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RESEARCH ARTICLE

Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level

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SUMMARY

In fishes, performance failure at high temperature is thought to be due to a limitation on oxygen delivery (the theory of oxygen and capacity limited thermal tolerance, OCLTT), which suggests that thermal tolerance and hypoxia tolerance might be functionally associated. Here we examined variation in temperature and hypoxia tolerance among 41 families of Atlantic salmon (*Salmo salar*), which allowed us to evaluate the association between these two traits. Both temperature and hypoxia tolerance varied significantly among families and there was a significant positive correlation between critical maximum temperature (CT_{max}) and hypoxia tolerance, supporting the OCLTT concept. At the organ and cellular levels, we also discovered support for the OCLTT concept as relative ventricle mass (RVM) and cardiac myoglobin (Mb) levels both correlated positively with CT_{max} (R^2=0.21, P<0.001 and R^2=0.17, P=0.003, respectively). A large RVM has previously been shown to be associated with high cardiac output, which might facilitate tissue oxygen supply during elevated oxygen demand at high temperatures, while Mb facilitates the oxygen transfer from the blood to tissues, especially during hypoxia. The data presented here demonstrate for the first time that RVM and Mb are correlated with increased upper temperature tolerance in fish. High phenotypic variation between families and greater similarity among full- and half-siblings suggests that there is substantial standing genetic variation in thermal and hypoxia tolerance, which could respond to selection either in aquaculture or in response to anthropogenic stressors such as global climate change.

Key words: critical maximum temperature, enzyme activity, heat shock protein, heritable, oxygen and capacity limited temperature tolerance, heart.

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INTRODUCTION

Models of the earth's climate predict an increase of 1–7°C in mean global temperature over the next hundred years (e.g. Ficke et al., 2007). Associated with future increases in global mean temperature, the occurrence of extreme climate events is predicted to increase (e.g. Ficke et al., 2007; Doney et al., 2012). In addition, eutrophication as a result of anthropogenic effects is already causing an increased prevalence of aquatic hypoxia in many areas (Diaz, 2001). Together, changes in water temperature and oxygenation will pose a substantial challenge for aquatic organisms including fish.

When temperature increases beyond the optimum temperature range or oxygen declines below optimal levels, growth, development and reproductive capacity decrease and susceptibility to disease increases in fish (Brander, 2007; Pörtner and Knust, 2007). As a result, changes in climate are predicted to have detrimental effects on fish populations both in nature and in aquaculture settings (Brander, 2007). Fish may be capable of adjusting, at least in part, to these changing environmental variables through phenotypic plasticity in their responses to high temperature and hypoxia. Acclimation, a form of reversible phenotypic plasticity, is known to modify the maximum temperature tolerance and hypoxia tolerance of many, but not all, fish species (e.g. Rees et al., 2001; Ford and Beitinger, 2005; Eme and Bennett, 2009; Fu et al., 2011; Petersen and Gamperl, 2011). For example, salmonids appear to have fairly limited capacity for acclimation of these traits as maximum temperature tolerance changes only a few degrees in response to acclimation (Elliott, 1991; Baroudy and Elliott, 1994). If environmental change exceeds the capacity of fish to respond *via* these mechanisms, selection might act on these traits, allowing populations to respond *via* evolutionary adaptation. However, for selection to act over ecologically relevant time scales, thermal and hypoxia tolerance must be genetically determined and sufficiently variable among individuals to provide the standing genetic variation on which evolution can act (Boulding and Hay, 2001).

Unfortunately, little is known about the genetic basis and extent of inter-individual variation in thermal tolerance or hypoxia tolerance, and thus the potential for adaptive change in these traits is difficult to estimate. Temperature-at-death has been shown to be significantly heritable in least killifish (*Heterandria formosa*) (Doyle et al., 2011) and mosquitofish (*Gambusia holbrooki*) (Meffe

et al., 1995), suggesting that there is a genetic component of maximum temperature tolerance and the potential for selection to act on this trait at least in some fish species. In rainbow trout (*Oncorhynchus mykiss*), the existence of a quantitative trait locus (QTL) associated with maximum thermal tolerance also suggests a genetic basis for this trait (Perry et al., 2001). Hypoxia tolerance may also be genetically determined, at least in part, in fishes. For example, hypoxia tolerance has been shown to have significant heritability in common carp (*Cyprinus carpio*) (Nagy et al., 1980). Together these data suggest that temperature and hypoxia tolerance may have a significant heritable component in fishes, but the capacity for selection to act on these traits depends in part on whether these traits are positively or negatively associated. For example, if there is a functional or genetic trade-off between hypoxia tolerance and thermal tolerance, it would be difficult for selection to simultaneously increase both traits. In contrast, if there is a positive functional or genetic association between the traits, then selection on one trait will also act positively on the other trait.

There is some theoretical and empirical evidence to suggest that thermal tolerance and hypoxia tolerance may be positively associated, at least in some species of fishes (including salmonids). The theoretical basis for this possibility stems from the concept of oxygen and capacity limited thermal tolerance (OCLTT), proposed by H. Pörtner (for reviews, see Pörtner and Knust, 2007; Pörtner, 2010). The basis for the concept comes from the early findings of Fry (Fry, 1947), who observed that while routine metabolic rate increases exponentially with temperature, maximum metabolic rate, after first increasing exponentially with temperature, reaches a plateau and then starts to decline at high temperatures. The temperature at which the maximum aerobic scope (the difference between routine and maximum rates) is reached is called the optimum temperature (T_{opt}). The decline of maximum performance above T_{opt} is suggested to result from mismatch between the demand for oxygen and the capacity to supply oxygen to tissues (Pörtner and Knust, 2007; Pörtner, 2010). There is good empirical evidence supporting the validity of this concept in a variety of fish species, including salmonids (Farrell, 2009; Eliason et al., 2011). Furthermore, Steinhausen with her co-workers (Steinhausen et al., 2008) observed a failure to increase maximum cardiac output above T_{opt}, suggesting that limitations at the level of the heart were the cause of limited oxygen supply with increasing temperature. This limitation in oxygen supply and constant increase with oxygen demand could lead to functional tissue hypoxia at temperatures beyond T_{opt} (Farrell, 2002), limiting temperature tolerance of the individual. However, at the tissue and molecular levels, less is known about the properties that make some individuals more tolerant than others and whether these differences support the OCLTT concept.

The objectives of the present study were thus to: (1) determine the extent of variation in temperature and hypoxia tolerance within and between families of Atlantic salmon, (2) determine whether there is a positive association between these traits, and (3) examine variation in physiological organ- and cellular-level factors in these families that would be hypothesized to account for some of the tolerance variation, based on the predictions of the OCLTT concept.

MATERIALS AND METHODS

The research was conducted at the Fisheries and Oceans Biological Station at St Andrews, NB, Canada, with 1+ juvenile Atlantic salmon (*Salmo salar* L.) in January 2011 (mean fork length and mass were 163.2±0.9 mm and 47.9±0.8 g, respectively). The fish came from experimental brood stock maintained at Cooke Aquaculture, Oak Bay Hatchery, NB, Canada. Forty-one families of Atlantic salmon were produced from a group of 70 parental fish. Each family had different dam (i.e. 41 female Atlantic salmon were used to produce the families), but only 29 sires were used; eight sires fertilized eggs from two different dams (resulting in eight pairs of half-sibling families) and two sires fertilized eggs from three different dams (resulting in two half-sibling groups of three families each), while the remaining 19 sires were crossed with independent dams (resulting in 19 unrelated families). This breeding design allowed us to estimate the genetic component of the observed phenotypic variation, and by using independent dams with the same sire to generate the half-sibling families (rather than independent sires with the same dam) we are able to exclude maternal effects as a possible cause of similarity in phenotype between half-sibling families. In order to keep track of the family background of the fish, families were kept in separate tanks at the National Cold Water Marine Aquaculture Center (Franklin, ME, USA) until the fork length of fish exceeded 12 cm and fish could be tagged with passive integrated transponder (PIT) tags. After PIT-tagging, the fish were transferred to St Andrews and kept together in common tanks. In order to partially control for tank effects that might occur before tagging, the progeny from each family was divided across two separate tanks. Each tank was split to contain two different randomized families. All tanks were identical flow-through tanks of the type used in aquaculture. The fish were kept at a natural photoperiod (9 h:15 h light:dark) and water temperature (4.0±0.1°C) at time of the experiments. Fish were fed *ad libitum* once a day with commercial feed (Skretting, St Andrews, NB, Canada) and were fasted 24 h before experiments. Treatment of all experimental animals was in accordance with the approved University of British Columbia animal care protocol A07-0288-A002.

Temperature tolerance experiment

The temperature tolerance of fish was tested with a critical maximum temperature (CT_{max}) protocol that warmed the fish until they exhibited sustained loss of equilibrium (LOE) and lost the capacity to escape conditions that would eventually lead to death (Beitinger et al., 2000). Briefly, each evening one fish from each family was randomly selected and transferred from the rearing tanks into an experimental tank (88 l) with constant flow-through freshwater at the holding temperature (4°C). Two identical setups were used. Because of possible diurnal light cycle effects on temperature tolerance of fish (Bulger, 1984; Healy and Schulte, 2012), all the experiments were performed between 09:45 and 13:45 h for each day. After an overnight acclimation period in the experimental tank, the water flow was turned off and the temperature of the water was increased at a constant rate of 0.3°C min^{-1} to 22°C, and at a rate of 0.1°C min^{-1} thereafter until the fish exhibited LOE. Water temperature was controlled with a circulating 1100 W heater (1160S, VWR, Mississauga, ON, Canada) and five submersible aquarium heaters (100 W, Marineland Aquarium Products, Cincinnati, OH, USA). Water homogeneity and oxygenation were assured by bubbling air vigorously into the tanks, keeping oxygenation level above 80% saturation throughout the experiments. After a fish lost equilibrium, it was quickly removed from the tank, identified (by reading the PIT tag) and placed in a recovery tank at the acclimation temperature. After the experiment, all the fish were lightly anesthetized with 50 p.p.m. MS-222 (Sigma-Aldrich, Oakville, ON, Canada) buffered with sodium bicarbonate, and the mass and length of the fish were recorded before returning the fish back into the rearing tank. Twelve fish per family were tested for CT_{max} (total 492 fish) and only three mortalities (0.6%) were recorded in the days that followed the CT_{max} determinations.

Hypoxia

To test hypoxia tolerance, one fish per family was transferred to same experimental tanks as used in CT_{max} experiment and allowed to adjust to new conditions overnight. The flow of water into the tank was shut down on the following morning and thereafter the water temperature was kept constant at 4°C with a circulating chiller (1160S, VWR). The level of oxygen in the water was then decreased at the constant rate of 1.5% air saturation per minute until 10% air saturation (1.3 mg l^{-1}) was reached by bubbling nitrogen in the tank. The time until the fish exhibited LOE after the 10% air saturation level was reached was measured. After a fish lost equilibrium, it was removed from the tank, identified (by PIT tag reading) and placed into a recovery tank. After the experiment, all the fish were lightly anesthetized as before, so that mass and length could be recorded before returning the fish back into the rearing tank. Hypoxia tolerance was tested in eight fish per family (328 fish in total) with no mortality in the days that followed the experiment. In a pilot experiment, 87.5% of fish exhibited LOE within 1.5 h. Therefore, to complete these hypoxia assessments within a similar time window as the CT_{max} measurements, the hypoxic duration was set to 2 h, once 10% air saturation was reached. However, during the actual measurements some of fish did not exhibit LOE after 2 h.

Sampling

Two weeks after the CT_{max} experiment, six fish from eight extreme families (the four most temperature tolerant families and the four least tolerant families) were euthanized with a lethal dose of buffered MS-222 (200 p.p.m., Sigma-Aldrich) (N=48). The PIT tag of the fish was read to identify the individual, the mass and length of the fish were measured, cardiac muscle was removed and the ventricle was weighed and frozen with liquid nitrogen. Relative ventricle mass (RVM) was calculated using the formula $RVM=(M_v/M_b)\times1000$, where M_v is ventricle mass (mg) and M_b is body mass (g). Tissue samples were stored at –80°C prior to analyses. Half of the ventricle (cut longitudinally) was used for western blotting analyses and the other half was used for enzyme activity analyses.

Western blotting

Cardiac muscle samples were homogenized by sonication in six volumes of ice-cold homogenization buffer (62.5 mmol l^{-1} Tris-HCl, pH 6.8) containing protease inhibitors (Protease inhibitor cocktail, Sigma-Aldrich). Aliquots (5 µl) of homogenized tissue were removed for protein analyses performed according to Bradford (Bradford, 1976). The samples were denatured according to Laemmli (Laemmli, 1970) in 1:1 v/v of sodium dodecyl sulphate (SDS) sample buffer by heating the samples for 7 min at 70°C. The proteins were separated with SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) as previously described in Towbin et al. (Towbin et al., 1979). For myoglobin (Mb) determinations, 5 µg of protein was loaded into each lane and 15% polyacrylamide gels were used, whereas for heat shock proteins (HSPs) the protein amount was 60 µg and 7.5% polyacrylamide gels were used. An identical control sample was included on each gel to control for gel-to-gel variation. Immunodetections of Mb, HSP 90β and heat shock cognate protein (HSC) 70 were performed in Tris-buffered saline (pH 7.5) plus 0.05% Tween 20 (TBST). Blots were blocked using 5% (w/v) non-fat powdered milk in TBST. The membranes were incubated overnight at 4°C in TBST containing 5% bovine serum albumin (BSA) and primary antibody [1:1000 polyclonal rabbit antimyoglobin antibody, M8648, Sigma-Aldrich (according to Faust et al., 2004), 1:1000 mouse monoclonal HSP 90β antibody, ab53497, Abcam, Cambridge, MA, USA, or 1:5000 rabbit polyclonal HSC 70 antibody, ab79857, Abcam]. After washing in TBST, the membranes were incubated in TBST containing 5% BSA and 1:2000 alkaline phosphatase conjugated secondary antibody (either goat polyclonal secondary antibody to mouse IgG, ab6790, Abcam, or goat polyclonal secondary antibody to rabbit IgG, 7054, Cell Signaling Technology, Danvers, MA, USA) for 2 h. The proteins were visualized by incubating membranes in substrate solution containing 0.17 mg ml^{-1} bromo-4-chloro-3-indolyl phosphate mono-(-toluidinium) salt and 0.49 mg ml^{-1} Nitro Blue Tetrazolium (both agents from Fisher Scientific, Nepean, ON, Canada). The intensities of the detected bands were analyzed with a FluorChem 8800 Imager (Alpha Innotech, San Leandro, CA, USA) using AlphaEase FC software (v. 3.1.2; Alpha Innotech). Protein amount was expressed relative to total protein loaded into each well and normalized to the control sample.

Enzyme activities

The other half of the ventricle was homogenized in 20 volumes of ice-cold homogenization buffer (50 mmol l^{-1} Hepes, 1 mmol l^{-1} EDTA, 0.1% Triton X-100, pH 7.4) in 4 ml Wheaton glass homogenizers kept on ice. The homogenates were kept at –80°C before analyses. The analyses of citrate synthase (CS; EC 2.3.3.1) and lactate dehydrogenase (LDH; EC 1.1.1.27) were performed according to Dalziel et al. (Dalziel et al., 2012) at 23°C, which was the temperature at which first LOE for CT_{max} was observed. The measurements were taken using a plate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA) and the final substrate concentrations followed the optimizations performed by Dalziel et al. (Dalziel et al., 2012). For CS, the concentrations were 0.15 mmol l^{-1} DTNB, 0.15 mmol l^{-1} acetyl CoA and 0.5 mmol l^{-1} oxalacetic acid in 50 mmol l^{-1} Tris (pH 8), and for LDH the concentrations were 5 mmol l^{-1} NADH and 25 mmol l^{-1} pyruvate in 50 mmol l^{-1} Tris (pH 7.4). Triplicate assays were used for each sample and background reaction rate was subtracted. The activities of enzymes were calculated to gram of ventricle.

Statistics

The variability of CT_{max} and hypoxia tolerance LOE time among families was tested with a one-way ANOVA and a Kruskal–Wallis test, respectively. Comparisons of variability of CT_{max} between half-sibling and unrelated families were made with independent-sample t-tests that compared the mean difference among unrelated families with the mean difference among related families. The correlation analysis between CT_{max} and hypoxia time to LOE was performed using a Spearman's correlation test with the families ranked according to tolerances of the individuals in each family. Thus, these correlations were made at the family level.

Other correlations were examined at the individual level. Pearson's correlation test was used to look for correlations between CT_{max} and the following variables: size, RVM, relative Mb, HSC 70 and HSP 90β protein levels, and enzyme activities of sampled individuals. Because only individuals from the upper four and lower four families from the CT_{max} experiment were sampled for protein analyses, the comparison of level of cellular parameters was not made for hypoxia tolerance. The effect of sex on each parameter measured was analyzed with a t-test. The values are presented as means ± s.e.m. if not stated otherwise.

RESULTS

CT_{max} and hypoxia tolerance

Both CT_{max} and hypoxia time to LOE varied significantly between families (P<0.001 for both traits; F=4.6 for CT_{max}; χ^2=72.8 for time

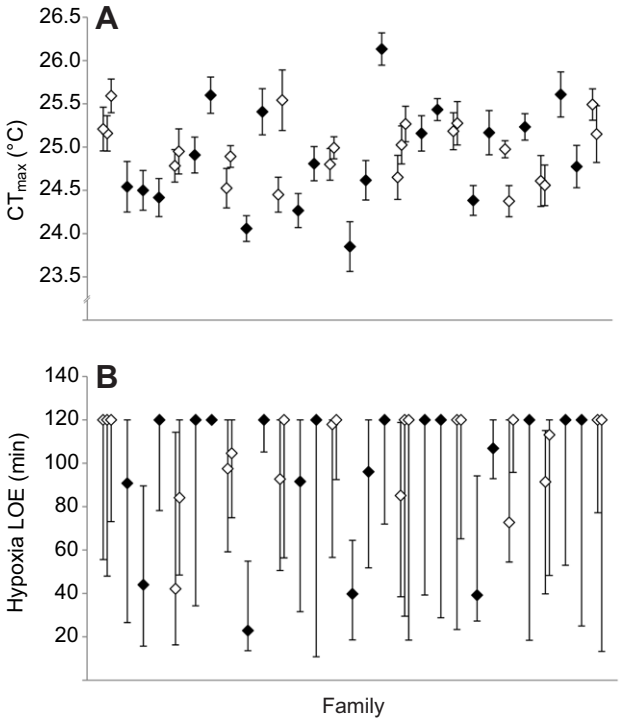

Fig. 1. (A) Mean (±s.e.m.) critical maximum temperature (CT_{max}) and (B) median (±quartiles) loss of equilibrium (LOE) time during progressive hypoxia for 41 families of Atlantic salmon. For each family, 12 individuals were tested for CT_{max} and eight individuals for hypoxia LOE. The half-sibling families with the same sire but different dams are presented beside each other and marked with open diamonds.

to LOE; Fig. 1). Family mean values ranged between 23.9±0.3 and 26.1±0.2°C for CT_{max} and median values for hypoxic time to LOE ranged from 22.9 to 120 min among the families. At the individual level, CT_{max} showed a range of 5.2°C (between the highest and lowest performing individuals in the complete data set) and time to LOE in hypoxia showed a range of 114.8 min. Moreover, there was a significant correlation between CT_{max} and hypoxia time to LOE at the family level (Spearman's rank order correlation, ρ=0.69, P<0.0001; Fig. 2). Neither length nor mass of the fish was correlated with CT_{max} or time to LOE (R^2=0.02–0.09, P=0.18–0.59, N=492 fish for CT_{max} and 328 fish for hypoxia).

For CT_{max}, half-sibling families (families that had same sire and different dams) resembled each other significantly more than did un-related families. The difference in mean CT_{max} between half-sibling families was 0.36±0.07°C while it was 0.55±0.01°C between unrelated families (t=2.5, P=0.02).

Ventricle mass

Relative ventricle mass explained 21% of the variability in CT_{max} between individuals (Fig. 3). Thus, there was a significant positive correlation between RVM and CT_{max} (P<0.001). A significant positive correlation also existed between total ventricle mass and CT_{max} (R^2=0.11, P=0.02). However, individual fish size had an effect on the correlation between total ventricle mass and CT_{max}. Because ventricle mass was significantly correlated with body mass (M_v=0.78M_b+1.02; R^2=0.86, P<0.001), this relationship was used to predict individual ventricle mass based on the body mass of fish, which was then compared with observed ventricle mass. A significant (P<0.001) positive correlation existed between

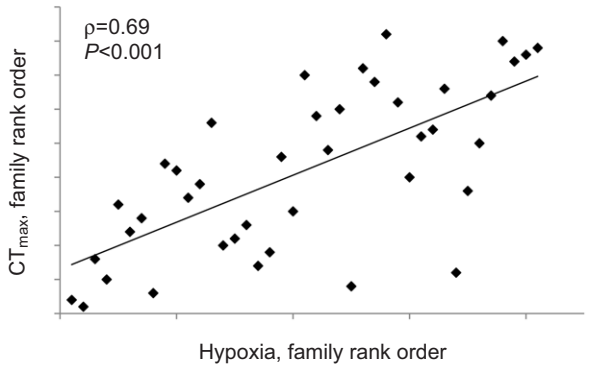

Fig. 2. The correlation between critical maximum temperature (CT_{max}) and hypoxia tolerance of 41 families of Atlantic salmon, as analyzed with Spearman's correlation. The linear regression follows the equation y=0.69x+6.5.

CT_{max} and observed minus predicted ventricle mass [CT_{max}=0.11($M_{v,observed}$–$M_{v,predicted}$)+25.0; R^2=0.19, P=0.002], such that a fish with a smaller ventricle than predicted according to its body mass had a lower CT_{max} than a fish with a larger ventricle. Neither total mass nor relative ventricle mass differed significantly with gender (P=0.13 and 0.67, respectively, N=25 males and 23 females).

Protein levels

A significant positive correlation was discovered between relative Mb level and CT_{max} (P=0.003), and relative Mb level explained 17% of the variability in CT_{max} among individuals (Fig. 4). An estimate of total ventricular Mb level (relative Mb level multiplied by ventricle mass) also correlated significantly with CT_{max} [CT_{max}=0.04(total Mb level)+24.0; R^2=0.22, P<0.001]. There was no significant difference in Mb level between genders (P=0.55). Neither relative HSC 70 nor HSP 90β level correlated with CT_{max} (Fig. 5).

Enzyme activities

The activities of CS and LDH as expressed per gram of ventricle did not correlate with CT_{max} (Fig. 6). However, there was a significant positive correlation between total activity of CS in the

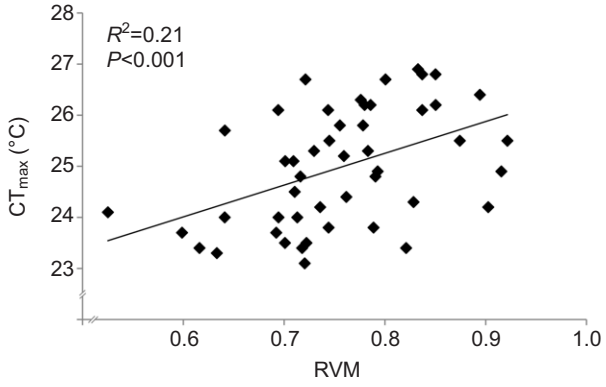

Fig. 3. The correlation between relative ventricle mass and critical maximum temperature (CT_{max}) in Atlantic salmon. The linear regression line follows the equation y=6.2x+20.3 (N=48).

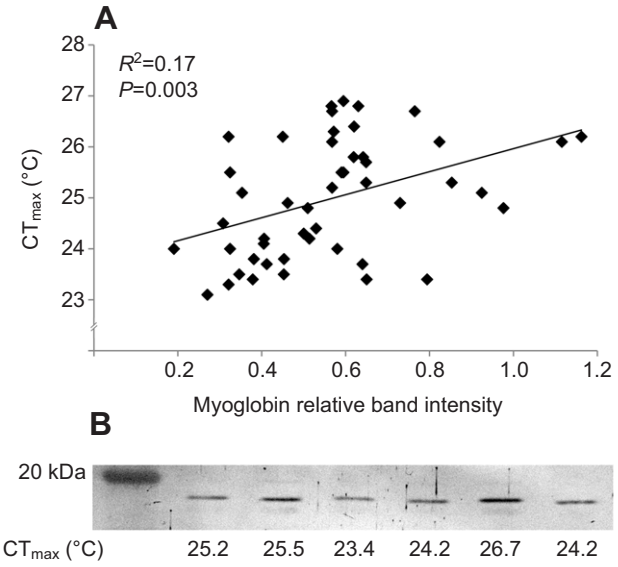

Fig. 4. (A) The correlation between relative ventricular myoglobin level and critical maximum temperature (CT_{max}) in Atlantic salmon. The linear regression line follows the equation y=2.3x+23.7 (N=48). (B) A representative western blot membrane for myoglobin from six individuals with a different CT_{max}.

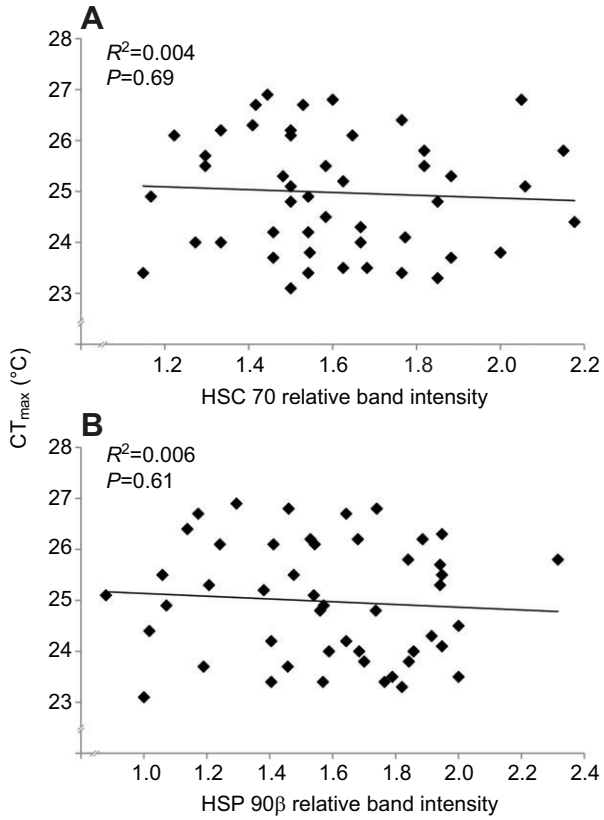

Fig. 5. The correlation between critical maximum temperature (CT_{max}) and the relative levels of (A) HSC 70 and (B) HSP 90β in Atlantic salmon. The linear regression lines follow equations y=–0.28x+25.4 and y=–0.27x+25.4 (N=48).

ventricle (the activity per gram of ventricle multiplied by ventricle mass) and CT_{max} (CT_{max}=1.98CS+23.9; R^2=0.09, P=0.04). For hypoxia tolerance, no individuals were sampled, thus we were not able to directly correlate CS or LDH activity to hypoxia tolerance.

DISCUSSION

The three most significant findings of the present study were that: (1) both temperature and hypoxia tolerance varied significantly among families of Atlantic salmon; (2) phenotypic variation in these traits was positively correlated at the family level; and (3) temperature tolerance was positively associated with variation in relative ventricle mass and ventricular Mb levels at the individual level. Together these results demonstrate that thermal and hypoxia tolerance in Atlantic salmon exhibit high phenotypic variation that has a significant genetic basis, and therefore has the potential to respond to artificial selection in aquaculture or allow adaptation in response to environmental factors such as climate change. In addition, our results are consistent with the OCLTT concept (Pörtner, 2010) and suggest that traits at the level of the heart are important in determining the thermal tolerance at the level of the whole organism.

By comparing the temperature tolerance among a large number of individuals from a large number of different families of Atlantic salmon, we characterized how this trait varies significantly among families and among individuals within families. Here, CT_{max} ranged from a low of 22.3°C to a high of 27.5°C. High CT_{max} variability was also observed in cutthroat trout, *Oncorhynchus clarkii* (Underwood et al., 2012), with tolerance varying 8°C among individuals as compared with 5.2°C here. However, in cutthroat trout the size of the fish varied, which explained some of the phenotypic variation found, as CT_{max} correlated significantly and negatively with the size of the fish. In the present study we observed no such correlation between size and CT_{max}. In fact, in the present study body size was quite similar between individuals. Thus, our data indicate that even within the same size group CT_{max} varies significantly. This is an important finding because phenotypic variation among individuals is the raw material upon which selection can act. However, in order for natural selection to act on a trait, the trait must be also be heritable, which we also clearly demonstrate in Fig. 1, which shows that full-siblings within families are more similar to each other than they are to other families, and that the half-sibling families are also remarkably similar to each other as compared with the variance in the whole population. As our experimental design involved half-sibling families from the same sire, but with different dams, these data are not consistent with a maternal effect, and instead suggest that there is a significant genetic component underlying CT_{max} in salmon, a finding consistent with the fact that a QTL for thermal tolerance has been discovered in rainbow trout (Perry et al., 2001). Indeed, in the context of previous findings in other species (Meffe et al., 1995; Doyle et al., 2011), we hypothesize that thermal tolerance has a strong genetic basis in many fish species.

For hypoxia tolerance, the phenotypic variance among families was also remarkably high, a result that is again consistent with previous work on intraspecific variability in cardiac hypoxia tolerance in rainbow trout (Faust et al., 2004). However, here we ended the tolerance test 2 h after the 10% saturation was reached, based on the results of preliminary experiments. Because a significant portion of the fish had a greater hypoxia tolerance, we were unable to obtain an estimate of the full variance in hypoxia tolerance among these families. This meant that the hypoxia experiment did not have the statistical power to detect differences

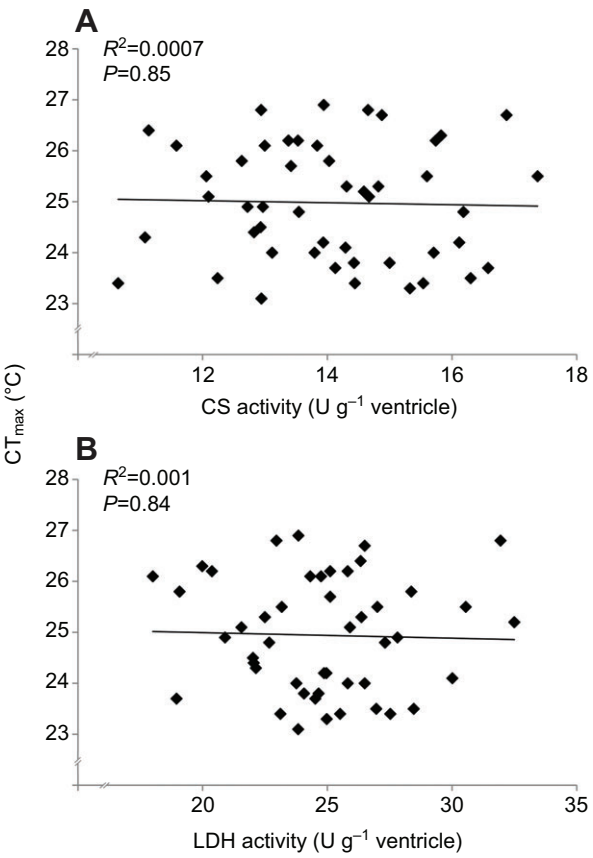

Fig. 6. The correlation between critical maximum temperature (CT_{max}) and (A) citrate synthase (CS) and (B) lactate dehydrogenase (LDH) activity in Atlantic salmon. The linear regression lines follow equations y=–0.02x+25.3 and y=–0.01x+25.2 (N=48).

between half-siblings and unrelated individuals. Even though the heritability of hypoxia tolerance was not detected here, Nagy et al. (Nagy et al., 1980) have shown that hypoxia tolerance has a genetic component in fish.

We discovered an extremely important correlation between temperature and hypoxia tolerance at the family level (Fig. 2). Such cross-tolerance has been suggested previously interspecifically but not intraspecifically at the family level. For example, Smale and Rabeni (Smale and Rabeni, 1995) showed a weak correlation between hypoxia and temperature tolerance among 36 fish species from a Missouri stream, and cross-tolerance exists within other taxa from mammals to plants (e.g. Wu et al., 2002; Banti et al., 2008; Tetievsky et al., 2008). Moreover, acclimation to low oxygen levels increases not only hypoxia tolerance (e.g. Rees et al., 2001) but also maximum temperature tolerance of cardiac tissue (Burleson and Silva, 2011). Cross-tolerance between hypoxia and temperature tolerance, of course, lends support the OCLTT hypothesis (Pörtner and Knust, 2007; Pörtner, 2010), which proposes that failure to survive at high temperatures is a result of insufficient oxygen supply to meet increasing tissue oxygen demand associated with higher temperature.

Here, we provide additional support for the OCLTT hypothesis with our observations at the tissue and molecular levels. We show for the first time that fish with larger ventricles have a higher capacity to tolerate elevated temperatures. Indeed, both total and relative ventricular mass correlated with CT_{max}, such that a fish with a small heart relative to body mass had a significantly lower CT_{max}. That variation in ventricle mass could explain 21% of the variation in the CT_{max} was an unexpected finding. It has been shown previously that fish with larger ventricles have larger stroke volumes (Franklin and Davie, 1992), and because cardiac output is a product of stroke volume and heart rate, fish with larger ventricles could have the capacity for a higher cardiac output, which in turn might support a better oxygen supply to tissues at high temperature. This would then translate to a higher temperature tolerance as observed here. This connection lends supports the hypothesis that maximum temperature tolerance is indeed capacity and oxygen limited. Interestingly, thermal acclimation studies with salmonids consistently show that cold-acclimated fish possess a larger RVM but their upper temperature tolerance is reduced (e.g. Farrell et al., 1988; Klaiman et al., 2011), which is somewhat contradictory to the current results. In cold-acclimated fish, increased ventricle mass, which involves increases in connective tissue and spongy myocardium (Klaiman et al., 2011), is thought to compensate for reduced heart rate by increasing stroke volume. Perhaps the association between RVM and CT_{max} involves a greater percentage of compact myocardium among individuals more tolerant of warm temperature given that warm-acclimated fish increase the amount of compact myocardium, which is thought to be a compensatory response to high temperature compromising oxygen supply to the heart itself (Farrell et al., 1996; Klaiman et al., 2011).

CT_{max} was also positively correlated with the Mb level in the heart, which could be similarly connected to the OCLTT concept and oxygen supply to the heart itself. In salmonids, the majority of the ventricle is devoid of coronary circulation, and oxygen is instead obtained from venous blood *via* diffusion (Farrell, 2002). Mb both stores oxygen and facilitates oxygen transfer from venous blood to tissue (Wittenberg et al., 1975). Both functions could be extremely beneficial if oxygen supply to the heart does indeed become problematic at high temperature, which could then extend the heart's capacity to support aerobic activity at elevated temperatures as well as temperature tolerance. Poupa et al. (Poupa et al., 1981) observed that the Mb level increased with size in tuna, *Thunnus thynnus*, and that this rate of Mb increase was elevated as the fish grew bigger, also supporting the function of Mb in facilitating the diffusion of the oxygen to tissues as the diffusion distance increases with the increased size of fish. High Mb levels have also been shown to be connected to elevated myocardial performance in hypoxic conditions (Driedzic et al., 1982), although there are also studies showing that acclimation to hypoxic conditions, for instance, does not increase the Mb level in the heart, even though it does in other tissues (e.g. Cossins et al., 2009). The present study is, however, the first to show that there is a connection between Mb level and the upper temperature tolerance of fish. We suggest that the reason for this may be because Mb protects the heart from functional hypoxia. Although the linkage between cardiac Mb and CT_{max} could protect cardiac tissues from functional hypoxia at elevated temperatures, an alternative explanation is that elevated Mb is beneficial because it scavenges reactive oxygen species and nitric oxide under conditions of reduced oxygen supply (e.g. Flögel et al., 2004; Merx et al., 2005), as high temperature stress increases oxygen radicals (e.g. Heise et al., 2006). Of course, Mb could simultaneously protect the cardiac tissue from heat stress as an antioxidant as well as being a facilitator of oxygen supply.

The absence of correlations between CT_{max} and either aerobic or anaerobic enzymes is interesting. Only a small amount of the variance in CT_{max} was significantly correlated with total cardiac CS activity. Previous studies have shown that cold acclimation increases

the activity of aerobic enzymes (e.g. Guderley, 1990), including in the northern subspecies of killifish (*Fundulus heteroclitus*), which tolerates cold better than the southern subspecies (Fangue et al., 2009; Dhillon and Schulte, 2011). Perhaps aerobic enzyme activities have a larger role in cold rather than warm tolerance. The lack of correlation between LDH activity and CT_{max} is interesting because LDH catalyses the conversion of pyruvate to lactate and of NADH to NAD^+, which are important in sustaining glycolytic flux during cellular hypoxia. High LDH activities are also connected to increased hypoxia tolerance (e.g. Almeida-Val et al., 2000); thus, while we expected fish with a higher CT_{max} to have a higher activity of LDH, this was not the case at the individual level.

Another interesting finding was the lack of correlation between resting levels of both HSP 90β and HSC 70 and CT_{max}. Multiple studies have shown that heat stress induces expression of the HSPs at the mRNA and protein levels under physiologically and environmentally relevant conditions (e.g. Podrabsky and Somero, 2004; Hori et al., 2010; Currie, 2011). For example, increased expression of HSC 70 is thought to be related to high temperature tolerance in killifish (Fangue et al., 2006). Instead, our results are consistent with those of a recent study of LeBlanc et al. (LeBlanc et al., 2012), who found that rainbow trout with a low cortisol response to stress did not increase HSP/HSC expression after heat stress as much as fish with a high cortisol response and, even though there was significant difference in HPS/HSC levels, CT_{max} values were similar. Perhaps in salmonids, the resting HSP 90β and HSC 70 levels are not as important in defining CT_{max} *per se* as other cellular and physiological traits.

In summary, we show that temperature tolerance and hypoxia tolerance of Atlantic salmon is highly variable at the family level and that these phenotypes appear to have a significant genetic component, which means that these individuals possess the raw material on which natural selection can act. As a result, Atlantic salmon may possess at least some capacity to adapt to environmental changes such as global warming. In addition, we suggest that individuals or populations with a larger heart and higher myocardial Mb levels might be selected under such conditions. Collectively, our data lend strong support the concept of OCLTT from the whole animal to the tissue and subcellular levels.

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LIST OF SYMBOLS AND ABBREVIATIONS

BSA	bovine serum albumin
CS	citrate synthase
CT_{max}	critical maximum temperature
HSC	heat shock cognate
HSP	heat shock protein
LDH	lactate dehydrogenase
LOE	loss of equlibrium
Mb	myoglobin
M_b	body mass
M_v	ventricle mass
OCLTT	oxygen and capacity limited thermal tolerance
PIT	passive integrated transponder
QTL	quantitative trait locus
RVM	relative ventricle mass
SDS	sodium dodecyl sulphate
TBST	Tris-buffered saline with Tween 20
T_{opt}	optimum temperature

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