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# The effect of temperature acclimation on thermal tolerance, hypoxia tolerance and aerobic scope in two subspecies of sheepshead minnow; *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi*

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A B S T R A C T

The freshwater teleost *Cyprinodon variegatus hubbsi* (*Cvh*) diverged from its euryhaline relative *Cyprinodon variegatus variegatus* (*Cvv*) ~150 kya and these subspecies are physiologically distinct in their osmoregulatory capabilities. *Cvv* inhabits intertidal estuaries and saltwater marshes along the Gulf of Mexico and Atlantic coast, where they experience a broad temperature range from −1.9 to 43 °C and frequent bouts of hypoxia. In contrast, *Cvh* lives in several lakes in central Florida, where temperature is more stable (12–31 °C) and hypoxia is uncommon. To assess whether relaxed selective pressure on *Cvh* has resulted in reduced temperature and hypoxia tolerance, a comparative study on the effects of acclimation to 25, 30 and 35 °C on critical thermal tolerance (CTMax), hypoxia tolerance, and aerobic scope was performed. The CTMax was similar between subspecies and positively correlated with acclimation temperature. Neither subspecies, however, could survive at 38 °C for a prolonged period of time. In general, *Cvv* displayed greater hypoxia tolerance and aerobic scope relative to *Cvh* over the range of acclimation temperatures. Routine metabolic rate was significantly lower while maximum metabolic rate and aerobic scope were significantly higher in *Cvv*, but only in fish acclimated to 30 °C. Overall, the different responses of *Cvh* to relaxed selective pressure suggest these traits are weakly linked physiologically in these fishes.

## 1. Introduction

The euryhaline sheepshead minnow, *Cyprinodon variegatus variegatus* (*Cvv* hereafter; Lacépède 1803) inhabits the Gulf of Mexico and Atlantic coasts of North America. A closely related freshwater subspecies, *Cyprinodon variegatus hubbsi* (*Cvh* hereafter; Carr 1936), diverged from *Cvv* ~150 kya and inhabits seven lakes in central Florida (Darling, 1976; Guillory and Johnson, 1986). Previous studies suggest a taxonomic revision of these subspecies into separate species as they show significant pre- and post-zygotic isolation (Brix and Grosell, 2013) and have significant, genetically-based differences in osmoregulatory capabilities (Brix et al., 2015; Brix and Grosell, 2012).

In addition to salinity, the habitats of *Cvv* and *Cvh* differ in temperature and oxygen availability, abiotic characteristics known to greatly impact the physiology of fishes. *Cvv* is highly eurythermic experiencing temperatures ranging from −1.9 to 43 °C across its geographic distribution, with diurnal fluctuations as large as 15 °C (Bennett and Beitinger, 1997). In contrast, *Cvh* occurs in a comparatively stenothermic environment and experiences annual temperature fluctuations ranging from 12–31 °C with limited diurnal fluctuations. *Cvv* typically lives in euryoxic salt marshes with dissolved oxygen levels ranging from complete anoxia to hyperoxia. In contrast, oxygen levels in the lakes to which *Cvh* is resident are generally normoxic (> 16.5 kPa; fully saturated $pO_2$ at sea-level = 21 kPa), although urban and agricultural development has led to some eutrophication of the lakes and sporadic periods of hypoxia (4.1–6.2 kPa) have been observed for short periods of time (days to weeks) (monitoring data at http://www.lake.wateratlas.usf.edu).

Using the temperature tolerance polygon method, *Cvv* has been previously characterized as the most eurythermal fish studied to date with a maximum thermal tolerance (CTMax) of 45.1 °C (Beitinger and Bennett, 1999; Bennett and Beitinger, 1997). *Cvv* is also known to be relatively hypoxia tolerant, able to survive hypoxic conditions (5.3 kPa at 30 °C) for at least 24 h and has been observed to occur in hypoxic saline springs at even lower oxygen tensions (< 2.4 kPa) (Odum and Caldwell, 1955; Peterson, 1990). To the best of our knowledge, the temperature and hypoxia tolerance of *Cvh* has not been previously investigated.

These differences in temperature and oxygen regimes between the two subspecies led us to hypothesize that *Cvh* may have evolved reduced tolerance to high temperatures and low oxygen tensions. To test this hypothesis, we conducted a series of experiments with *Cvv* and *Cvh* to measure several physiological parameters that, in combination, can characterize the temperature and hypoxia tolerance of an aquatic organism. Specifically, we measured Critical Thermal Maximum (CTMax) as a proxy for maximum temperature tolerance, Time to Loss of

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Equilibrium (LOE) at 0.43 kPa as a proxy for hypoxia tolerance, and aerobic scope (AS), as the difference between maximal and resting metabolic rate. For both subspecies, each of these parameters was measured in fish acclimated to 25, 30, or 35 °C to evaluate how ambient temperature influenced physiological performance.

## 2. Material and methods

*Cyprinodon variegatus variegatus* (Mean ± SEM; 1.13 ± 0.03) were collected from a tidal pond on Virginia Key, Florida, and *Cyprinodon variegatus hubbsi* (1.09 ± 0.03) were collected from Lake Dora, in central Florida. They were air-shipped to the Department of Zoology at the University of British Columbia, where all experiments were conducted. Upon arrival, the subspecies were held separately in 60-L freshwater filled glass aquaria (20 fish/aquaria) outfitted with charcoal filters. The water consisted of dechlorinated City of Vancouver tapwater ($[Na^+]$ = 170 uM, $[Cl^-]$ = 210 uM, hardness, 30 mg/L $CaCO_3$ (Ou et al., 2015)) supplemented with salts (0.76 mM $CaSO_4(2H_2O)$, 0.3 mM $CaCl_2$, 0.51 mM $NaHCO_3$, 0.05 mM $KHCO_3$, 0.2 mM $MgSO_4(7H_2O)$). This water was then adjusted with NaCl to 7 mM $Na^+$, which is the lower osmoregulatory limit for *Cvv* (Brix and Grosell, 2013). All aquaria were maintained in a common environmental chamber on a 12 L:12D photoperiod at 25 °C. Fish were fed daily to satiation (Tetramin™ Tropical Flakes, VG, USA). Under these common garden conditions, fish were bred as described in Brix and Grosell (2013). $F_1$ fish from each subspecies were raised to the adults and used in all experiments.

Fish were acclimated to temperatures of 25, 30 or 35 °C for 4 weeks prior to experiments. Acclimation to 30 or 35 °C was achieved by increasing water temperature from 25 °C by 1 °C/day using submersible electric heaters plugged into temperature controllers (Fisherbrand™ Traceable™ Digital Temperature Controller, ON, Canada) until the acclimation temperature was reached. In pupfishes (*Cyprinodon artifrons* and *Cyprinodon dearborni*), acclimation to elevated temperatures for as little as 3 days has resulted in an increase in CTMax (Chung, 1981; Heath et al., 1993). Thus, we assume that the 4-week duration used here is sufficient for these fishes to acclimate to new temperatures. We also attempted to acclimate both subspecies to 40 °C, however, a noticeable decrease in activity and body mass were observed by the time fish reached 38 °C and at this temperature, 40% morbidity was observed within 3 days. Consequently, the highest acclimation temperature used in this study was 35 °C.

After 4 weeks of acclimation to 25, 30 or 35 °C, aerobic scope was measured. Fish were then allowed a week of recovery at their respective acclimation temperatures and then divided randomly into groups for assessment of either thermal tolerance or hypoxia tolerance. All fish were fasted for 36–48 h prior to experimentation. Treatment of all experimental animals was in accordance with the UBC animal care protocol #A11-0235.

### 2.1. Aerobic scope

After 4 weeks of acclimation, 8 fish were transferred to individual 250-mL glass stop-flow respirometers held within a 100-L recirculating system at the respective acclimation temperature and allowed to recover overnight (~12 h). In the morning, 3–4 respirometers were sealed and a Presense FibreOptic oxygen probe (PreSens Precision Sensing, Regensburg, Germany) was inserted into each chamber and the decline in oxygen levels were recorded every 3 s. Fish remained quiescent during the routine metabolic rate (RMR) measurement, which lasted ~20–30 min until water oxygen levels were reduced to ~16.5 kPa. Then, flow to the respirometers was re-instated and fish were allowed to recover for an hour in fully oxygenated water.

After recovery, each fish was removed from the respirometer, placed in a 10-L bucket with the same water conditions and hand-chased until exhaustion. The point of exhaustion was determined by the inability of the fish to actively swim away when turned upside down. Preliminary trials demonstrated that these species do not swim in a swim tunnel. Previous studies in other species have found results from chase protocols to yield similar or higher maximum metabolic rates (MMR) than those from alternative methods such as a swim tunnel (Norin and Clark, 2016; Reidy et al., 1995; Sylvestre et al., 2007). Therefore, we assumed MMR measured using a chase protocol is representative of the maximum capacity of oxygen delivery and use, providing an adequate estimation of aerobic scope.

The fish was then quickly transferred back into the respirometer (< 10 s) and the decline in water oxygen levels was recorded until ~18.5 kPa to measure maximum metabolic rate (MMR). The initial 10–15 s were discarded due to unstable traces during probe insertion and sealing of the respirometer. For all fish, the total duration of MMR measurement was < 10 min. The oxygen consumption for every minute was calculated and the average of the 3 highest rates – which all occurred within the initial 5 min – was used to calculate MMR to minimize variability. The mass-specific metabolic rates (RMR and MMR) were calculated with the collected oxygen saturation data, wet weight of the fish, oxygen solubility coefficient at the appropriate salinity and temperature, and volume of the respirometer (Benson and Krause, 1984; Fangue et al., 2009; Sloman et al., 2008). Aerobic scope (AS) is the difference between maximum and minimum metabolic rates, which can be influenced by ambient temperature in ectotherms (Fry, 1947). Here, we calculated aerobic scope by subtracting RMR (a commonly used estimate of the standard metabolic rate which represents the minimum oxygen consumption rate required to sustain life; SMR), from MMR and hence termed it A*Sr*. The factorial aerobic scope (FA*Sr*; MMR divided by the RMR) was also calculated and compared between subspecies and temperature treatment groups. The "*r*" subscript is to acknowledge that routine rather than standard metabolic rates were used in our calculation.

Metabolic rate measurements were always measured during the day (9:00–14:00 h) to minimize the effect of daily cycle dependent metabolic activity (Brett and Zala, 1975; Steffensen, 1989). The respirometers were rinsed after each trial with 90% ethanol and an empty respirometer was included in every metabolic rate run to account for possible bacterial respiration, which was always found to be negligible.

### 2.2. Thermal tolerance

To measure and compare subspecies' critical maximum thermal tolerance (CTMax) following acclimation to 25, 30 and 35 °C, water was slowly heated until loss of equilibrium (LOE: inability to actively right itself) was observed. Fish were transferred from their acclimation aquaria to 20-L fibreglass aquaria and allowed an hour to recover ($n$ = 8). Aquaria were initially held at the respective acclimation temperature and then increased by 0.3 °C/min (Becker and Genoway, 1979; Cox, 1974). At LOE, the temperature was noted (indicative of CTMax), and the respective fish was rapidly removed and weighed, with care not to disturb the remaining fish. Throughout the trial, water was constantly aerated to ensure air equilibrated oxygen levels and to avoid thermal stratification. This procedure continued until all fish in the tank experienced LOE.

### 2.3. Hypoxia tolerance

To measure and compare the hypoxia tolerance of each subspecies' following acclimation to temperatures of 25, 30 and 35 °C, the water oxygen levels were reduced to $PO_2$ of 0.43 kPa and then time to LOE was measured. Eight fish were initially weighed and transferred to individual 473-ml mesh sided chambers submerged in a 50-L Plexiglas tank. The system was maintained at the respective acclimation temperature with electric heaters and controllers described above. During the 1-h acclimation, the water within the tank was continuously aerated and circulated with two submersible water pumps to ensure adequate water flow into all chambers. These pumps were situated under a false

bottom to prevent the generated water current from directly affecting the fishes.

Hypoxia was initiated by bubbling nitrogen gas into the water at a constant rate to reduce oxygen levels by ~1.08 kPa/min, reaching the target of 0.43 ± 0.1 kPa within 20 min. The oxygen was monitored continuously using a NeoFox oxygen probe (Ocean Optics, Dunedin, FL) that was calibrated before every trial. As soon as the target of 0.43 kPa was reached, time to LOE was measured as the end point of hypoxia tolerance. At LOE (as indicated by the inability to maintain dorso-ventral orientation even with a gentle movement of the container), the chamber was removed without disturbing other fish, and the fish was euthanized and weighed.

### 2.4. Statistical analysis

Statistical analyses were performed using R software (version 0.98.1091), and graphs were created with Graphpad Prism software (version 5.0a). All data have been expressed as mean ± SEM. We used log-transformed oxygen consumption rate and mass data to build ANCOVA models for each temperature treatment group. A regression line derived from the ANCOVA was used to calculate the residuals of the logarithm of oxygen consumption. The two subspecies were assumed to have the same effect of weight on oxygen consumption rate. Thus, the $MO_2$ data reported here are all normalized for weight.

Data were analyzed using a type III two-way ANOVA with subspecies and temperature treatment as factors. Data were separated and analyzed using one-way ANOVA for each subspecies with treatment as factor or at each treatment with subspecies as factor. *Post hoc* tests were then carried out using Tukey's HSD. In all cases, differences were considered statistically significant at $p \leq .05$.

## 3. Results

### 3.1. Thermal tolerance

The two subspecies, *C.v.variegatus* and *C.v.hubbsi*, had very similar critical thermal maxima (CTMax) in all three treatments (Table 1; $p > .05$). There was no significant difference in weight between subspecies across all temperatures ($p > .05$), and there was no effect of weight on CTMax for either subspecies ($p > .05$). The CTMax significantly increased after 4 weeks of acclimation to higher temperatures ($p < .001$). When acclimated to 35 °C, the CTMax of both subspecies was up to 44.6 °C.

### 3.2. Hypoxia tolerance

The two subspecies had significantly different hypoxia tolerance in all treatments; Overall, *Cvv* was more hypoxia tolerant than *Cvh* (Fig. 1). Acclimation to higher temperatures for 4 weeks significantly decreased the ability to tolerate hypoxia ($PO_2$ = 0.43 kPa) in both subspecies. At 25 °C, *Cvv* could tolerate 0.43 kPa 6 times longer than *Cvh*. When acclimated to 30 °C and 35 °C, there was a 54% and 93% reduction in time to LOE for *Cvv,* and 58% and 96% *Cvh*, respectively. At 35 °C *Cvh* was very hypoxia intolerant, with most individuals losing equilibrium before the target saturation ($PO_2$ of 0.43 kPa) was reached, which were denoted to have hypoxia LOE of 0 min. There was a significant difference in weight between 25 and 30 °C treatment groups of *Cvh* (25 °C: 0.94 ± 0.03 g; 30 °C: 1.21 ± 0.05 g; $p = .002$), but overall there was no effect of weight on time to LOE for either subspecies ($p > .05$).

### 3.3. Aerobic scope

Acclimation to higher temperatures had a significant effect on aerobic scope of both *Cvv* and *Cvh* ($p = .0006$; $p = .0246$ respectively). For both subspecies, RMR significantly increased (*Cvv*: $p < .0001$; *Cvh*: $p = .0008$) while MMR decreased (*Cvv*: $p = .0096$; *Cvh*: $p = .0840$) following acclimation to higher temperatures (Fig. 2A, B). As a result, aerobic scope and factorial aerobic scope were the lowest at 35 °C for both subspecies (Fig. 2C, D). Following 30 °C acclimation, *Cvv* had lower RMR and higher MMR than *Cvh*, leading to a greater aerobic scope. However, there was a greater increase in RMR and a more prominent decrease in MMR in *Cvv* after acclimation to 35 °C, while MMR of *Cvh* remained similar relative to 30 °C. Thus, the aerobic scopes of two subspecies were similar at the highest acclimation temperature of 35 °C.

**Table 1**
Critical thermal tolerance (CTMax) of two subspecies of *Cyprinodon variegatus* (*C.v.*) measured in this study following 4 weeks of acclimation to 25, 30 or 35 °C and CTMax of other closely related thermal tolerant fishes.

| Acclimation Temperature (°C) | Species | CTMax (°C) |
|---|---|---|
| 25[a] | *C.v.variegatus* | 41.7 ± 0.1 |
| | *C.v.hubbsi* | 41.8 ± 0.2 |
| 30[b] | *C.v.variegatus* | 43.4 ± 0.1 |
| | *C.v.hubbsi* | 43.1 ± 0.2 |
| 35[c] | *C.v.variegatus* | 44.6 ± 0.1 |
| | *C.v.hubbsi* | 44.6 ± 0.1 |
| 38[1] | *C.v.variegatus* | 44.2 ± 0.1 |
| 37–42 daily thermoperiod[1] | *C.v.variegatus* | 45.1 ± 0.1 |
| 26–40 daily thermoperiod[2] | *Cyprinodon artifrons* | 45.3 |
| Field collection in June ~40°C[3] | *Cyprinodon macularius* | 44.6 ± 0.05 |
| Field collection 40–41[4] | *Alcolapia grahami* field | 45.6 ± 0.1 |

Data are mean ± SEM ($n = 8$). There was no significant difference in CTMax between subspecies measured in this study at any acclimation temperature. Letters that differ indicate significant difference between CTMax at acclimation temperatures of both subspecies.

[1] Measurement of CTMax in laboratory after 30 days of acclimation to 38 °C or daily thermocycling between 37–42 °C (Bennett and Beitinger, 1997).

[2] Measurement of Belizian pupfish *Cyprinodon artifrons* CTMax in laboratory after 2 or more days of acclimation to daily thermocycling between 26–40 °C (Heath et al., 1993).

[3] Measurement of desert pupfish *Cyprinodon marcularius* CTMax in the field in June where maximum temperature recorded was 40 °C (Lowe and Heath, 1969).

[4] Measurement of CTMax in the field of Lake Magadi tilapia (*Alcolapia grahami*) fish caught from water at 40–41 °C (Wood et al., 2016).

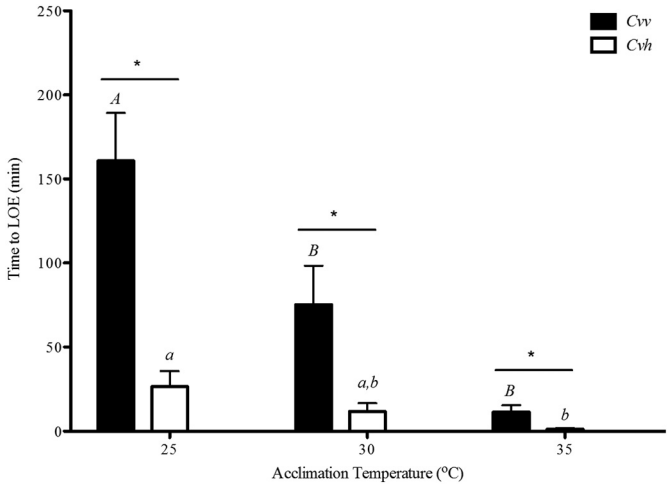


**Fig. 1.** Time to loss of equilibrium (LOE) at 0.43 kPa for *C.v. variegatus* (*Cvv*) and *C.v. hubbsi* (*Cvh*) following 4 weeks of acclimation to 25, 30 or 35 °C. Data are mean ± SEM ($n = 6–8$). Asterisk indicates a significant difference between subspecies within an acclimation temperature. Letters that differ indicate significant difference between acclimation temperatures, where uppercase letters refer to *Cvv* and lowercase to *Cvh*.

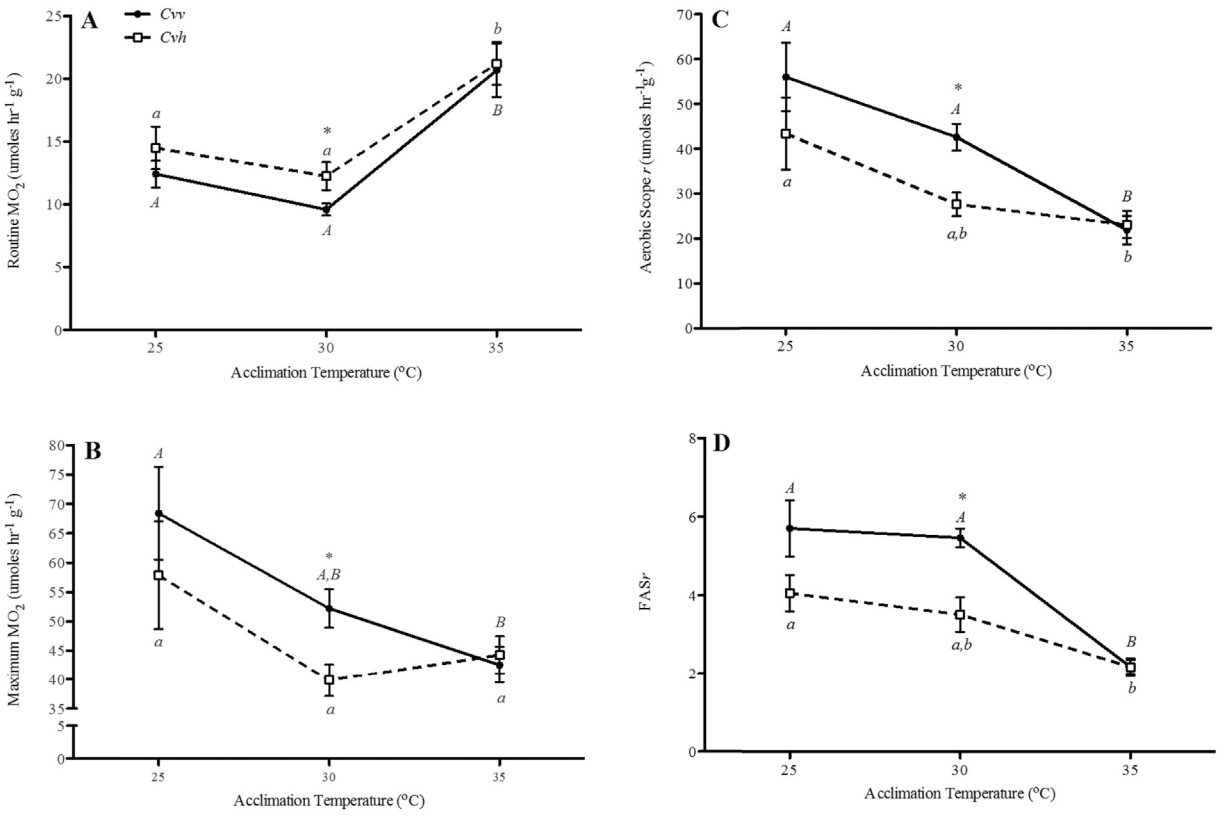


**Fig. 2.** Mass specific (A) Routine metabolic rate; (B) Maximum metabolic rate; (C) Aerobic Scope (AS*r* = MMR-RMR) and (D) Factorial aerobic scope (FAS*r* = MMR/RMR) of *C.v. variegatus* (*Cvv*) and *C.v. hubbsi* (*Cvh*) following 4 weeks of acclimation to 25, 30 or 35 °C. Data are mean ± SEM ($n$ = 7–13). Asterisk indicates significant difference between subspecies within an acclimation temperature. Letters that differ indicate significant differences between acclimation temperatures, where uppercase letters refer to *Cvv* and lowercase to *Cvh*.

## 4. Discussion

### 4.1. Thermal tolerance

Results from the current experiments demonstrate that *Cvv* and *Cvh* have comparable CTMax values across a range of acclimation temperatures. These results are similar to previous studies with *Cvv* and other *Cyprinodon* species that have been acclimated to similar temperatures (Table 1). For example, Bennett and Beitinger (1997) acclimated *Cvv* to 38 °C for 30 days and observed a CTMax of 44.2 °C, similar to the CTMax of 44.6 °C observed for both *Cvv* and *Cvh* acclimated to 35 °C in our study. In another experiment on *Cvv*, Bennett and Beitinger (1997) observed a slightly higher CTMax of 45.1 °C following 30 days of acclimation to daily temperature cycling between 37 and 42 °C. Diurnal temperature cycling is also associated with the highest CTMax observed in a fish of 45.6 °C in the Lake Magadi tilapia (*Alcolapia grahami*) (Wood et al., 2016). The rate of heating can affect CTMax values, as sufficient time must be given for the deep body temperature to match the ambient environment temperature. The heating rates of 0.5–1.5 °C/min that we used here has often been used by others (Lutterschmidt and Hutchison, 1997) and we have restricted comparisons of our CTMax data on *Cvv* and *Cvh* to other species that used a similar heating rate (Bennett and Beitinger, 1997; Healy and Schulte, 2012; Heath et al., 1993; Lowe and Heath, 1969; Wood et al., 2016). The principal mechanism for LOE during CTMax is unclear, but the extreme temperatures can denature proteins, impair enzyme function, increase membrane leakiness, and cause a failure of the central nervous system (Friedlander et al., 1976).

The ability to rapidly acclimate to elevated temperatures (Chung, 1981; Heath et al., 1993) and tolerate acute exposure to extreme temperatures are adaptive traits in the habitats of *Cvv* where significant temperature fluctuations can occur diurnally and maximum temperature can exceed 35 °C. In contrast, *Cvh* experiences relatively stable diurnal temperatures and more moderate maximum temperatures (31 °C) in its natural habitat. Despite relaxation of this selective pressure, *Cvh* appears to have retained the maximum thermal tolerance of its ancestral form, *Cvv*.

It is unclear why we were unable to acclimate fish to 38 °C as accomplished by Bennett and Beitinger (1997), but note that their study was conducted with fish acclimated to seawater (35 PSU) which may be less stressful for *Cvv* than freshwater (Brix and Grosell, 2012). We did not attempt to acclimate fish to 36 or 37 °C and thus can only define the long-term upper thermal limit as > 35 and < 38 °C in freshwater. Future studies should explore whether there is a salinity and temperature interaction associated with the long-term upper thermal limit of *Cvv* and *Cvh*.

### 4.2. Hypoxia tolerance

We observed that *Cvv* is more hypoxia tolerant based upon time to LOE at a $PO_2$ of 0.43 kPa than *Cvh* at all three acclimation temperatures, consistent with our initial hypothesis. Unlike thermal tolerance, *Cvh* has a reduced ability to tolerate hypoxia compared to the ancestral *Cvv*, which appears to be very hypoxia tolerant. For example, *Cvv* exhibited a slightly longer time to LOE at 25 °C than the southern population of *Fundulus heteroclitus* evaluated at 23 °C using the same protocol (McBryan et al., 2016). These similarities in hypoxia tolerance are not surprising given these species often co-occur and consequently are subjected to similar selective pressures. The somewhat better performance in hypoxia of *Cvv* as a function of temperature is consistent with it having a wider, and in particular, more southern distribution than *F. heteroclitus*. The reduced hypoxia tolerance of *Cvh* is also consistent with their occurrence in lakes that are only rarely subjected to

relatively mild hypoxia.

A previous study showed that *Cvv* has a greater gill ionocyte density and area relative to *Cvh* to actively uptake osmolytes at the salinity used in this study ($[Na^+]$ = 7 mM) (Brix and Grosell, 2012). These morphological changes in the gill could create an osmorespiratory compromise that hinders gas exchange by reducing the surface area and thickening the epithelium (Randall et al., 1972). This is consistent with observations by Nordlie et al. (1991) who demonstrated ~20% reduction in RMR in freshwater compared to seawater (35 PSU) for *Cvv*. However, despite this potential constraint, *Cvv* exhibited significantly longer time to LOE and thus hypoxia tolerance than *Cvh*, suggesting that morphological limitations at the gill are not unduly constraining oxygen uptake in *Cvv* under hypoxic conditions or that the superior hypoxia tolerance of *Cvv* is the result of other physiological processes such as anaerobic capacity.

Hypoxia tolerance in both subspecies decreases with acclimation temperature, likely due to a mismatch between oxygen supply and demand. The gas solubility decreases with increasing water temperature, resulting in a lower amount of oxygen per given volume and partial pressure (Benson and Krause, 1984). In addition to the reduced oxygen supply and delivery to respiring tissues, the amount of oxygen required to carry on regular functions and thus RMR increases in warmer water as shown in this study, further constraining the fish in hypoxia and contributing to the decrease in time to LOE.

The physiological mechanisms resulting in LOE during hypoxia in these subspecies is currently unknown. The onset of anaerobic metabolism, magnitude of this recruitment, and oxygen tension at which it is initiated are species-specific (Pörtner and Farrell, 2008; Verberk et al., 2013) and some combination of these factors likely explains the differences in time to LOE observed in this study. Further study into these mechanisms in these two subspecies is warranted to shed insight into the basis of hypoxia tolerance differences.

### 4.3. Aerobic scope

The Oxygen and Capacity- Limited Thermal Tolerance (OCLTT) hypothesis posits that the thermal limit is controlled by an organism's inability to supply and deliver sufficient oxygen to the respiring tissues (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). According to the hypothesis, as temperature increases above the pejus temperature ($T_p$) for an organism, the SMR or RMR will begin increasing faster than MMR (which may begin to actually decrease). The net effect is a reduction in AS which approaches zero at the critical temperature ($T_c$) (Fry and Hart, 1948; Pörtner et al., 2017). In theory, this represents the upper thermal limit of the organism with respect to physiological traits such as long-term survival, growth, and reproduction.

For both subspecies in this study, RMR increased and MMR decreased with an increase in acclimation temperature (Fig. 2). Although *Cvv* exhibited greater AS*r* and FAS*r* at 30 °C, it was more responsive to temperature than *Cvh*, resulting in the same AS*r* and FAS*r* at 35 °C. As we noted above, we were unable to acclimate either subspecies to 38 °C, suggesting that $T_c$ is > 35 and < 38 °C. Although based on extrapolation, the slopes for declining AS*r* and FAS*r* suggest that $T_c$ would be reached at ~37–38 °C, consistent with the OCLTT.

### 4.4. Relationship between temperature and hypoxia tolerance

The results of our study, suggest the physiological traits that confer temperature and hypoxia tolerance are different in these fish as evidenced by an evolved divergence in hypoxia tolerance without concurrent divergence in thermal tolerance. This result is consistent with recent observations in the related killifish, *Fundulus heteroclitus* (Healy et al., 2018), where no overlap in SNPs associated with hypoxia and thermal tolerance of different populations of *F. heteroclitus* were observed. It differs, however, from observations among rainbow trout populations (*Oncorhynchus mykiss*) where temperature and hypoxia tolerance were relatively strongly correlated (Spearman's rank order, $p$ = .69) (Anttila et al., 2013) and from a study evaluating 35 temperate fish species in Missouri (USA) where these parameters were more weakly correlated ($r^2$ = 0.17) (Smale and Rabeni, 1995). It is difficult to reconcile these disparate results given the limited number of studies and different methodologies used across studies. What is clear, is that additional studies using consistent methodologies are needed to evaluate a wider range of species.

## 5. Conclusion

Along with the previously found differences in osmoregulatory capacities, this study found that the subspecies of sheepshead minnow *C.v. variegatus* and *C.v. hubbsi* also differ in their ability to tolerate hypoxia (as indicated by time to LOE at 0.43 kPa), but are quite similar in their maximum thermal tolerance (as indicated by CTMax) despite reduced selective pressure on both traits for *Cvh*. Consequently, our current results are consistent with the contention that these two populations differ sufficiently in fundamental physiological processes to be classified as separate species (Brix and Grosell, 2013). As has been demonstrated in other species, thermal tolerance, hypoxia tolerance and aerobic scope were all sensitive to acclimation temperature. The lack of concurrent changes in temperature and hypoxia tolerance between these subspecies suggests that these traits may not be physiologically linked (Healy et al., 2018). Our data, however, contributes to the observed correlation across species between hypoxia and thermal tolerance. Additionally studies on a wider range of species should be undertaken to further investigate these inconsistencies.

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## Author contributions

K.V.B. and C.J.B. devised the study.

E.H.J. conducted the experiments, analyzed data and wrote the article.

All authors designed the experiments and contributed to intellectual input, edited and approved this manuscript.

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