BRIEF COMMUNICATION

Hematocrit Is Associated with Thermal Tolerance and Modulated by Developmental Temperature in Juvenile Chinook Salmon

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ABSTRACT

To evaluate whether oxygen-carrying capacity influences thermal tolerance in fishes, we reared four Chinook salmon families in present-day (+0°C) and possible future (+4°C) temperatures and assessed the response of hematocrit (Hct) to acute temperature stress. In the +4°C treatment, Hct increased above control levels when juvenile fish were exposed to their critical thermal maximum (CT_{max}). Conversely, no effect of temperature stress on Hct was found in the +0°C treatment. Hct was positively associated with CT_{max} ($r^2 = 0.12$; $n = 66$), contributing to the CT_{max} of the +4°C treatment being significantly higher than that of the +0°C treatment (mean ± SD, 26° ± 0.6°C and 25° ± 0.5°C, respectively). The association between CT_{max} and Hct found here supports the hypothesis that thermal tolerance is affected by oxygen supply to tissue. Moreover, the developmental plasticity of CT_{max} and Hct could represent an adaptive mechanism for salmon faced with climate change.

Keywords: climate change, oxygen transport, thermal tolerance, fish, phenotypic plasticity, developmental acclimation.

Introduction

Rising global temperatures associated with climate change are projected to cause widespread change in biological communities due to the fundamental effect that temperature has on organisms (Dillon et al. 2010). Individuals will have to cope with both warmer temperatures throughout their ontogeny and increases in the frequency and severity of acute changes in temperature. Over the longer term, populations will likely have to evolve physiological mechanisms to contend with the increases in temperature projected to occur over the course of this century. Evaluating the adaptive potential of populations faced with climate change thus requires an understanding of the potential for acute, developmental, and evolutionary responses to warm temperatures.

The traditional index for measuring acute thermal tolerance in aquatic ectothermic organisms such as fish is the critical thermal maximum (CT_{max}), which is the temperature at which an individual loses a directed locomotor capacity and their righting response (Lutterschmidt and Hutchison 1997). Measuring CT_{max} for large numbers of individuals is relatively simple and provides a useful index of relative thermal tolerance among groups of organisms. However, CT_{max} represents a functional collapse of the whole animal, and other key physiological systems can collapse before the collapse of the whole animal (Farrell et al. 2009; Farrell 2016). Indeed, the physiological basis of the realized limits of thermal tolerance has been subject to much research and debate. A commonly supported yet controversial hypothesis is the oxygen- and capacity-limited thermal tolerance hypothesis (OCLTTH), which posits (in part) that an insufficient supply of oxygen to tissue limits upper thermal tolerance (Pörtner and Knust 2007; Pörtner and Farrell 2008). However, the generality of the OCLTTH has been called into question (Clark et al. 2013; Clark and Mark 2017). If an insufficient supply of oxygen to tissue does indeed limit upper thermal tolerance in fishes, arterial oxygen convection (i.e., the product of cardiac output and arterial oxygen content) should become constrained as fish are warmed, while increased oxygen-carrying capacity of the blood should be associated with higher thermal tolerance. A seemingly universal finding in fishes is that cardiac function (i.e., cardiac output, heart rate, and mitochondrial metabolic capacity) peaks and then declines at temperatures lower than CT_{max} (Farrell et al. 1996; Gollock et al. 2006; Hilton et al. 2010; Mendonça and Gamperl 2010; Eliason et al. 2013; Iftikar and Hickey 2013; Ferreira et al. 2014; Muñoz et al. 2014; Penney et al. 2014; Rodnick et al. 2014; Ekström et al. 2017). Conversely, tests of the relationship between CT_{max} and oxygen-carrying capacity have yielded mixed results.

A common measure of oxygen-carrying capacity is hematocrit (Hct), which is the proportion of red blood cell volume to total blood volume. Two main approaches have been used to test the relationship between CT_{max} and Hct: (1) experimental manipulation of oxygen availability or transport capacity within in-

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dividuals of the same species (Wang et al. 2014; Brijs et al. 2015) and (2) comparative studies of interspecific variation in these traits (Beers and Sidell 2011). Experimentally induced anemia did not affect CT_{max} in both European perch (*Perca fluviatilis*; Brijs et al. 2015) and sea bass (*Dicentrarchus labrax*; Wang et al. 2014), providing evidence against the OCLTTH. Conversely, an interspecific comparison of five Antarctic notothenioid fishes found a strong, positive relationship between CT_{max} and Hct at the species level, supporting the OCLTTH (Beers and Sidell 2011). An alternative approach is to use natural, intraspecific variation in CT_{max} and Hct to evaluate their functional relationship. Such use of interindividual variation to assess functional, ecological, or evolutionary relationships among physiological traits was advocated for 30 yr ago in an influential article by Bennett (1987). Although the use of individual variation as a tool in ecological physiology remains an "underutilized resource," recent review articles have emphasized the power of this approach (Williams 2008; Killen et al. 2016; Roche et al. 2016; Ward et al. 2016). Still, individual variation in CT_{max} and Hct has yet to be described.

The aim of this study was to assess whether Hct explains variation in CT_{max} among juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and to evaluate the potential for acute and developmental responses of Hct to high temperatures. Chinook salmon populations have been reported to experience increased rates of mortality in high river temperatures (Crozier and Zabel 2006) and are predicted to be vulnerable to the projected increases in temperature associated with climate change (Muñoz et al. 2015). We reared fertilized Chinook salmon eggs in one of two developmental temperature treatments and measured the acute response of Hct in hatched, juvenile fish following exposure to their CT_{max}. We used a total of 187 individuals from four different families in the experimental design. We predicted that Hct would be positively associated with CT_{max}.

Methods

The fish collection and rearing protocol are detailed in Muñoz et al. (2015). In brief, gametes from wild Chinook salmon captured in the Quinsam River in northeast Vancouver Island, British Columbia, were transported to a nearby aquaculture facility (Yellow Island Aquaculture, Quadra Island) for fertilization on October 23, 2012. Four unique families were produced by crossing four males with four females (i.e., unique paternity and maternity of each family), with each cross replicated four times. Two of the replicates of each family were reared in temperatures that simulated the current thermal conditions of the Quinsam River (termed the +0°C developmental treatment; mean ± SD temperature throughout incubation, 5.6° ± 1.7°C; minimum, 3.6°C; maximum, 10.1°C), whereas the other two replicates were reared in ~4°C warmer temperatures than the +0°C group to simulate possible future conditions (the +4°C developmental treatment; mean ± SD temperature throughout incubation, 10.0° ± 1.5°C; minimum, 7.9°C; maximum, 12.3°C).

The conclusion of the endogenous feeding stage (i.e., emergence as free-swimming juveniles) occurred ~90 and ~140 d postfertilization in the +4°C and +0°C groups, respectively. The mass of offspring in these two groups averaged 0.56 ± 0.08 and 0.54 ± 0.07 g, respectively. At this time, hatched offspring were subjected to one of two acute treatments: the control treatment, whereby fish were sampled directly from their incubation environment without exposure to an acute temperature change, and the CT_{max} treatment, whereby fish were sampled following an acute increase in temperature and exposure to their CT_{max}. Hct was used as an index of oxygen-carrying capacity that could be easily measured for large numbers of fish in a field setting. Hct was measured in fish from both the control treatment ($Hct_{control}$) and the CT_{max} treatment (Hct_{CTmax}). In the control treatment, individuals from each family and developmental treatment (n = 109 fish in total, 9–18 fish per family per treatment) were anesthetized in 100 mg L^{-1} MS-222 (Sigma-Aldrich, St. Louis, MO), weighed (± 0.001 g), and sampled for their blood by severing the caudal peduncle and filling a heparinized capillary tube (Fisher Scientific, Waltham, MA) as blood flowed out of the caudal vein. The tubes were then sealed and centrifuged in a Hct centrifuge for 5 min at 3,000 rpm. The proportion of red blood cell volume to whole blood volume was measured twice per sample using digital calipers (± 0.01 mm); the mean value was used as Hct.

Fish in the CT_{max} acute treatment group were moved directly from their incubation tray to a 50-L insulated tank. Offspring from the same family were placed into a floating, cylindrical container within the tank (volume of container, 2.0 L). Two or three containers were used per trial, with three or four offspring within each container. Fish were left in the containers for 1 h before the trial, with the water maintained at the acclimation temperature of the fish (6°C for the +0°C group, 10°C for the +4°C group) via an in-line chiller (Fisher Scientific) that recirculated aerated water through the tank. After 1 h, temperature was acutely increased at a rate of 0.3°C min^{-1}. Fish were monitored and removed from the tank at their CT_{max} (i.e., immediately after loss of their righting response). At this time, fish were anesthetized, weighed, and sampled for their Hct_{CTmax} as described above. The CT_{max} of 78 fish was measured, using 6–15 fish per family per treatment. The Hct_{CTmax} of 12 individuals could not be measured; these individuals were therefore removed from all analyses involving Hct.

A family ID term was included in statistical analyses to capture family-level differences, which includes any additive genetic, nonadditive genetic, and maternal effects. CT_{max} was analyzed using an ANOVA model with developmental treatment and family as fixed factors, as well as their interaction. To assess how Hct responds to high temperatures both developmentally and acutely, the $Hct_{control}$ and Hct_{CTmax} data were combined. A three-way ANOVA model of Hct was used with developmental treatment, acute treatment (i.e., control or CT_{max} treatment), and family as fixed factors, as well as the interactions of each factor. All statistical analyses were performed using JMP (ver. 12; SAS, Cary, NC). All means are reported ±1 SD.

Results

In the +0°C and +4°C developmental treatments, $Hct_{control}$ averaged 34% ± 5% and 36% ± 7%, respectively, whereas Hct_{CTmax} averaged 36% ± 6% and 41% ± 6%, respectively (fig. 1). Hct

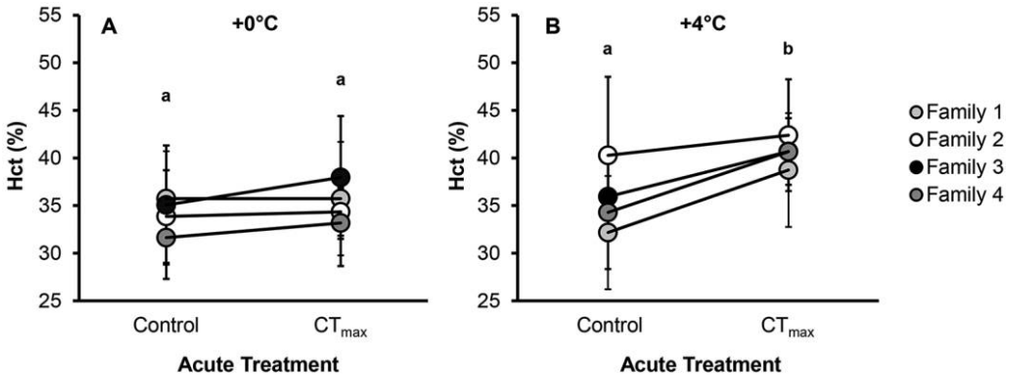

Figure 1. Developmental, acute, and family effects on hematocrit (Hct) in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). The offspring of four families developed in current (+0°C; *A*) or future (+4°C; *B*) temperature conditions. Hct was measured in fish that were not exposed to an acute temperature change (control) and in fish that were exposed to their critical thermal maximum (CT_{max}) following an acute increase in temperature. Values are mean ± SD. Different letters above treatment groups indicate a significant ($P < 0.05$) difference across developmental and acute treatments according to a Tukey's post hoc test. A color version of this figure is available online.

was weakly yet significantly associated with body mass ($r^2 = 0.08$, $P < 0.001$); mass-independent residuals of Hct were thus used in subsequent analyses. Across both $Hct_{control}$ and Hct_{CTmax} measurements, there was a significant interaction between the acute and developmental treatment effects (table 1). Indeed, only fish from the +4°C developmental treatment group had significantly increased Hct_{CTmax} levels relative to their $Hct_{control}$ (fig. 1). There was also a significant interaction between family and developmental treatment (table 1) driven by family 2, which after development at +4°C had relatively high $Hct_{control}$ levels and only a minor increase in Hct following acute temperature stress (fig. 1). With family 2 excluded from the analysis, the family × developmental treatment interaction effect was nonsignificant ($F_{2, 119} = 2.69$, $P = 0.07$), whereas the acute × developmental treatment effect was stronger ($F_{1, 119} = 4.46$, $P = 0.037$).

CT_{max} was significantly higher in the +4°C developmental treatment group than in the +0°C group, averaging 26.0° ± 0.6°C and 25.0° ± 0.5°C, respectively (fig. 2). There was a significant effect of the interaction between family and developmental treatment on CT_{max} (table 2). Again, this interaction was driven by family 2, whose CT_{max} was relatively unaffected by developmental temperature (fig. 2); with this family excluded from the analysis, both the family effect ($F_{2, 51} = 0.76$, $P = 0.473$) and the family × developmental treatment effect ($F_{2, 51} = 0.41$, $P = 0.664$) were nonsignificant, whereas the developmental treatment effect remained highly significant ($F_{1, 51} = 154$, $P < 0.001$). A regression analysis revealed a significantly positive relationship between Hct_{CTmax} and CT_{max} ($r^2 = 0.12$, $P = 0.004$; fig. 3). The strength and significance of this relationship were unaffected by the use of mass-independent residuals of Hct ($r^2 = 0.11$, $P = 0.007$).

Discussion

The OCLTTH provides a mechanistic framework for understanding the limits of thermal tolerance in aquatic ectothermic organisms. We found support for the OCLTTH in that thermal tolerance was positively associated with the oxygen-carrying capacity of blood, with Hct_{CTmax} explaining 12% of the variation in CT_{max} across 66 individual Chinook salmon. This result is in accord with an interspecific comparison of CT_{max} and Hct among five species of notothenioid fishes (Beers and Sidell 2011). It is unclear why this link between Hct and CT_{max} was not similarly found in studies of experimental anemia in European perch and sea bass (Wang et al. 2014; Brijs et al. 2015). However, an important consideration is that the effect size of the Hct-CT_{max} relationship is small ($r^2 = 0.12$ here, $r^2 = 0.23$ in Wang et al. 2014), making the effect difficult to detect with small sample sizes. Moreover, the large amount of variation in CT_{max} that has not been explained by Hct suggests that there are factors that limit acute thermal tolerance more significantly than the oxygen-carrying capacity of blood. These factors could include other mechanisms involved in the cardiorespiratory oxygen cascade (reviewed in Farrell 2009). Alternatively, oxygen limitation (i.e., tissue hypoxia) could play only a minor role in inducing the loss

Table 1: Results of a three-way ANOVA describing acute, developmental, and family effects on the hematocrit of juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

	df	SS	*F*	*P*
Acute treatment	1	329	10.7	**.001**
Developmental treatment	1	393	12.8	**.001**
Family	3	202	2.19	.091
Acute × developmental	1	120	3.90	**.050**
Acute × family	3	42	.45	.715
Developmental × family	3	344	3.73	**.013**
Acute × developmental × family	3	51	.55	.647
Residual	158	4,854	...	...

Note. Boldface type indicates statistical significance ($P < 0.05$).

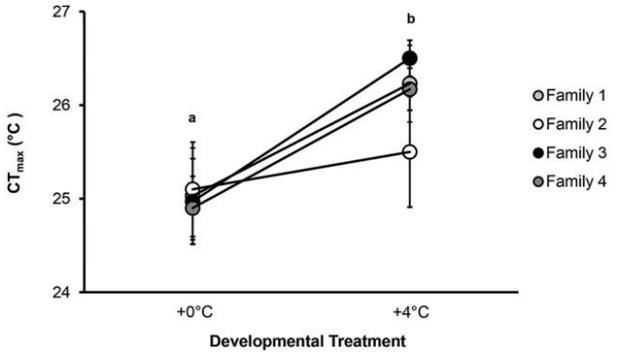

Figure 2. Developmental and family effects on critical thermal maximum (CT_{max}) in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). The offspring of four families developed in current (+0°C) or future (+4°C) temperature conditions, and the CT_{max} of offspring from each family and developmental treatment was measured. Values are mean ± SD. Different letters above treatment groups indicate a significant ($P < 0.05$) difference between treatments according to a two-way ANOVA. A color version of this figure is available online.

of locomotor capacity that occurs at CT_{max}; instead, factors such as protein denaturation, oxidative stress, or effects on membrane fluidity could be key contributors (e.g., Lushchak and Bagnyukova 2006). Indeed, more research is needed on the extent of oxygen limitation that occurs before acutely lethal temperatures are reached as well as on how other physiological functions affect performance during thermal challenges (Clark et al. 2013). Nevertheless, there is evidence that the OCLTTH explains physiological principles that have been maintained across phyla (Pörtner and Giomi 2013), and our study provides another line of evidence that thermal tolerance is affected by oxygen supply to tissue in fish.

We used Hct as a proxy for oxygen-carrying capacity, an approach that has been widely used but is not without its limitations (for a detailed discussion, see Gallaugher and Farrell 1998). Hct increases rapidly in response to acute stressors such as handling, air exposure, exercise, and anesthesia (Pearson and Stevens 1991; Gallaugher et al. 1992; Biron and Benfey 1994). Such rapid increases in Hct can occur due to increases in both the number and the size of red blood cells; fish spleens contain reservoirs of erythrocytes that can be released into the blood within minutes of an acute stress (Pearson and Stevens 1991), while erythrocytic swelling due to elevated blood CO_2 also increases Hct following acute stress (reviewed in Weber and Jensen 1988; Gallaugher and Farrell 1998). Blood samples obtained via acute venesection, as employed here, result in Hct values that are higher than those obtained from cannula due to cannulation techniques being less stressful for fish (Wells and Weber 1991). Thus, the Hct values from both the control treatment (34% ± 5% and 36% ± 7% for the +0°C and +4°C groups, respectively) and the CT_{max} treatment (36% ± 6% and 41% ± 6% for the +0°C and +4°C groups, respectively) are likely higher than basal Hct values of resting fish due to splenic release of erythrocytes and erythrocytic swelling. Indeed, the Hct values found here are similar to values previously found for Chinook salmon sampled via venesection (37%–39%; Brauner et al. 1993) and are higher than samples from cannulated fish (23%–30%; Thorarensen et al. 1993; Gallaugher 1994). Because all individuals in our study were handled similarly, erythrocytic swelling should not have differentially affected the Hct values of any one treatment and would therefore not affect our statistical comparisons. Thus, we interpret differences in Hct as numerical differences in red blood cells.

The developmental modification of the Hct acute stress response and of CT_{max} could represent adaptive mechanisms by which salmon can cope with warm temperatures. Increasing Hct in deleteriously high temperatures could help maintain oxygen delivery after cardiac function collapses, which occurs at temperatures cooler than CT_{max} (Eliason et al. 2013; Muñoz et al. 2014). Likewise, the increase in CT_{max} from 25.0° ± 0.5°C in the +0°C developmental treatment to 26.0° ± 0.6°C in the +4°C treatment could represent a small yet important increase in thermal tolerance via developmental plasticity. The maximum temperature of the stream in which the study population resides during their juvenile stage is projected to surpass 24.6°C by 2100 under the maximum global warming scenario (Muñoz et al. 2015), suggesting that individuals in this population will require the full breadth of their acute thermal tolerance. We also found a significant effect of family ID on CT_{max} as well as family × developmental treatment effects on both CT_{max} and Hct. This variation among families could represent heritable (i.e., additive) genetic variation, which would be evidence for evolutionary potential of Hct and CT_{max} in this population. However, the design used in our study prevents distinguishing additive genetic effects from maternal or nonadditive genetic effects. Studies of the heritability of thermal tolerance in Chinook salmon have found significant maternal effects on CT_{max} and the arrhythmic temperature of the heart as well as additive genetic effects on measures of cardiac capacity (Muñoz et al. 2014, 2015). Continued integration of physiological and quantitative genetic data, whereby the extent of physiological variation explained by heritable genetic variation is explicitly assessed, is a promising approach to understanding the potential for evolutionary responses to climate change (e.g., Muñoz et al. 2015; Munday et al. 2017).

The data presented here represent the first association of CT_{max} and Hct using natural, individual variation within a species, finding support for the hypothesis that upper thermal tolerance is affected by oxygen supply to tissue. We also found significant acute, developmental, and family-specific responses to high temperature stress, indicating a variety of processes that could contribute to the adaptive mechanisms available to salmon

Table 2: Results of a two-way ANOVA describing developmental and family effects on the critical thermal maximum of juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

	df	SS	*F*	*P*
Developmental treatment	1	24	119	**<.001**
Family	3	2.0	3.26	**.026**
Developmental × family	3	3.1	5.09	**.003**
Residual	76	20	...	...

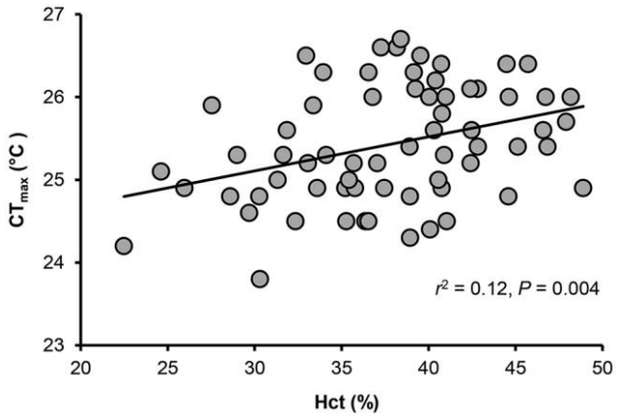

Figure 3. Relationship between hematocrit (Hct) and critical thermal maximum (CT_{max}) in individual Chinook salmon (*Oncorhynchus tshawytscha*; $n = 66$).

populations challenged by climate change. Continued study of the mechanistic basis of thermal tolerance across taxa is necessary for our understanding of which traits will be limiting the viability of individuals and populations in warmer environments as well as the potential for these traits to adaptively respond to such change.

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