



Local adaptation to osmotic environment in killifish, *Fundulus heteroclitus*, is supported by divergence in swimming performance but not by differences in excess post-exercise oxygen consumption or aerobic scope

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ABSTRACT

Regulation of internal ion homeostasis is essential for fishes inhabiting environments where salinities differ from their internal concentrations. It is hypothesized that selection will reduce energetic costs of osmoregulation in a population's native osmotic habitat, producing patterns of local adaptation. Killifish, *Fundulus heteroclitus*, occupy estuarine habitats where salinities range from fresh to seawater. Populations inhabiting an environmental salinity gradient differ in physiological traits associated with acclimation to acute salinity stress, consistent with local adaptation. Similarly, metabolic rates differ in populations adapted to different temperatures, but have not been studied in regard to salinity. We investigated evidence for local adaptation between populations of killifish native to fresh and brackish water habitats. Aerobic scope (the difference between minimum and maximum metabolic rates), excess post-exercise oxygen consumption, and swimming performance (time and distance to reach exhaustion) were used as proxies for fitness in fresh and brackish water treatments. Swimming performance results supported local adaptation; fish native to brackish water habitats performed significantly better than freshwater-native fish at high salinity while low salinity performance was similar between populations. However, results from metabolic measures did not support this conclusion; both populations showed an increase in resting metabolic rate and a decrease of aerobic scope in fresh water. Similarly, excess post-exercise oxygen consumption was higher for both populations in fresh than in brackish water. While swimming results suggest that environmentally dependent performance differences may be a result of selection in divergent osmotic environments, the differences between populations are not coupled with divergence in metabolic performance.

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1. Introduction

Salinity is one of the main factors limiting species distributions in aquatic organisms and most large clades of fishes are restricted to either marine or freshwater environments (Kolar and Lodge, 2002; Lee and Bell, 1999; Nelson, 2006; Vega and Wiens, 2012; Whitehead et al., 2011). However, some groups of fishes can tolerate broad ranges of salinity and have repeatedly diversified across osmotic boundaries (Dunson and Travis, 1991; Plaut, 1998; Whitehead, 2010). It is hypothesized that species possessing plastic, euryhaline phenotypes are those most able to invade novel osmotic niches (Bamber and Henderson, 1988). For fishes, this suggests that estuarine and

diadromous species are those most likely to invade and diversify into different osmotic habitats (Bamber and Henderson, 1988; Schultz and McCormick, 2013).

Species within the genus *Fundulus* harbor an ancestral marine physiology but occupy the continuum of osmotic niches following multiple independent freshwater invasions (Whitehead, 2010). *Fundulus heteroclitus* is particularly flexible in its osmotic physiology and occurs in fresh, brackish, and marine habitats (Hildebrand and Schroeder, 1928). The species is distributed continuously, given appropriate marsh habitat, from coastal marine sites, through estuaries, and into freshwater habitats. Along a salinity gradient within the Chesapeake Bay watershed a steep genetic discontinuity separates upstream freshwater populations from their downstream brackish counterparts (Whitehead et al., 2011). Transcriptomic and physiological data collected during acute salinity challenges have revealed differences between populations where physiological plasticity has expanded in the freshwater populations to accommodate low salinities

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(Whitehead et al., 2011) but is coupled with a reduced ability to tolerate higher salinity challenges (Brennan et al., 2015), consistent with local adaptation.

Patterns of local adaptation take many forms (Blanquart et al., 2013; Kawecki and Ebert, 2004) and how local adaptation is defined can influence the ability to detect its signal. Local adaptation can be identified when local populations show higher fitness in their native habitat relative to populations from other (foreign) habitats (Kawecki and Ebert, 2004). This definition, known as the “local vs. foreign” criterion, results in the classic fitness-by-environment interaction pattern seen in Fig. 1A and B. It is important to note that absolute fitness does not need to be highest in a population's local habitat to detect local adaptation (Fig. 1B) as long as the relative difference between populations within a habitat fits the criterion (i.e., Anacker, 2014; Brady, 2012). Conversely, in low quality habitats with no evidence for local adaptation parallel losses of fitness would be expected for all populations (Fig. 1C). In the case of *F. heteroclitus*, because the freshwater phenotype is derived from a saltwater ancestor, it is not unexpected for absolute fitness to be lower in fresh water than in brackish while still being locally adapted.

In fresh water (hypo-osmotic conditions) fish must expend energy to maintain osmotic homeostasis in the face of loss of ions through diffusion and passive uptake of water through osmosis. Conversely, in higher salinities (hyper-osmotic conditions) ions must be actively excreted and water retained as ions are passively gained while water is lost (Evans and Claiborne, 2006; Hwang et al., 2011). Therefore, immersion within an osmotic environment that differs from the internal osmotic state of a fish requires greater expenditure of energy to maintain internal homeostasis as compared to environments of similar osmolarities as the fish's internal state (Tseng and Hwang, 2008). Theory predicts, given appropriate conditions of limited gene flow and sufficient population size, natural selection will maximize the fitness of a population in its native environment relative to foreign populations (Fig. 1A, B; Kawecki and Ebert, 2004). Thus, we predict that natural selection should modify killifish osmoregulatory abilities to match a population's native salinity such that energy usage to maintain osmotic homeostasis, measured as an individual's resting metabolic rate, will be lower for native as compared to non-native populations at this salinity (Boeuf and Payan, 2001; Tseng and Hwang, 2008).

The energy required to maintain homeostasis, including both aerobic and anaerobic capabilities, can negatively affect whole organism performance and can be estimated based on metabolic capacity. One common method to estimate aerobic capacity is aerobic scope (AS), which is the difference between resting (RMR) and maximum (MMR) metabolic rates (Clark et al., 2013). Aerobic scope reflects whole organism performance and reductions in AS constrain functions such as growth, reproduction, and muscular activity (Pörtner and Knust,

2007). Therefore, individuals with low RMR and high MMR possess large AS and are presumably most fit. Additionally, the anaerobic contributions to activity can be assessed by measuring the total amount of oxygen required for recovery of an individual following exhaustive exercise, known as excess post-exercise oxygen consumption (EPOC) or oxygen debt (Hill and Lupton, 1923; Scarabello et al., 1991). During strenuous exercise, muscle requires more energy than can be supplied by aerobic respiration alone and anaerobic metabolism is employed, which results in the depletion of phosphocreatine, ATP, and glycogen stores, the buildup of lactic acid, and changes in acid–base balance (Milligan, 1996; Scarabello et al., 1991). Following exercise, an individual must clear metabolic waste products and lactic acid, replenish energy and oxygen stores, and recover acid–base balance, resulting in elevated oxygen consumption that can result in metabolic constraints and reduced fitness (Lee, 2003; Milligan, 1996; Svendsen et al., 2010). The rate at which oxygen use is restored to resting levels reflects the ability of an individual to recover from activity and lower EPOC values represent increased performance (Svendsen et al., 2010).

While there is much evidence for biochemical and molecular divergence between populations of *F. heteroclitus* native to different environments, less is known about how these differences may manifest at the whole organism level (Brennan et al., 2015; Scott et al., 2004; Scott and Schulte 2005; Whitehead et al., 2011). Here, we use metabolic measures and swim performance as proxies for whole organism performance and fitness to explore the nature of local adaptation between populations and provide insight into the mechanisms that may restrict gene flow and maintain distinct populations of *F. heteroclitus* that are locally adapted to alternate osmotic environments. This whole organism approach builds on previous biochemical and molecular work to provide a biologically relevant summary of performance at varying salinities. We conducted chase trials at different salinities to compare the time and distance required for FW-native and BW-native populations of *F. heteroclitus* to reach exhaustion. Swim ability is an important performance indicator that is relevant for animal fitness as it influences the ability to find mates, obtain food, and avoid predators (Plaut 2001). We measured oxygen consumption and EPOC to investigate how metabolic factors may influence swim performance and overall fitness. We hypothesized that RMR would be lower for native (i.e., FW-native in fresh water) as compared to foreign populations (i.e., FW-native in brackish water) at a given salinity because costs associated with osmoregulation would be higher for non-native individuals. We also predicted that swim performance would be different between populations within a salinity where native individuals outperformed foreign individuals. Because active metabolic rate and aerobic scope typically correlate with swim performance, we predicted that MMR

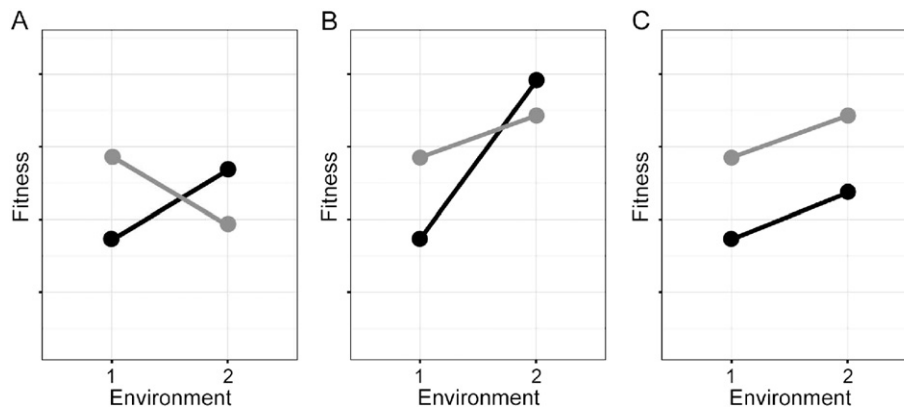


Fig. 1. Hypothetical patterns of fitness of two populations acclimated to two different environmental conditions. Gray points represent the population sampled from environment 1 while black is the population from environment two. (A) A typical situation of local adaptation where each local population is most fit in its home environment as compared to the away environment. Within each environment, the local population is more fit than the foreign population. (B) An alternative example of local adaptation where local populations are more fit than foreign populations within an environment. However, both populations are more fit in environment 2 than in environment 1. (C) A scenario where local adaptation is not present and both populations show an increase in fitness in environment 2. The gray population is more fit across both environments. Figure adapted from Kawecki and Ebert 2004.

and AS would be higher for the native than the foreign population at a given salinity. Finally, it was predicted that EPOC would be lower for the native population following exhaustive exercise as these individuals would be able to recover most efficiently at their home salinity.

2. Methods

In July 2013 adult killifish, *F. heteroclitus*, were collected from populations native to fresh water (FW-native) and to brackish water (BW-native). FW-native fish were sampled from the Potomac River at Piscataway Park, near Accokeek, Maryland (38°41'42.18"N, 77°3'10.38"W), where salinity has not exceeded 0.12 ppt since it was first monitored in 1986 (chesapeakebay.net). Salinity less than 0.5 ppt is considered dilute fresh water (Cowardin et al., 1979). BW-native fish were collected from the Chesapeake Bay at Point Lookout State Park, near Scotland, Maryland (38°3'10.90"N, 76°19'34.38"W), where the 25 year average salinity is 11.8 ppt (approximately 1/3 the salinity of marine waters) with annual variation ranging from 5.9 to 17.6 ppt. Fish were held at the UC Davis Center for Aquatic Biology and Aquaculture in static tanks equipped with biological filtration at a density of less than one fish per gallon. Each population (BW-native and FW-native) was split into two tanks (to account for tank effects) at each of two salinity treatments: fresh water (0.3 ppt) and brackish water (15 ppt). All individuals were allowed to acclimate to their treatment salinity for a minimum of 3 weeks at 21 °C before the start of data collection. To maintain fish in a non-reproductive state, light cycle was 10 h light and 14 h dark. Untreated well water was used for fresh water while brackish water consisted of well water mixed with Instant Ocean Aquarium Salt. Ammonia, nitrite, and nitrate waste were monitored regularly with commercial test kits (API, Mars Fishcare Inc., Chalfont, PA) and water changes were performed as necessary.

At each salinity, oxygen consumption was measured using custom 250 ml respirometers placed in a circulating water bath. Water temperature varied between 19.1 and 20.2 °C. Water was continuously aerated and each respirometer was held in a 10 gal tank within the bath with blacked out sides and a partial lid to minimize disturbance and limit activity (Cech, 1990). A Loligo 4-channel oxygen regulator system with galvanic oxygen probes (Loligo Systems, Tjele, Denmark) was used to measure oxygen consumption. Oxygen probes were calibrated prior to each set of fish following the manufacturer's instructions: oxygen free water was achieved using a 0.16 M sodium sulfite solution while saturated water was established by vigorously bubbling the sample water with atmospheric air. To minimize background respiration, respirometers were cleaned twice weekly with 90% ethanol. With this system, four fish could be measured simultaneously and two fish from each population were randomly assigned to each chamber. Three different metabolic rates (MR) were collected for each fish: resting (RMR), maximum (MMR), and recovery. For RMR, fish were fasted for 24 h before introduction into each respirometer between 5:00 and 6:00 PM. Oxygen concentrations were measured once per second. Resting data were collected overnight for approximately 14 h using intermittent flow respirometry with repeated periods of 10 min measure, 15 min flush, and 1 min wait, resulting in 32 measure periods per individual. Preliminary measures over 24 h indicated that this time frame was sufficient to achieve minimum oxygen consumption. MR dropped to minimum values within approximately 9 h and did not decrease further. The following morning, each fish was individually chased to exhaustion with a gloved hand in a 54.6 cm diameter tub with a 20.3 cm center barrier, consistent with established methods (Hardewig et al., 1998; Healy and Schulte, 2012; Reidy et al., 1995; Soofiani and Priede, 1985; Sylvestre et al., 2007). Distance to exhaustion, defined as the number of complete laps swum by each fish, as well as time to exhaustion, were recorded. Given the dimensions of the chase tub, one complete lap consisted of a fish traveling a maximum distance of 171.5 cm, depending on the path chosen within the tub. Exhaustion was characterized by a lack of response to a gentle pinch to the tail and a delayed

righting response upon inversion (Healy and Schulte, 2012). Immediately following exhaustion, the fish was placed back into the respirometer to measure post-chase oxygen consumption (MMR). Data were collected for 10 min or until oxygen levels dropped to 80% saturation. Previous studies show that manual chase protocols result in similar or greater estimates of MMR compared to measures based on maximum sustained swimming (U_{crit}) and are typically representative of maximum oxygen consumption (Soofiani and Priede, 1985; Sylvestre et al., 2007). Following the chase of all four fish, recovery MR was assessed over the next 20 h using the same approach as with RMR. Mass (g) and total length (cm) were then measured and each fish was placed into a recovery tank.

For all MR measures, MR ($\mu\text{mol/h}$) was calculated using the slope of the decrease in O_2 during each measure interval. Data were checked to ensure that oxygen consumption was consistent through time with an R-squared value of each measure interval above 0.95, indicating no or minimal spontaneous activity during the measure period. RMR values were determined by fitting a double Gaussian distribution to the frequency distribution of MR values using the function `normalmixEM` in the R package 'mixtools' (Benaglia et al., 2009). This method fits two Gaussian distributions to the data, with the lower distribution representing the minimum MR, and has been applied to fishes in previous studies (Herskin, 1999; Steffensen et al., 1994; Svendsen et al., 2012). Because RMR measures can be artificially inflated due to handling stress when introducing fish into respirometers as well as spontaneous activity during the measuring period, this method addresses inaccurately elevated MR by excluding those elevated measures from RMR calculations. We also calculated RMR using the lowest 15% of measurements for each individual (Clark et al., 2013). While this method does result in slightly lower absolute resting values, relative differences did not change and the significance of downstream analyses was the same between both methods. Therefore, we only include analyses using RMR values obtained from fitting the double Gaussian distribution.

To calculate MMR, the first 30 s of post chase measures were discarded to allow for oxygen probe stabilization. In six cases where 30 s was not sufficient to allow for probe stabilization, the amount of data discarded was extended to between 31 and 42 s, depending on the individual. Extending this time period did not significantly alter the mean MMR relative to individuals without extended time (t -test, $p = 0.27$). The 90 s following the stabilization period were then used to calculate MMR. Aerobic scope (AS) was then determined as the difference between MMR and RMR and factorial aerobic scope (FAS) was calculated as the quotient of MMR and RMR. Finally, recovery MR was assessed for each interval following the same method as RMR.

EPOC uses the difference between MR following exercise and RMR to quantify the energy required to clear oxygen debt. Time to recovery was determined as the point where two consecutive RMR values fell below the upper 95% confidence interval of resting MR (Svendsen et al., 2012). Individuals that did not recover within 20 h were assigned a recovery time of 20 h, which is equal to the minimum recovery time collected for all individuals. This approach is conservative in that it minimizes differences by limiting the upper values obtained for EPOC. Following Svendsen et al. (2012), the subsequent model was fit to the data for each individual to determine the relationship between MR and time:

$$Y = ae^{k_1 t} be^{k_2 t} + c,$$

where e is the base of the natural logarithm, t is time in hours, c represents the RMR value for each individual, and a , b , k_1 , and k_2 , are constants estimated using non-linear regression implemented using the R package 'nls2' (Grothendieck, 2007). MMR was included in the model at $t = 0$. Each individual recovery model was then integrated to obtain the area beneath the curve until the calculated point of recovery using the R package 'AUC' (Ballings and Van den Poel, 2013). The area due

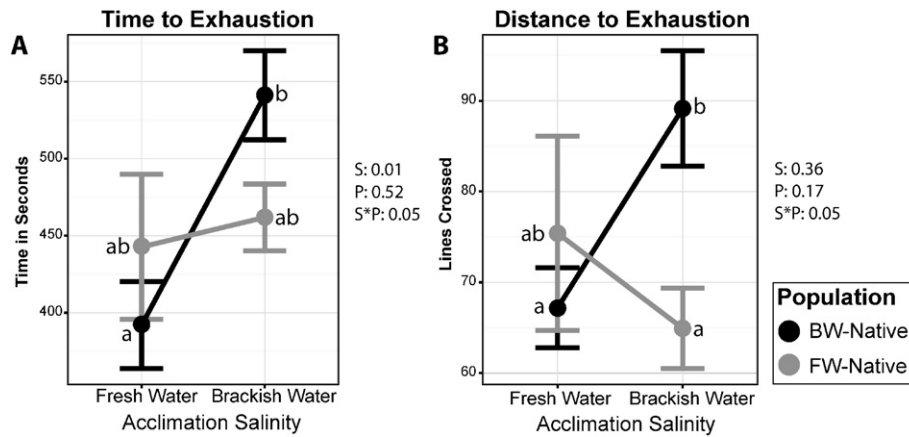


Fig. 2. (A) Time to exhaustion (seconds) and (B) distance to exhaustion (complete laps swum) after a manual chase for two populations of *F. heteroclitus* native to either fresh or brackish water and acclimated to fresh or brackish water. Within each graph, points are mean values \pm s.e.m. Black points correspond to the BW-native population while gray is the FW-native population ($n = 9$ – 12 per population per acclimation salinity). For statistical analysis S and P (to the right of each graph) represent results from (A) univariate ANOVA and (B) randomization test, with the main effects of salinity treatment and population, respectively, while S * P is the interaction term. Lower case letters associated with each treatment represent post-hoc tests where different letters show statistically significant differences ($p \leq 0.05$) between treatments.

to the RMR was then subtracted to obtain the total area contributing to EPOC.

2.1. Statistics

All statistical analyses were performed in R (R Core Team, 2015). Complete data were collected for the following number of individuals per treatment: 11 freshwater acclimated BW-native fish, 9 freshwater acclimated FW-native fish, 12 brackish water acclimated BW-native fish, and 12 brackish water acclimated FW-native fish. The effect of fish mass on MR was assessed using multiple methods. We first tested for differences in mass between groups using ANOVA. Next, a log regression was used to test for significant correlation between mass and all response variables. Analysis of covariance (ANCOVA) was implemented with salinity, population, and population * salinity as main effects and mass as a covariate. Using ANCOVA accounts for the non-independence of mass with response variables and is commonly used in studies of metabolic rate (Capossela et al., 2012; Helland et al., 2011; Rasmussen et al., 2012; White et al., 2007;). Using AIC, model fit for each ANCOVA was compared to a full-factor ANOVA that did not include the covariate of mass. The model that resulted in the lowest AIC score was chosen for statistical analysis. Response variables were transformed as necessary to meet the assumptions of normality. Of our response variables, time to exhaustion, distance to exhaustion, RMR, and EPOC showed no significant effect of mass using log regression. Model fit was also best when mass was not included as a covariate. Therefore, these response variables, with the exception of distance to exhaustion, were analyzed using ANOVA with salinity and population as main effects as well as an interaction term (see below for details of distance to exhaustion statistics). Regression showed a significant effect of mass for MMR, AS, and FAS and model fit improved when mass was included as a covariate. Therefore, ANCOVA was used to analyze these data. Where ANCOVA was implemented, group means are presented after adjusting for the effect of mass, which were obtained using the *ef* command in the 'Effects' package in R (Fox 2003). Though appropriate for MMR, AS, and FAS, adjusting for mass did not change our general conclusions; however for reference we include raw data that is not adjusted for mass in Supplemental Fig. 1. Tukey's HSD was used for post-hoc comparisons. These same response variables were analyzed using a multivariate analysis of variance (MANOVA) with salinity and population as main effects. Results from the univariate and multivariate approach did not differ and only the univariate results are reported in the main text.

Distance to exhaustion did not meet the assumptions of homogeneity of variance. Therefore, randomization tests were implemented following Logan (2011). This analysis involved random shuffling of observations among treatments in order to obtain a null distribution of F-ratios to which the observed F-ratio can be compared and is robust to data with unequal variance between groups (Logan 2011). Post-hoc tests were performed using randomization for each pairwise comparison to generate a null distribution and calculate a p-value for each comparison.

To compare the proportion of fish recovering in fresh versus brackish water, a proportions test was implemented using the *prop.test* function. Because not all individuals recovered within the 20 h time period, non-recovering individuals resulted in right censoring of the data and represented a type of missing data. To account for this, a survival analysis using the Cox proportional-hazards model was implemented using the 'survival' package in R (Therneau, 2015). This method typically models the relationship between survival and covariates, though it can be modified for any time dependent response, and attempts to determine the degree to which the covariates influence the probability, or hazard, of an outcome (Therneau and Grambsch, 2000). Salinity, population, and their interaction were included as predictor variables in the model.

3. Results

There were no significant differences in fish mass among groups ($p = 0.12$) and individuals had an overall mean mass of 5.29 ± 0.169 g (all measures reported as mean \pm s.e.m). Time to exhaustion showed significant treatment effects for both salinity and salinity-by-population interaction (Fig. 2A). Overall, fish in brackish water took a significantly longer time to reach exhaustion than fish in fresh water (502 ± 19.5 vs. 413 ± 28.1 s; $p = 0.01$). BW-native fish, in particular, swam longer in brackish water than in fresh water (541 ± 28.9 vs. 392 ± 28.3 s; $p = 0.01$). Total distance to exhaustion varied between 25 and 141 total laps, corresponding to an approximate minimum of 42.9 m and a maximum of 241.8 m. These distances are upper estimates as fish varied their path within the chase tub. Total distance showed a salinity-by-population interaction ($p = 0.05$) where the BW-native population swam further than the FW-native population in brackish water (89 ± 6.4 vs. 65 ± 4.4 lines; $p < 0.01$; Fig. 2B), whereas in fresh water the populations performed similarly. The BW-native population also swam significantly further in brackish than in fresh water ($p < 0.01$). These differences in exercise performance measures suggest that BW-native fish have reduced performance in fresh water. Similarly,

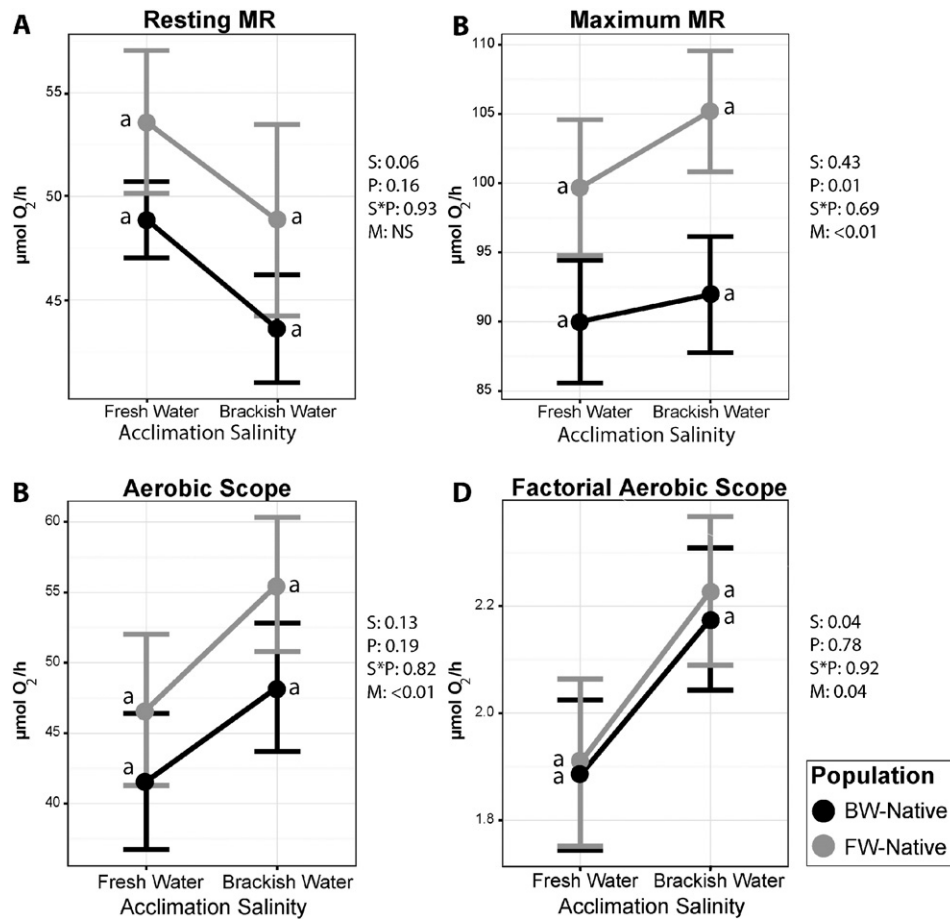


Fig. 3. Metabolic rates (MR, $\mu\text{mol O}_2/\text{h}$) for FW-native (gray) and BW-native (black) *F. heteroclitus* acclimated to fresh water or brackish water conditions. MR values are shown as mean \pm s.e.m. ($n = 9\text{--}12$ per population per acclimation salinity) and displayed as (A) resting MR (RMR), (B) maximum MR (RMR), (C) aerobic scope (AS), (D) and factorial aerobic scope (FAS). Letters to the right of each graph represent results from statistical analysis. Where ANCOVA was implemented (B, C, D), means are displayed after adjusting for mass and M represents the covariate of mass with main effects of salinity and population and an interaction term $S * P$. Where ANOVA was implemented, M was not included in the model. Lower case letters associated with each treatment represent post-hoc tests where different letters show statistically significant differences ($p \leq 0.05$) between points.

FW-native individuals have reduced performance in brackish water compared to the BW-native population. However, within fresh water fish from the two populations performed similarly. These results suggest improved performance of populations in their native salinity, consistent with predictions of local adaptation to environmental salinity.

The patterns in MR across treatments differed from the swimming performance results described above. Where salinity-by-population interactions were significant for some swim performance endpoints, no MR measurements showed salinity effects that differed between populations. However, RMR showed a salinity effect, where individuals had a higher RMR in fresh water than in brackish water (51.0 ± 1.87 vs. $46.2 \pm 2.64 \mu\text{mol O}_2\text{h}^{-1}$; $p = 0.06$; Fig. 3A). MMR demonstrated a population effect where FW-native individuals had a higher MR than BW-native individuals (102.7 ± 3.18 vs. $91.1 \pm 3.04 \mu\text{mol O}_2\text{h}^{-1}$; $p = 0.01$; Fig. 3B) FAS showed a significant effect of salinity where the brackish water treatment had higher values than the freshwater treatment (2.2 ± 0.10 vs. $1.9 \pm 0.11 \mu\text{mol O}_2\text{h}^{-1}$; $p = 0.04$; Fig. 3D) and no population effect was present ($p = 0.92$). Similarly, AS trended towards a salinity effect with higher values in brackish water, but these differences were not significant ($p = 0.13$). These results do not parallel the swimming performance data and do not support local adaptation, but are consistent with reduced metabolic performance of both populations in low salinity environments.

Time to recovery was determined as the point at which two consecutive recovery MR measures fell below the upper 95% confidence interval for RMR. Fish that did not fully recover to resting values within 20 h were assigned a recovery time of 20 h, which is equivalent to the

minimum amount of recovery data available for all individuals. Recovery analysis showed no significant effect for the interaction of population and salinity ($p = 0.82$). This interaction was removed from the model due to its insignificance and because the AIC of the model with no interaction was lower than the model where it was included (188.8 vs. 186.8). The reduced model, including population and salinity, showed a significant effect for salinity ($p = 0.03$) but not population ($p = 0.23$). Acclimation to fresh water reduced the probability of recovery at any time period by a factor of 0.42 and increased the median time to recovery from 6.33 h to 16.72 h (Fig. 4A), demonstrating that the probability of recovery in fresh water is reduced relative to brackish water. Additionally, considering the relative proportion of individuals recovering to resting values, fewer freshwater acclimated individuals were able to regain RMR compared to fish acclimated to brackish water (0.5 vs. 0.75; $p = 0.08$). EPOC values showed a similar trend as time to recovery (Fig. 4B). Again, there was no significant population effect, but individuals in fresh water showed a larger EPOC than those in brackish (188.7 ± 22.0 vs. $115.8 \pm 18.42 \mu\text{mol O}_2$; $p = 0.02$). These findings do not support local adaptation, but rather indicate that individuals from both populations recover more slowly in fresh water compared to in brackish water.

4. Discussion

The objective of these experiments was to test for evidence of local adaptation in metabolic and performance traits between two populations of killifish native to different osmotic niches. The salinities chosen,

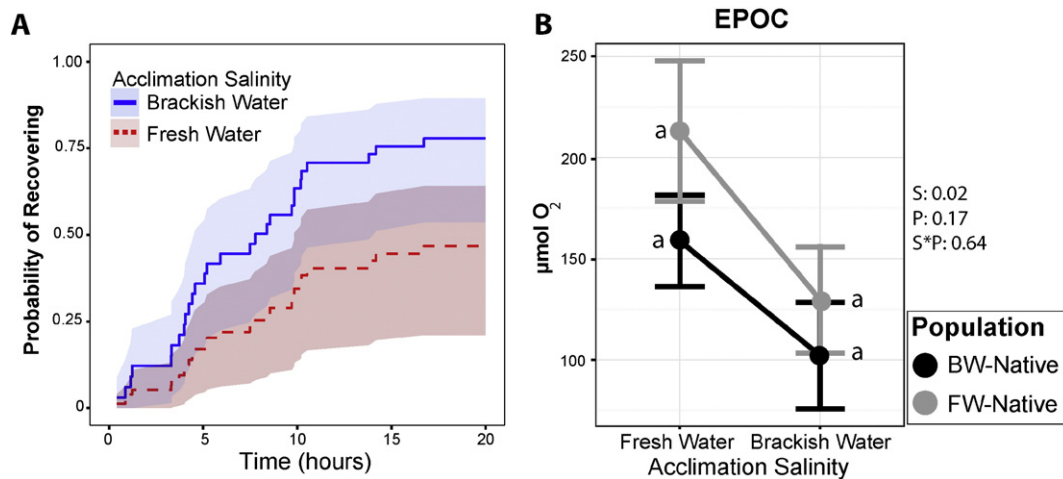


Fig. 4. Metabolic recovery following an exhaustive chase of FW-native and BW-native populations of *F. heteroclitus* acclimated to fresh water and brackish water. (A) Probability of recovery over time for individuals acclimated to fresh water (red dashed line) and brackish water (blue solid line) as estimated from a Cox proportional-hazards model. Shaded area represents the 95% confidence interval. Recovery curves were significantly different at $p = 0.03$. (B) excess post-exercise oxygen consumption ($\mu\text{mol O}_2$). Values are shown as mean \pm s.e.m. ($n = 9$ –12 per population per acclimation salinity). Gray points represent FW-native fish while black points represent BW-native fish. ANOVA results are represented to the right of each graph where S and P represent the main effects of salinity treatment and population, respectively, while S * P is the interaction term. Lower case letters associated with each treatment represent post-hoc tests where different letters show statistically significant differences ($p \leq 0.05$) between treatments.

0.3 and 15 ppt, both differ from the fish's iso-osmotic point of ca. 11 ppt such that maintenance of internal osmotic homeostasis should require physiological regulation (Buckling et al., 2012). The results of time and distance to exhaustion support our hypothesis of local adaptation (Fig. 1). Time to exhaustion showed that FW-native fish maintained performance across salinities while BW-native fish performed significantly worse in fresh water (Fig. 2A). This result suggests that FW-native fish have evolved physiological compensations that maintain exercise performance in dilute fresh water. Additionally, distance to exhaustion indicated that both populations performed similarly in fresh water but the BW-native population outperformed the FW-native population in brackish water (Fig. 2B). This suggests that evolution in a dilute freshwater habitat isolated from salt-water influence has contributed to the deterioration of some aspects of high-salinity performance in the FW-native population.

Changes in external salinity can lead to alterations in internal plasma ion concentrations which may reduce the force of muscle contraction (Homsher et al., 1974). As such, salinity challenges have been found to reduce maximum swimming speed in sturgeon (*Acipenser naccarii*, McKenzie et al., 2001) and salmon (Brauner et al., 1994). However, because *F. heteroclitus* shows an ability to acclimate and maintain plasma ion concentrations in response to a broad range of salinities (Brennan et al., 2015; Griffith 1974; Scott et al., 2004; Scott and Schulte, 2005; Whitehead et al., 2011; 2012) it seems unlikely that pre-exercise alterations in plasma ions are responsible for the observed differences in performance. Exhaustive exercise can result in disturbances in ionic, osmotic, and fluid volume homeostasis (Kieffer, 2000; Wang et al., 1994). If BW-native fish are more prone to disruption of internal osmotic balance in fresh water than FW-natives, the resulting change in homeostatic balance may result in reduced swimming performance at low salinity relative to higher salinity. We observe that BW-native fish show a decrease in the time to reach exhaustion in fresh water (Fig. 2A). Taken together, these results may be indicative of changes in internal osmotic balance during exercise for both populations in non-native salinities. Future experiments should measure internal ion concentrations pre- and post-exercise to determine the effect of internal osmotic balance on swim performance.

Contrary to our predictions to our findings in swim performance, the metabolic results do not support the hypothesis of local adaptation of each population to its native osmotic niche. Rather, we observed that both populations performed similarly poorly in fresh water compared

to brackish water (Figs. 3, 4). The increase of RMR and EPOC and decrease of FAS and AS in fresh water suggests that there are higher metabolic demands or limitations in fresh than in brackish water, consistent with the idea that brackish water is closer to the iso-osmotic point than fresh water. These results agree with previous work focusing on a coastal population of *F. heteroclitus* that predicts brackish water to be less osmotically stressful and less energetically costly than fresh water (Kidder et al., 2006). *F. grandis*, which has similar osmotic physiology and niche as its sister species *F. heteroclitus* (Griffith, 1974), also performs markedly worse in fresh versus brackish water (Kolok and Sharkey, 1997).

The MR data suggests that FW-native fish have elevated MMR, RMR, and AS relative to BW-native fish (Fig. 3A, B, C). FW and BW native populations within the Chesapeake Bay share population genetic affinity with northern coastal and southern coastal clades of *F. heteroclitus*, respectively (Adams et al., 2006; Duvernell et al., 2008). Fangue et al. (2009) found that northern populations of *F. heteroclitus* held at 20 ppt possessed significantly higher RMR than southern populations. Healy and Schulte (2012) also found that northern fish possess higher RMR and MMR than southern fish. Our results are consistent with these studies; the higher MR that we observe in the FW-native population relative to the BW-native are consistent with fish from northern genetic backgrounds possessing higher MR than those from southern genetic backgrounds. Moreover, absolute values for MR and AS that we obtained are similar to the two aforementioned studies. These results suggest that genetic background has a significant effect on metabolic physiology even after adaptive divergence into a novel osmotic niche.

Previous studies focused on other measures of performance (i.e., not AS) of marine or anadromous fishes that have invaded freshwater niches have identified environmentally dependent differences, where freshwater populations show highest performance in their native low salinity niche as compared to higher salinities. This is consistent with our swim performance data but not our MR data. This pattern has been found in three-spined stickleback (DeFaveri and Merilä, 2013), Arctic char (Staurnes et al., 1992), and alewives (Velotta et al., 2014; 2015). The contribution of osmoregulation to RMR is estimated to be low (<4%, Morgan and Iwama, 1998). Ern et al. (2014) demonstrated that metabolic costs are typically minimized at a fish's native salinity, suggesting that RMR may generally be responsive to selection. However, this is not always the case. The authors analyzed 24 studies on 22 different species and found no universal indicator of the salinity at which

metabolic costs are lowest. While many studies show minimal costs at native habitat salinity (De Boeck et al., 2000; Morgan and Iwama, 1998; Toepfer and Barton, 1992), some implicate salinities near the iso-osmotic point as optimal (Farmer and Beamish, 1969; Nordlie, 1978; Pérez-Pinzón and Lutz, 1991). In a minority of studies, maximal efficiency of oxygen uptake has been found in the salinity opposite to a species' native environment; i.e., in seawater for freshwater native fish or fresh water for a seawater native fish (Gracia-López et al., 2006; Plaut, 1999). *F. heteroclitus* typically inhabits brackish or marine niches, coastal populations prefer salinities of about 22 ppt, and plasma salts are iso-osmotic with the external environment at 10–12 ppt (Buckling et al., 2012). Therefore maintaining minimum RMR at brackish salinities is reasonable for BW-native fish since those salinities are similar to their native habitat and near to the iso-osmotic point. While the FW-native population does not have its minimum RMR matched to its native environment, this population invaded fresh water recently (Duvernell et al., 2008) and it is possible that variation in characters other than RMR were recruited upon adaptation to freshwater environments and insufficient time has elapsed for RMR to similarly evolve to match this new osmotic niche (Lande, 2009). Furthermore, from this experiment the effects of iso-osmotic point and native habitat salinity may be confounded in the BW-native fish as these salinities are similar for this population. Therefore, RMR may be minimized for both populations in salinities near the iso-osmotic point rather than in native ancestral salinities. Further experiments utilizing marine populations would allow for the clarification of this issue.

Similar to the previously discussed MR results, the EPOC results do not support local adaptation to external salinity. Here, both populations take longer to recover and show a larger EPOC in fresh versus brackish water, which indicates a common reduction of physiological performance in fresh water (Fig. 4). While many studies have focused on the effects of external factors such as temperature (Lee, 2003), diet (Fu et al., 2007), and hypoxia (Maxime et al., 2000; Svendsen et al., 2012), to our knowledge no studies have investigated the effects of salinity on EPOC. Studies on other stressors have generally found that deviation from optimal conditions may inhibit recovery (Svendsen et al., 2012). Accordingly, the increased recovery time and EPOC for fish in fresh water suggest that this osmotic environment is sub-optimal for both populations of *F. heteroclitus*. This reduction of AS and increase of EPOC may increase the risk of mortality as fish become more susceptible to metabolic constraints (Priede, 1977).

EPOC measures also suggest an interesting role of aerobic and anaerobic contributions to swim performance in fresh versus brackish water. RMR, AS, and FAS show evidence for increased aerobic capacity in brackish water compared to fresh water (Fig. 3). Conversely, the probability of recovery and EPOC both suggest increased anaerobic contributions to activity in fresh water relative to brackish water (Fig. 4). This inverse relationship implies that *F. heteroclitus* relies on different proportions of anaerobic and aerobic metabolism in fresh water versus brackish water. That is, the anaerobic contribution to activity depends on the external salinity and is greater in fresh water than brackish water. While the reduction of FAS and AS in fresh water likely leads to the reduction of swimming performance, this reduction may be tempered by anaerobic contributions that allow performance to be maintained to a degree, particularly in FW-native populations.

It is generally expected that EPOC scales positively with intensity of exercise (Børsheim and Bahr, 2003). Distance to exhaustion was not correlated with EPOC (Pearson's correlation, $r = -0.18$, $p = 0.25$) but time to exhaustion showed a significant negative relationship with EPOC (Pearson's correlation, $r = -0.31$, $p = 0.04$). This negative correlation was unexpected and suggests that individuals that swam further may have swum less vigorously and required a lower anaerobic contribution to exercise than those fish that reached exhaustion more quickly. Experiments utilizing swim chambers and determining critical swimming speed may help to quantify swimming effort and its contribution to metabolic measures.

It should be noted that EPOC measurements were limited to recovery over 20 h and not all individuals recovered. Therefore, these measures underestimate the EPOC values of those individuals that did not recover. Given that fewer freshwater acclimated individuals recovered as compared to brackish water acclimated (0.5 vs. 0.75), our EPOC values are conservative and actual differences in EPOC between salinities may be larger than reported here. Lactate concentrations were not measured, and as such, levels of anaerobic effort could not be directly estimated. Future studies should measure pre- and post-exercise lactate levels to quantify the differences in anaerobic effort between populations and treatments as this may be an important aspect of performance differences.

Metabolic performance data do not support the hypothesis of local adaptation in killifish from different osmotic niches. The number of possible traits that may support local adaptation is large (i.e., Sanford and Kelly, 2011) and any trait influencing fitness may play an important role. While we hypothesized that divergence in metabolic traits would be subject to selection due to the energetic demands that typically arise in non-native salinities (Ern et al., 2014; Kidder et al., 2006), it is possible that fitness differences that limit gene flow between FW and BW habitats are underpinned by other physiological or behavioral traits. For example, freshwater populations of threespine stickleback have repeatedly evolved from marine ancestors within the last ~20,000 years (Bell, 2001). Evidence for local adaptation of osmoregulation is evident for both freshwater and marine populations as each show improved fitness (growth, survival, or health index) in their native salinity relative to foreign environments (DeFaveri and Merilä, 2013; McCairns and Bernatchez, 2009). However, Grotan et al. (2012) tested oxygen consumption of a fresh, brackish, and marine population at 0, 15, and 30 ppt and found no differences between populations or treatments. Ongoing experiments in *F. heteroclitus* are focusing on more direct measures of fitness such as reproductive success, survival, and growth in order to identify evolutionarily-important differences between the populations.

Our data offer evidence for local adaptation in populations of *F. heteroclitus* as reflected by swimming performance differences in alternate osmotic environments. The BW-native individuals took significantly longer to reach exhaustion in brackish water than in fresh water. Similarly, BW-native individuals had greater swimming endurance in brackish than in fresh water. While local adaptation is not reflected in our measures of aerobic metabolism and recovery, other traits may contribute to local adaptation such as maintenance or recovery of ionic homeostasis, anaerobic metabolism, mating behavior, and reproductive success. Ongoing experiments seek to identify other locally adapted characteristics and clarify how these characteristics work to maintain divergence between the populations.

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