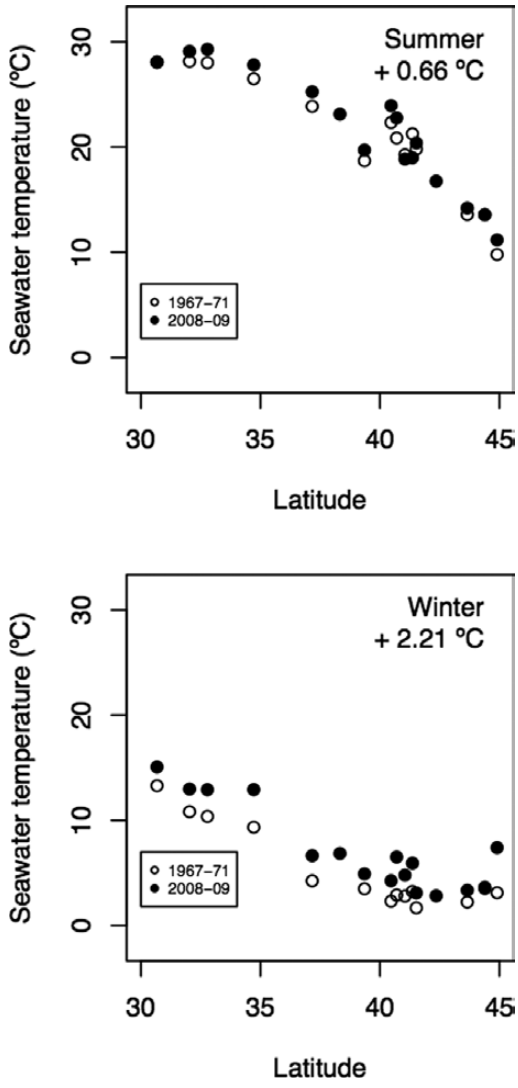

Figure 1. Average daily temperatures throughout the year from 13 tide stations along the East Coast, ranging from Beaufort, NC to Eastport, ME in early 1970s to late 2000s (2006–2009). Data available from NOAA's National Oceanic Service Center for Operational Oceanographic Products and Services at http://tidesandcurrents.noaa.gov

Methods

Sample Collection

We collected 20–30 *F. heteroclitus* from 13 locations along a latitudinal transect along East Coast of the United States in June 2010. Collection sites were loosely selected based on accessibility and as described by Powers and Place (1978). About 20–30 *F. heteroclitus* were collected using small aluminum minnow traps cast out at low tide and retrieved several hours past high tide. We anesthetized specimens in MS-222 and then preserved them in 95% ethanol. These preserved specimens were brought to Grice Marine Laboratory at the College of Charleston for molecular analysis.

Molecular Analyses

In order to describe latitudinal patterns in allele frequency, we isolated DNA using Qiagen DNeasy Kits. Using an alignment of GenBank sequences for a complementary DNA transcript of *Fundulus* LDH and sequence information from Powell et al. 1992, we designed custom primers and Blackhole Quencher SNP Probes (Biosearch Technologies, Novato, CA). Each set of bi-allelic SNP probes has its own dye color, and fluorescence of that color

is emitted only when a copy of that probe is incorporated during PCR. Probe and primer sequences were custom generated: forward primer (TCGGCAGCGTGGTGAAC); reverse primer (GGTGTCGGCGCTCTTCTT); probe-1 (FAM-ATGACCTGACGGACG); and probe-2 (CAL-Fluor Orange 560-ACCCTGACGGCCG). Following PCR on the ABI PRISM 7500 Fast Real-Time PCR System, an endplate reading of fluorescence allowed for assignment of individual genotypes. Each PCR reaction consisted of a 20-μL mixture of the following reagents: 0.25 μM of each primer, 0.25 μM of Biosearch SNP Genotyping Probe, 4 μL of Promega GoTaq 5× PCR buffer, 1 μL of 10× bovine serum albumin, 1 μL of dNTPs, and double-distilled H_20. Separate multiplex PCR reactions (involving 1 set of primers and 2 probes, 1 for each allele) were performed for each locus. PCR amplification occurred with 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Each set of 96 PCR reactions was run with 8 no template controls and 8 positive controls. Positive controls were obtained through cloning (Invitrogen-TOPO TA Cloning Kit) and sequencing of 8 individuals (6 homozygotes and 2 heterozygotes) from 3 populations (Portland, ME; Cape May, NJ; and Charleston, SC). Each sample was screened in 3 independent reactions to insure consistent genotype assignment. We determined the allelic composition of each sample using the allelic discrimination platform of the ABI PRISM Sequence Detection System Software. Analyses of deviations between frequency distributions of the allozyme SNP were performed using a G-goodness-of-fit test (with a Bonferroni correction: $\alpha = 0.004$). Deviations from Hardy–Weinberg equilibrium were assessed using Fisher's Exact test, and all populations (1970s and 2010) were found in equilibrium.

Cline Analysis

We fit generalized logistic functions to the frequencies of the "southern allele" identified by Powers and Place (1978) over the latitudinal cline. First, we fit 4-parameter models that estimated allele frequencies at each end of the cline as well as cline midpoint and width. We determined that 3-parameter models that fixed the allele frequencies at the northern end of the cline to 0 fit as well as the 4-parameter model using likelihood ratio tests (G = 1.38, degrees of freedom [*df*] = 1, $P = 0.24$ and G = 2.28, $df = 1$, $P = 0.13$ for 1978 and 2010 data, respectively). Therefore, the 3-parameter logistic function was used for all subsequent analyses of cline shape. All fits were performed in R 3.0.2 (R Development Core Team, 2013) using simulated annealing implemented in the function "optim."

Simulation Study

We estimated the range of possible 2010 allele frequencies resulting from genetic drift and sampling of finite numbers of individuals using an individual-based simulation implemented in Rmetasim (Strand 2002; Strand and Niehaus 2007). These simulations implemented a discrete-time approximation to the Wright–Fisher model (Fisher 1930; Wright 1931) with a single locus segregating for 2 alleles. To set up the simulations, we first estimated Ne from the change in allele frequencies between the 2 time points examined in this study using the temporal change approach (Waples 1989), using NeEstimator v2 (Do et al. 2014). Effective size estimates ranged from 230 (95% confidence interval [CI] 0.2–1156) to 1060 (95% CI 1.1–5279) across the 13 populations. These CIs are wide due to the use of a single gene locus in our estimates of Ne. Four sets of simulations were run, 2 sets with each population size set to 230 and 2 with populations sizes set to 1060. Within each set of effective sizes, we set initial conditions by either 1) taking the point estimates of allele frequencies for each population determined by Powers and Place (1978) or 2) randomly selecting allele frequencies for each population from a uniform distribution with minimum and maximum equal to the lower and upper 95% CIs for allele frequencies estimated by Powers and Place (1978). This design resulted in simulations that bracketed effects of drift and sampling uncertainty on allele frequencies from least conservative (low Ne and initial conditions including sampling error) to most conservative (high Ne and initial conditions based upon point estimates alone).

Each simulation was run for 35 years (corresponding to 35 generations in a Wright–Fisher model), and 1000 replicate simulations were conducted for each choice of Ne. At the end of each simulation, we randomly sampled 30 individuals (60 alleles) from each simulated population. We then calculated the 2.5 and 97.5 percentiles of allele frequencies across simulations for each of the original populations surveyed by Powers and Place (1978). In addition, we estimated cline midpoint and width from each simulated data set using the same approach outlined previously. In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

Results and Discussion

Allele frequencies between the 2010 and 1978 collections were indistinguishable for 12 of the 13 sites (Figure 2, Table 1). The exception was at Portland, Maine, where the 1970s samples were exclusively LDH_N, whereas the 2010 samples were ~7% LDH_S ($P < 0.003$). The midpoints and cline widths estimated for 1978 were 40.7°N and 1.15°; the estimates for the 2010 data were 40.8°N and 0.9°. There was no support, however, for differences between these cline shapes. A likelihood ratio test comparing functions fit to each year compared with an overall fit was not significant (G = 1.68, $df = 3$, $P = 0.64$). The similarity of these fits is apparent in Figure 2 (see inset). A comparison of empirical data with a simulation study indicated that differences in allele frequency between temporal samples could be easily explained by sampling error and genetic drift in the intervening 35 generations (Figure 2). For example, simulations that produced the least amount of stochastic variation (dark shaded region in Figure 2) resulted in a range between the lower 2.5% and upper 97.5% of cline midpoints and widths of 39.7–41.45 and 0.81–1.5, respectively. Thus, if directional selection operates at this LDH SNP, its effects are swamped by even the least extreme of the stochastic effects simulated in this paper.

The sedentary LDH cline was somewhat surprising given that water temperatures have increased substantially in these estuaries (Figure 1), and the ranges of several species dramatically expanded or contracted during this same timespan (Nye et al. 2009). We propose at least 3 possible explanations for the LDH stasis. First, increases in water temperature were principally during the winter months (Figure 1), and it is possible that thermal selection on this portion of the *Fundulus* life cycle is not strong. With dispersal distances averaging 36 m over a 90-day period, a preference for subtidal marshy creeks, and a strong local retention of larvae, *F. heteroclitus* has aptly been described as one of the most stationary of estuarine fishes (Bigelow and Schroeder 1953; Lotrich 1975). Despite this, selection on LDH is not apparently strong enough to shift its cline along the East Coast of the United States.

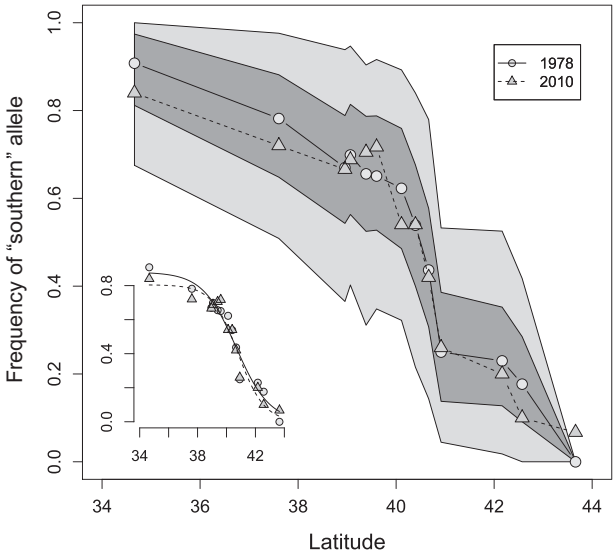

Figure 2. Simulation results and analysis of cline shape. Individual points correspond to point estimates of allele frequencies at each population sampled by Powers and Place (1978; circles) and this study (triangles). The shaded regions correspond to the central 95% of allele frequencies at all populations after 35 generations of drift. The lighter, outer region is the result of 1000 simulations with Ne = 230 for each population and initial allele frequencies chosen while including sampling error (see Methods for details). The darker, inner region corresponds to 1000 simulations with Ne = 1060 and assumes that the initial allele frequencies were identical to the point estimates produced by Powers and Place (1978). The inset shows the results of fitting logistic functions to the same data in the larger figure.

Second, LDH may not be as sensitive to temperature as in vitro empirical data suggest. In the most conclusive field test of the fitness of LDH alleles, Mitton and Koehn (1975) showed that *F. heteroclitus* from localities warmed by effluent from power plants experienced a 12 °C increase in temperature from the mean for that area, but that the LDH_S increased only slightly in comparison to cooler sites nearby (i.e., frequencies of 0.26 and 0.20, respectively; Mitton 1973). Thus, despite the long list of molecular, cellular, and organismal experiments that indicate that LDH should be under thermal selection (Mitton and Koehn 1975; Brown and Chapman 1991; DiMichele and Powers 1991; DiMichele et al. 1991; Powers et al. 1991; Schulte 2001), it remains possible that LDH alleles are functionally equivalent across the range of temperatures tested here and in Mitton and Koehn (1975). One piece of evidence supporting this possibility is that expression of the LDH gene also differs between northern and southern latitudes (Crawford and Powers 1992) and may serve as a buffer to selection directly upon the protein itself.

Of course, lack of change of LDH allele frequencies does not rule out evolutionary response to temperature. For example, the differences in gene expression across latitude could have an adaptive origin or selection could be targeting a completely different enzyme system. Malate dehydrogenase (MDH), another locus that exhibited latitudinal changes in frequency in Powers and Place (1978), may represent such an enzyme. Indeed, in contrast to their results with LDH, Mitton

Table 1 The frequency of LDH isozymes in *Fundulus heteroclitus* along the East Coast of the United States

		1970–1972		2010	
Site	Latitude	Southern allele frequency	Number of individuals	Southern allele frequency	Number of individuals
1. Portland, ME	43.661	0.000	58	0.067	30
2. Manchester, MA	42.572	0.177	65	0.100	30
3. Marshfield, MA	42.163	0.230	82	0.200	25
4. Stony Brook, NY	40.918	0.250	60	0.260	25
5. Captree, NY	40.664	0.437	48	0.420	25
6. Atlantic Highlands, NJ	40.394	0.539	76	0.540	25
7. Manasquan, NJ	40.11	0.623	75	0.540	25
8. Tuckerton, NJ	39.601	0.651	48	0.717	30
9. Atlantic City, NJ	39.387	0.656	48	0.705	22
10. Stone Harbor, NJ	39.067	0.699	73	0.688	24
11. Cape May, NJ	38.956	0.670	47	0.666	24
12. Wachapreague, VA	37.607	0.782	174	0.720	25
13. Charleston, SC	34.661	0.908	87	0.840	25

and Koehn (1975) found that killifish populations next to power plants showed dramatic shift in MDH_S frequency (0.27 vs. 0.05 in warm vs. cool water sites, respectively).

Finally, it is possible that *F. heteroclitus*, as predicted by its highly eurythermal tolerance and an extremely broad geographic extent, is able to tolerate these thermal changes through alteration in behavior or other phenotypic means. Genetic adaptation is less likely when individuals themselves have a thermal tolerance that is extremely broad. We conclude that despite the numerous factors suggesting that would be an obvious target for selection, demonstrating evolutionary response to environmental change requires skeptical examination.

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