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Temperature tolerance and oxygen consumption of two South American tetras, *Paracheirodon innessi* and *Hyphessobrycon herbertaxelrodi*

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ABSTRACT

Temperature is a primary factor affecting species' ability to thrive in a particular ecological niche, but thermal conditions have changed dramatically in recent decades. Fishes shift their thermal tolerance range with a maximum and minimum temperature correlated to their recent thermal acclimation history, and species can show a reduced temperature quotient (Q_{10}) following chronic thermal acclimation. Neon tetra (*Paracheirodon innesi*) and Black Neon tetra (*Hyphessobrycon herbertaxelrodi*) are popular hobbyist aquarium fishes, and both species are examples of freshwater teleosts native to South American river systems that are potentially affected by changing thermal conditions. We acclimated these species to three different constant temperatures (26 °C, 29 °C, and 31 °C) for 15.4 ± 2.1 days, then measured acute critical thermal maxima (CTMax) and acute oxygen consumption rate ($\dot{M}O_2$) at each acclimation temperature. We also estimated chronic lethal thermal maximum (CLT) for both species following a 2-week acclimation to 30.4 °C. Mean CTMax of both species were found to increase with acclimation temperature from 38.5 to 39.6 °C for Neon tetra and from 39.5 to 41.0 °C for Black Neon tetra, gaining 0.24 (Neon tetra) or 0.29 °C (Black Neon tetra) of tolerance per 1 °C of acclimation. However, Black Neon tetra demonstrated consistently higher CTMax (1.0–1.4 °C). CLT was lower for Neon tetra (33.5 °C), compared to Black Neon tetra (35.9 °C). Mean $\dot{M}O_2$ were statistically similar across acclimation temperatures within species; Q_{10} between 26-31 °C were 1.92 and 1.22 for Neon and Black Neon tetra, respectively. Neon and Black Neon tetras physiologically acclimated to changing thermal demands, and although they demonstrate robust CTMax responses, CLT responses indicated both species are unable to survive temperatures 4–5 °C above current average natural values. The demonstrated metabolic plasticity and CTMax values provide a moderate cushion for both species to combat changing temperatures due to climate change, but CLT values suggest vulnerability to projected climate trends.

1. Introduction

Physiology and behavior of fishes are heavily linked to body temperature (Huey and Stevenson, 1979). Poikilothermic fish are thermal conformers that rely on physiological acclimatization (adjustment to natural environmental conditions) or acclimation (adjustment to environmental conditions within a laboratory setting) in response to changing temperatures. Many poikilothermic fish species, especially small fish, have body temperatures that fluctuate exactly with ambient temperature. Temperature, therefore, affects all aspects of these species' biology, including metabolic responses and thermal tolerance. An organism's thermal tolerance is the range of temperatures within which the organism can physiologically function. Organisms often demonstrate both preferred (e.g. Pérez et al., 2003) and optimal temperatures (Huey and Stevenson, 1979) within their thermal tolerance range. At the extreme of this spectrum reside critical thermal limits known as critical thermal maximum (CTMax) and critical thermal minimum (CTMin). CTMax and CTMin are measurements of acute temperature tolerance. Fish are able to acclimate to new temperatures within their thermal tolerance by reconstructing their physiology on a cellular (Chadwick et al., 2015; Hazel, 1984; Lu and Hsu, 2015) to whole animal level, including shifted metabolic or growth processes or morphological changes such as alterations to gill surface area (Sandersfeld et al., 2015; Sollid and Nilsson, 2006). For example, thermal fluctuations may trigger changes in protein activity or expression and cellular pathways (Todgham et al., 2017; Windisch et al., 2014). Critical thermal limits are measured by exposing the animal to a linear temperature change fast enough to prevent acclimation and slow enough to track the animal's

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body temperature and not trigger immediate thermal shock (Beitinger et al., 2000). Critical thermal limits use common endpoints such as onset of spasms or loss of righting response or equilibrium (Lutterschmidt and Hutchison, 1997a; 1997b) that represent an inability to survive or escape these immediate thermal conditions.

Another measure of thermal tolerance, chronic lethal thermal (CLT) limits, quantifies a species' thermal range as survival during chronic (days - weeks) exposure to changing temperatures. CLT methods use endpoints such as death or cessation of eating to mark when physiological organization becomes unsustainable (Beitinger and Bennett, 2000; Eme and Bennett, 2008; Fry, 1947). Critical Thermal Methodology (CTM) is used to understand what temperatures species can withstand on an acute time scale (minutes to hours), but CTM temperatures are often too extreme for sustained periods of acclimation (Ford and Beitinger, 2005; Treanor et al., 2013). CLT can closely mimic longer-term natural thermal fluctuations, such as when temperatures change up to a few degrees Celsius per day, for example.

Oxygen consumption rate ($\dot{M}O_2$) responses to thermal changes provide additional insight into thermal physiology. $\dot{M}O_2$ may be linked to many factors of species' fitness and is affected by a diverse set of environmental conditions, including temperature (Burton et al., 2011). Metabolic measurements can translate into understanding species' performance over a range of different temperatures. Fish generally prefer and perform best at temperatures in which their factorial aerobic scope is optimized (factorial change between maximum and routine aerobic metabolism; Beitinger and Fitzpatrick, 1979; Clark et al., 2013). However, thermal acclimation can potentially change where scope is maximized at preferred temperatures (Kelsch and Neill, 1990). Species can show a reduction in metabolic rate at increased temperatures following long-term acclimation to higher temperatures, possibly through optimized enzyme-substrate affinity *via* construction of allozyme or isozymes and/or decreased membrane fluidity through an increase in lipid saturation (Bullock, 1955; Hazel, 1984; Logan and Somero, 2011; Somero, 1969).

Temperature changes on a global scale are driven in large part by anthropogenic global climate change (Malhi and Wright, 2004), and it has become increasingly likely that many species will continue to experience temperatures at or near their thermal limits. Species more tolerant to environmental changes may be more equipped to adjust to altered native climate. However species demonstrating less flexibility in temperature tolerance and aerobic metabolism will be less prepared to acclimate and adapt to environmental changes. Tropical fish may be exposed to local climates that rapidly heat during the day and cool at night, making it advantageous to retain a measure of maximum thermal resistance as a buffer against fluctuating daily temperatures (Chung, 2000). Freshwater systems in South America, such as the Amazon basin, are largely dominated by a hot and humid climate (Gloor et al., 2015; Malhi et al., 2008; Malhi and Wright, 2004; Salati and Vose, 1984) and are integrated with surrounding forest ecosystems. These systems typically display high oxygen content with relatively limited thermal variation (Costa et al., 2015; Espírito-Santo et al., 2009; Malhi and Wright, 2004). While South American freshwater river temperatures are infrequently documented, relatively recent studies of the Amazon basin have recorded typical temperatures ranging between 25 and 28 °C (Costa et al., 2015; Espírito-Santo et al., 2009). Approximately 50 years ago, water temperatures in the Amazon river were recorded as high as 29–30 °C (The Amazon, measuring a mighty river, 1967). More recently, nearby sea surface temperatures at large river outlets including the Amazon River (O°, 50°W) showed average temperatures between 26.5 and 28 °C throughout an annual cycle (Ternon et al., 2000).

Neon tetra (*Paracheirodon innessi*) and Black Neon tetra (*Hyphessobrycon herbertaxelrodi*) are popular, colorful aquarium species that are freshwater teleosts indigenous to South American freshwater environments (Solimões River and tributaries – Neon tetra, Paraguay – Black Neon tetra) (Marshall et al., 2011). Neon tetra have been studied for notable light-sensitive iridophores and color-change capabilities (e.g. Clothier and Lythgoe, 1987), but the thermal physiology of both species has not been explored. Relatively little is known about the thermal biology or metabolic responses of tetra species generally, however, similar species like Cardinal tetra (*Paracheirodon axelrodi*) and Green Neon tetra (*Paracheirodon simulans*) show increasing CTMax measurements correlated to increased acclimation temperatures and the ability to withstand temperatures in excess of 35 °C (Campos et al., 2017; Pérez et al., 2003). These species are native to slightly warmer (~2 °C) albeit geographically adjacent environments including the Negro River. The ability to thrive in nutrient-poor environments like the Amazon Basin (Salati and Vose, 1984) and in hobbyist aquariums suggests tetra species may be tolerant to environmental changes, however, it may be that some species are more tolerant than others to increased temperatures. We hypothesized that both Neon tetra and Black Neon tetra would demonstrate critical thermal limits near 40 °C similar to other related species such as *P. simulans* and *P. axelrodi* (Campos et al., 2017), and that measurements of CTMax would increase with increased acclimation temperature. We hypothesized that acute oxygen consumption rates would reveal relatively low temperature quotient (Q_{10}) responses following chronic thermal acclimation. This hypothesis is based on metabolic responses of other South American species that demonstrate consistent metabolic rates even under extreme thermal fluctuation (Kochhann et al., 2015). For example, the Dwarf cichlid (*Apistogramma agassizii*) maintained metabolic rates around 400–500 mg O_2 $kg^{-1}h^{-1}$ even after rapid temperature increases of 3 °C from 26 °C to 29 °C. A relatively low Q_{10} response may support a species ability to mitigate increased energetic costs associated with increasing environmental conditions, especially when species are at or near thermal limits. It is necessary to measure multiple aspects of a fish's thermal tolerance, along with metabolic responses to changing temperature, in order to build a full picture of the species' thermal biology. This study is unique because it combines multiple measurements of thermal tolerance with the aerobic metabolic response for fishes from an understudied area. These data for Neon and Black Neon tetra provides insight into the upper limits and metabolic consequences of increased temperatures for South American fishes native to thermally stable environments near the equator.

2. Materials and methods

2.1. Acclimation and maintenance of fish

Experiments were performed on a total of 62 adult Neon tetras and 58 adult Black Neon tetras. Adult fishes, as denoted by coloration, were obtained from a local commercial distributor (Petco Animal Supplies, Inc., San Diego, CA), and species distributed separately among 4 replicate 25 L aquaria (N=4–7 fish per aquaria). All 4 replicate aquaria were situated within a single large water bath (230 L) heated by either a 150W or 300W aquarium heater (EHEIM Jager Aquarium Thermostat Heater, Deizisau, Germany). Fish were held for 1 week at 26 °C and observed to be healthy ('quarantine'), prior to any change in acclimation temperature. Temperatures were increased from 26 °C by 0.59 ± 0.08 °C day^{-1} (mean±s.e.m.) and held at one of three constant acclimation temperatures: 26 °C (26.2 ± 0.16 °C), 29 °C (29.0 ± 0.13 °C), and 31 °C (31.0 ± 0.12 °C; mean±s.e.m.). Average acclimation temperatures were calculated as the grand mean of daily averages, starting with the first day after achieving target temperature. Thermometers were calibrated against VWR® Traceable® Digital Thermometer (precision ±0.0001 °C; accuracy ±0.05 °C; Avantor, VWR, Radnor, PA). Temperatures in the 4 aquaria were monitored daily using a VWR® Traceable® Lollipop™ Water-Resistant Thermometer (precision ±0.1 °C; accuracy ±0.4 °C; Avantor, VWR). Tank temperatures were also tracked using iButton temperature loggers (precision ±0.1 °C; accuracy ±0.5 °C; Thermodata Corporation, Milwaukee, WI) set to record water temperature every 20 min. Fish were allowed to acclimate at the desired temperature for 13–20 days (15.4 ± 2.1; mean±SD) prior to thermal tolerance or oxygen consumption trials (Reber and Bennett, 2007; Eme and Bennett 2009a; Eliason et al. 2011; Dabruzzi et al. 2013). Aquaria were mechanically (2 L min^{-1}) and biologically filtered, and water changed 1–2 times per week with clean, like temperature water (15–30% volume) that had been carbon filtered for > 48 h. Water was monitored weekly and tested for pH, ammonia, nitrite, and nitrate levels. Water quality was maintained with a pH of ~7.7, and ammonia, nitrite, and nitrate values never exceeded 1 ppm, 5 ppm, and 80 ppm respectively. Fish were fed ground TetraMin® fish flakes (Spectrum Brands, Inc., Blacksburg, VA) once daily, except when fish were fasted for ~24h prior to any trials. Fish were maintained on 12:12 day/night cycle. All procedures were approved by California State University San Marcos Institutional Animal Care and Use Committee approved protocol 17-004.

2.2. Thermal tolerance trials

Critical Thermal Maximum (CTMax) was measured using 28 Neon tetras and 24 Black Neon tetras following Eme and Bennett (2009a). CTMax was measured for fishes at each acclimation temperature (26 °C, 29 °C, and 31 °C) for groups of N = 8–12 total individuals and was identified as the temperature at which fishes experienced loss of equilibrium (LOE). Fishes were randomly selected from the 4 replicate acclimation aquaria, then distributed individually into individual 250 ml Tri-Corner plastic chambers (Globe Scientific, Inc., Paramus, NJ). Chambers were suspended within a polystyrene frame situated in the 'CTMax tank' so that chamber tops were flush with the water surface. The CTMax tank was a 57 L polystyrene-insulated water bath filled with 19 L of water that was heated continuously during the trial by two 150 W aquarium heaters (EHEIM Jager). Temperature stratification of the bath water was prevented by continuous circulation using an aquarium pump (15 L min^{-1}, Lifeguard Aquatics Model 800, Cerritos, CA). Individual test chambers were covered with netting during the trial and equipped with an airline that allowed for moderate aeration to prevent thermal stratification of chambers. Water temperature was increased at a rate of 0.31 ± 0.04 °C min^{-1} (mean±s.e.m.). Heating rate was monitored throughout the trial in a representative blank chamber using an iButton (±0.1 °C; Thermodata Corporation) that recorded temperature every 20 s; all CTMax trials showed a linear change in temperature throughout the trial. Fish were monitored closely throughout the trial until LOE occurred, at which point the temperature in the respective chamber was promptly recorded using a calibrated Lollipop™ Thermometer (±0.1 °C). Subsequently, fish were quickly removed upon LOE, water was immediately exchanged with acclimation temperature water, and fish were monitored ≥ 1hr to note recovery. Average CTMax for each replicate aquaria was calculated from 2-3 fish measured at each acclimation temperature. The grand mean of the 4 replicate aquaria was taken as the mean CTMax for the species at a particular acclimation temperature.

Chronic Lethal Temperature (CLT) was measured using a separate group of fish, 10 Neon tetras and 11 Black Neon tetras. After fish were acclimated to 30.4 ± 0.05 °C (~31 °C; mean±s.e.m.) for 14 days prior to initiation of CLT. CLT was estimated by increasing temperature by 0.37 ± 0.10 °C day^{-1} (mean±SD) until loss of 50% or more of the population had occurred. Temperatures in the 4 replicate aquaria (2–3 fish per aquaria) were monitored daily using a VWR® Traceable® Lollipop™ Water-Resistant Thermometer (±0.1 °C; Avantor, VWR) in addition to an iButton data logger (±0.1 °C; Thermodata Corporation) set to record water temperature every 20 min. The grand mean of the collective replicate aquaria endpoints was taken as the mean CLT for the species following the 14 d acclimation to ~31 °C.

2.3. Oxygen consumption

Acute oxygen consumption rate ($\dot{M}O_2$) was measured using 24 Neon tetras and 23 Black Neon tetras (e.g., Eme et al., 2009b; Rowe et al., 2018; Rangel and Johnson, 2018; Scheffler et al., 2019). Our protocol was specifically interested in measuring acute oxygen consumption rates; it is important to note that our protocol does not account for handling stress, but that all fish were treated similarly at all measurement temperatures. $\dot{M}O_2$ was measured individually for fishes at each acclimation temperature (26 °C, 29 °C, and 31 °C) for groups of N = 7–8 individuals for each species. $\dot{M}O_2$ was determined as the decrease in partial pressure of O_2 (PO_2) measured via intermittent respirometry using Loligo® Systems glass chambers and software (Loligo® Systems, DK-8800, Viborg, Denmark). Water-filled glass chambers (8.2 ml) with connective tubing (1.4 ml) provided a 9.6ml closed system housed within a reservoir tank (12.5 L). Prior to trials, the tank system and chambers were filled with clean, dechlorinated, carbon-filtered water equilibrated to the appropriate acclimation temperature, then held constant by a recirculating chiller (VWR Signature Refrigerated/Heating Circulating Baths, 13 L, Model 1156, ±0.01 °C). Fishes were selected from the 4 replicate aquaria, placed in a chamber, and chambers were then sealed under reservoir water. Reservoir temperature was constantly monitored with a Pt1000 temperature probe (Loligo® Systems, DK-8800, Viborg, Denmark, ±0.15 °C). PO_2 was monitored using the Witrox 4 and fiber optic mini sensors paired with an O_2 sensor spot (Loligo® Systems) applied to the surface of each chamber. Each sensor spot was calibrated with an anoxic solution of 10 mg ml^{-1} sodium sulfite (0%) and air-equilibrated water (100%). Each trial was 180–210 min, and measurements began immediately, with average $\dot{M}O_2$ calculated from measurements taken after an initial 45 min acclimation period. O_2 levels were measured in repeating cycles of 500 s (7–11 individual measurements), between which the system was flushed until restoration of initial levels of O_2. Measurement time period was tailored to maintain PO_2 above 13 kPa. Average $\dot{M}O_2$ for each fish was taken as an average of individual measurements. $\dot{M}O_2$ was calculated as a function of decreased PO_2 over time, relevant oxygen capacitance, and volume of the chamber minus the volume of the fish (estimated in ml from grams of wet body mass) using the following equation:

$$\dot{M}O_2 = \left(\frac{PO_{2(t2)} - PO_{2(t1)}}{t_2 - t_1}\right) * \beta * V$$

where $PO_{2(t2)} - PO_{2(t1)}$ is the decrease in PO_2 (kPa) in the chamber over the time period ($t_2 - t_1$, h), β is the capacitance of the water at the relevant temperature (mg O_2 ml^{-1} kPa^{-1}), and V is the chamber volume minus the organism volume (ml, estimated from wet mass). $\dot{M}O_2$ for empty chambers was taken with similar parameters following representative trials to account for microorganism respiration. Empty-chamber $\dot{M}O_2$ values were subtracted from respective animal measurements to obtain corrected values. Wet mass of each animal was used to calculate wet mass-specific $\dot{M}O_2$ for each individual. Average $\dot{M}O_2$ for each replicate aquaria was calculated from the 1–2 fish measured at each acclimation temperature. The grand mean of the 4 replicate aquaria was taken as the mean $\dot{M}O_2$ for the species at a particular acclimation temperature.

2.4. Sacrifice and measurements

Following all CTMax and $\dot{M}O_2$ trials, fish were euthanized using an overdose of tricaine methanesulfonate (200–300 mg kg^{-1}). Immediately following sacrifice, standard length (±0.01 mm; electronic digital caliper) and wet body mass were measured on a scale (±0.01 mg, Model XS105, Mettler Toledo, Columbus, OH).

2.5. Statistical analyses

Statistical analyses were performed using JMP® (SAS Institute Inc., Version 14). For replicate aquaria data, the parametric assumptions of normality and homogeneity of variance were examined with Shapiro-Wilk W test and O'Brien test, respectively; all data met the assumptions. Separate, 2-way ANCOVA and 2-way ANOVA analyses were

Table 1
Mean acclimation temperature (°C), sample size (N), mean standard length (mm), and mean wet mass (g) for all individual fish in each acclimation treatment (±Standard Deviation). Values below are combined mean values for all fish used in CTMax and oxygen consumption experiments.

Common Name *Genus species*	Acclimation Temperature (±SD)	N	Standard Length (±SD)	Wet Mass (±SD)
Neon tetra	26.2 (±0.16)	16	20.21 (±0.94)	0.16 (±0.02)
Paracheirodon innesi	29.0 (±0.13)	16	20.91 (±0.88)	0.19 (±0.03)
	31.0 (±0.12)	20	20.61 (±1.10)	0.19 (±0.04)
Black Neon tetra	26.2 (±0.16)	16	21.31 (±1.97)	0.19 (±0.02)
Hyphessobrycon herbertaxelrodi	29.0 (±0.13)	15	22.47 (±2.60)	0.26 (±0.14)
	31.0 (±0.12)	16	24.18 (±3.59)	0.40 (±0.24)

Standard length and mass were not determined for fish in CLT trials, as those animals died during trials and were not viable for accurate measurements.

performed on replicate aquaria data to assess the main effects of acclimation temperature and species on CTMax and $\dot{M}\text{o}_2$, as well as the potential interaction between acclimation temperature and species. ANCOVA was performed on non mass-specific $\dot{M}\text{o}_2$ values, and ANOVA was performed on mass-specific $\dot{M}\text{o}_2$ values. Where significant ANOVA effects were found, Tukey's *post hoc* comparisons differentiated groups into statistically distinct subsets. ANCOVA analyses were used to account for potential effects of body mass on CTMax or $\dot{M}\text{o}_2$, using all individual fish across replicate aquaria to determine if body mass altered statistical conclusions drawn from ANOVA analyses outline above. Simple linear regression analysis (SLR) was used to model the relationship of CTMax on acclimation temperature for each species, separately. All statistical decisions were made based on $\alpha = 0.05$, and values are provided as mean±s.e.m., unless otherwise noted.

3. Results

3.1. Critical thermal tolerance

Wet body mass did not have a significant effect on CTMax within or between acclimation temperatures (2-way ANCOVA, $p > 0.05$, Table 1). Acclimation to elevated temperatures resulted in higher CTMax limits (SLR, $p < 0.001$; Fig. 1); for Neon or Black Neon tetra, fish gained 0.24 or 0.29 °C more thermal tolerance per degree of increased acclimation, respectively (Fig. 1). All fish survived the CTMax trial initially, and survival of individual fish following the trials for 60 min was 46 ± 11%. 2-way ANOVA showed a significant effect of species and acclimation temperature on CTMax, with no significant interaction between acclimation temperature and species (Table 2). *Post hoc* Tukey's test showed that Black Neon tetra had an overall higher CTMax than Neon tetra for all acclimation temperatures combined. *Post hoc* Tukey's showed that within both species, mean CTMax was identical for 29 °C and 31 °C groups, which were both higher than 26 °C group (Table 2, Fig. 1). Neon tetra acclimated to 29 °C (39.3 ± 0.22 °C, mean±s.e.m.) and 31 °C (39.6 ± 0.13 °C) displayed a significantly higher CTMax than Neon tetra acclimated to 26 °C (38.5 ± 0.30 °C; 2-way ANOVA; Tukey, $\alpha = 0.05$; Table 2, Fig. 1). Black Neon tetra acclimated to 29 °C (40.6 ± 0.19 °C) and 31 °C (41.0 ± 0.10 °C) showed a significantly higher CTMax than fish acclimated to 26 °C (39.5 ± 0.21 °C; 2-way ANOVA; Tukey, $\alpha = 0.05$; Table 2, Fig. 1).

3.2. Chronic lethal temperature

The mean CLT for Neon tetra occurred at 33.5 ± 0.26 °C (Fig. 2). At 33.5 °C, fish were observed to exhibit increased activity and food consumption, as well as extreme color loss. The CLT temperature for Neon tetras resulted in death of >80% fish within 4 d. Mean CLT for Black Neon tetra occurred at 35.9 ± 0.30 °C (Fig. 2). The CLT temperature for Black Neon tetra resulted in death of >70% of fish within 48 h. At 35.9 °C, fish were observed to exhibit increased activity and food consumption.

3.3. Oxygen consumption

Mass and length of fish are provided in Table 1, and wet body mass did not have a significant effect on oxygen consumption rate ($\dot{M}\text{o}_2$)

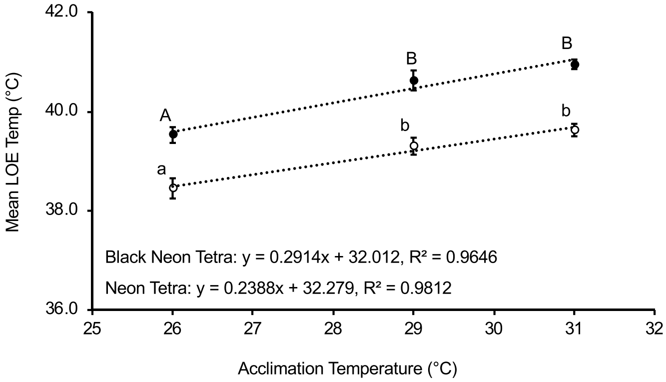

Fig. 1. Mean CTMax (LOE – loss of equilibrium) as a function of acclimation temperature for Neon tetra (open points) and Black Neon tetra (filled points). Each point represents mean CTMax from N = 4 replicate aquaria consisting of N = 8–12 fish each (2–3 fish per replicate), and error bars are s.e.m. The lines and equations represent significant simple linear regressions ($p < 0.001$). Uppercase and lowercase letters denote statistically distinct mean CTMax within Neon or Black Neon tetra, respectively (2-way ANOVA; $p < 0.001$; Tukey's *post hoc*; Table 2). 2-way ANOVA showed a significant effect of species and acclimation temperature, with no significant interaction between acclimation temperature and species (Table 2). *Post hoc* Tukey's test showed that Black Neon tetra had an overall higher CTMax than Neon tetra for all acclimation temperatures combined.

Table 2
Two-way ANOVA statistical results for CTMax and Wet Mass-specific Oxygen Consumption ($\dot{M}\text{o}_2$).

Predictor	Sum of Squares	df	Mean Square	F	P
Critical Thermal Maximum					
Model	33.4	5	6.67		
Error	10.3	46	0.22	29.94	<0.0001*
Species	19.6	1	19.59	87.89	<0.0001*
Acclimation Temperature	15.5	2	7.76	34.83	<0.0001*
Acclimation Temperature * Species	0.2	2	0.09	0.39	0.6822
Wet Mass-Corrected Oxygen Consumption					
Model	0.148	5	0.0296		
Error	0.261	41	0.0064	4.65	0.0019*
Species	0.0425	1	0.0425	6.67	0.0134*
Acclimation Temperature	0.0431	2	0.0215	3.38	0.0436*
Acclimation Temperature * Species	0.0602	2	0.0301	4.73	0.0141*

For CTMax, there was a significant effect (*$p<0.05$) of species and acclimation temperature, but no significant interaction between acclimation temperature and species; see Fig. 1 for Tukey's *post hoc* results. For $\dot{M}\text{o}_2$, there was a significant effect (*$p<0.05$) of species, acclimation temperature, and the interaction between acclimation temperature and species; see Fig. 3 for Tukey's *post hoc* results.

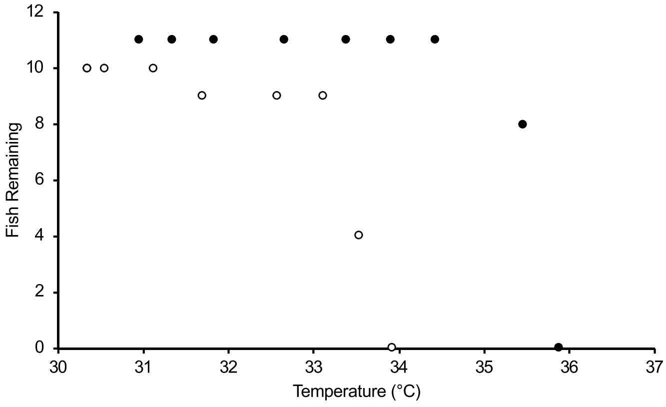

Fig. 2. Daily survival of Neon tetra (open points) and Black Neon tetra (filled points) throughout chronic lethal temperature trials as a function of temperature for each species. Both species were initially acclimated to ~31 °C for 14 days, after which the temperature was increased daily by 0.37 ± 0.10 °C day^{-1} (mean±SD). Neon tetra replicate tanks began the increased temperature protocol at a mean tank temperature of 30.3 °C, and Black Neon tetra tanks began at 30.9 °C. More than 50% population loss occurred for Neon tetra at 33.5 °C and for Black Neon tetra at 35.9 °C.

within or between acclimation temperatures (2-way ANCOVA, $p > 0.05$). 2-way ANOVA showed a significant effect of species, acclimation temperature, and the interaction between acclimation temperature and species (Table 2). *Post hoc* Tukey's test showed statistically identical $\dot{M}O_2$ across acclimation temperatures within species, with only the Neon tetra at 31 °C being statistically distinct from the Black Neon tetra's 26 °C and 31 °C group (Table 2, Fig. 3). There was no significant difference in the wet mass-specific $\dot{M}O_2$ of Neon tetra acclimated to 26 °C (0.26 ± 0.01 mg O_2 h^{-1} g^{-1}), 29 °C (0.28 ± 0.04 mg O_2 h^{-1} g^{-1}), and 31 °C (0.36 ± 0.04 mg O_2 h^{-1} g^{-1}). Similarly, there was no significant difference in wet mass-specific $\dot{M}O_2$ of Black Neon tetra acclimated to 26 °C (0.19 ± 0.02 mg O_2 h^{-1} g^{-1}), 29 °C (0.31 ± 0.02 mg O_2 h^{-1} g^{-1}), and 31 °C (0.21 ± 0.03 mg O_2 h^{-1} g^{-1}). Overall, the metabolic rate for Black Neon tetra showed a much lower Q_{10} of 1.22 between 26 – 31 °C, compared to a Q_{10} of 1.92 for Neon tetra between 26 – 31 °C.

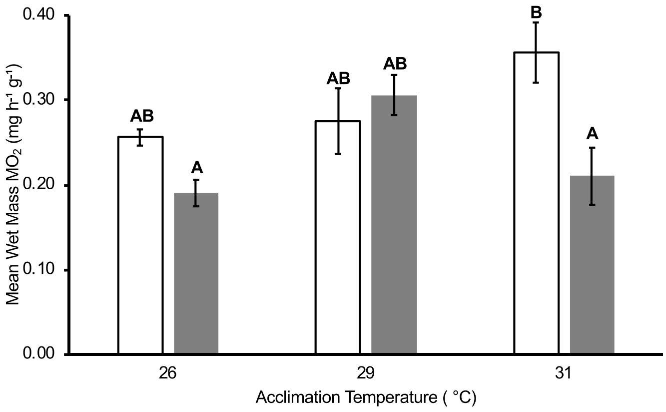

Fig. 3. Mean wet mass-specific oxygen consumption rates ($\dot{M}O_2$) as a function of acclimation temperature for Neon tetra (open bars) and Black Neon tetra (grey bars). Each point represents a mean $\dot{M}O_2$ from N = 4 replicate aquaria consisting of N = 7–8 fish (1–2 fish per replicate), and error bars are s.e.m. Uppercase letters denote statistically distinct mean $\dot{M}O_2$ within and between Neon and Black Neon tetra (2-way ANOVA; p=0.0138; Tukey's *post hoc*; Table 2). Uppercase letters show the results of *post hoc* Tukey's test following a 2-way ANOVA; there was a significant effect of the interaction between acclimation temperature and species. Both species showed statistically similar $\dot{M}O_2$ within acclimation temperatures.

4. Discussion

Measuring both CLT and CTMax, as well as metabolic responses to changing temperature, show that while Black Neon tetra and Neon tetra may show a modest Q_{10} effect and relatively high CTMax, CLT data indicate they may be susceptible to increased temperatures this century. Neon tetra and Black Neon tetra are resident to tropical river systems in South American climates with relatively little thermal fluctuation, and these species show moderately wide ranges of thermal tolerance, supporting our hypothesis. The linear relationship of CTMax to acclimation temperature and the resulting slope of the relationships reported here are similar to responses in other fishes (Currie et al., 1998; Eme and Bennett, 2009a; Ford and Beitinger, 2005; Rajaguru, 2002). Our data support those documented in other closely related species or species resident to thermally similar environments and exposed to similar thermal regimes (Chung, 2000; Oliveira et al., 2008). For example, Freshwater angelfish (*Pterophyllum scalare*) demonstrated CTMax values of 38.1 °C when acclimated to 26 °C that similarly increased and decreased with acclimation temperature (Pérez et al., 2003). Closely related *P. axelrodi* and *P. simulans* showed CTMax values near 40 °C when acclimated to temperatures comparable to those in our study (Campos et al., 2017). Our hypothesis regarding a lowered Q_{10} was especially supported for Black Neon tetra, which showed a relatively low Q_{10} response (1.22) following chronic thermal acclimation between 26-31 °C. In addition, there was no significant change in wet mass-specific $\dot{M}O_2$ between species, across the acclimation temperatures (26–31 °C), further supporting our hypothesis. Fishes typically show Q_{10} values of ~2.0 or greater following an acute change in temperature (Clarke and Johnston, 1999), and values markedly less than 1.5–2.0 indicate a relatively lower energetic cost following an increase in ambient temperature. Therefore, while CTMax data in the present and other studies may suggest a thermally robust profile, studies of multiple measured variables like those in the present study can provide a fuller picture of possible impacts on a specific species or group.

Many fish can modify their physiology across multiple levels of biological organization to suit the demands of their environment. Through thermal acclimation, fish physiology can be reconstructed on a cellular level to permit more efficient and prolonged function at higher temperatures and during minor thermal fluctuations (Todgham et al., 2017). Plastic physiological responses include synthesizing proteins better suited to new environmental temperatures, given appropriate time to manufacture new cellular machinery (Hazel, 1984). $\dot{M}O_2$ data in the present study suggest that two weeks is sufficient time for both species to acclimate to a moderate increase in temperature (3–5 °C). In addition, elevated temperatures can induce upregulated heat shock proteins, which augment additional thermal tolerance (Iwama et al., 1999; Logan and Somero, 2011; Madeira et al., 2013). Thermal tolerance attributed to acclimation and fluctuating exposure to elevated temperatures can be gained faster than lost and may be retained in some measure (Chung, 2000). Collapse of any single physiological system can be a significant cause for acute thermal death and often causes collapse of cardiac function at high temperatures (Somero, 2010). Neon tetras were unable to survive acclimation to 33.5 °C while Black Neon tetras could not survive acclimation to 35.9 °C, their respective CLT. Temperatures in the Amazon have historically risen by 0.25 °C per decade (Malhi and Wright, 2004), and if past trends continue, then it is likely that freshwater systems could reach temperatures detrimental to at least Neon tetra in the next century. A more rapid increase in temperatures of ≥3 °C could be driven by continued deforestation (e.g. Brazil up to ~25, 000 km^2 $year^{-1}$), and this could expedite the arrival of lethal thermal conditions by the end of the current century (Costa et al., 2015; Espírito-Santo et al., 2009; Malhi et al., 2008). Black Neon tetra are more robust in thermal tolerance and can tolerate a higher lethal thermal maximum temperature, possibly tied to physiological adaptation to the distinct thermal demands of their respective ecological niche (Marshall et al., 2011). Water temperatures in the Paraguay River are lower on average yet undergo more extreme seasonal thermal fluctuation than water temperatures in flooded areas of the Amazon. Nonetheless, it is likely that this species will face similar thermal pressures in addition to indirect effects of climate change.

Within each species, there was not a significant change in $\dot{M}O_2$ values across 26 °C to 31 °C following two weeks of acclimation to thermal conditions, with statistically indistinguishable $\dot{M}O_2$ within species across acclimation temperature groups (Fig. 3). The statistically indistinguishable change in $\dot{M}O_2$ reflected an increase in the standard deviation of $\dot{M}O_2$ values for both species at 29 and 31 °C. Our acute protocol could have increased the likelihood that handling stress was a factor in our experiment. However, our data in Fig. 3 also show a trend for $\dot{M}O_2$ to increase with temperature, which is expected, and handling stress would be likely to markedly increase $\dot{M}O_2$ at all measurement temperatures. Though it has been widely demonstrated that temperature can heavily influence poikilothermic species' acute and chronic metabolic processes, the literature has provided cases wherein animals tend to show markedly similar rates of metabolic variables across different thermal environments after short or long-term acclimation (Bullock, 1955; Chipps et al., 2000; Eme and Bennett, 2009b; Enzor et al., 2013; Guderley, 2004; Scheffler et al., 2019; St-Pierre et al., 1998). For example, results for Black Neon tetras closely coincide with metabolic tendencies in other related fish. *P. simulans* and *P. axelrodi* demonstrated mostly similar metabolic rates with acclimation around 500 mg O_2 kg^{-1} h^{-1} from 20 °C - 30 °C, with *P. simulans* showing drastically increased metabolic rates exceeding 1500 mg O_2 kg^{-1} h^{-1} at 35 °C (Campos et al., 2017). Poikilothermic species have long been shown to achieve relative metabolic independence using intracellular and organ reconstruction across multiple organizational levels (Bullock, 1955). Metabolic responses to thermal alteration occur over varying lengths of time when fish acclimate to new temperatures (Johnston and Dunn, 1987). Reasons for the responses observed in this study could include changes in metabolic enzyme expression and elevated saturated fatty acids in cell membranes at warmer temperatures to reduce fluidity and maintain homeoviscous adaptation (Hazel, 1984).

The selection of fish from commercial aquarium resources in this study could result in a discrepancy between the perceived and actual thermal limits of the two species. Selective breeding practices and inbreeding in commercial fisheries or the aquarium trade may promote the accumulation of deleterious mutations and reduce heterozygosity in a population (Ihssen, 1976). Selective breeding through many generations has conversely been shown to have the propensity to incite additionally plastic or extreme thermal tolerance responses in rainbow trout (Ineno et al., 2005). The full extent to which heritability and selective pressures influence thermal tolerance in aquarium fishes continues to be largely unknown. Thermal tolerance was found to differ between laboratory-reared and wild populations of zebrafish (*Danio rerio*), where a common acclimation temperature resulted in higher CTMax values for laboratory populations (Morgan et al., 2019). However, this significant increase in CTMax was relatively slight (~2.5% increase from 39.9 °C to 40.9 °C) and fish consistently retained this magnitude of thermal tolerance increase into F1 generation of domesticity. Therefore, Morgan et al. (2019)'s results suggest that fish from the aquarium trade do not have a large change in CTMax following laboratory acclimation, and that fish used in this study are likely a viable model for understanding the effects of climate change.

Overall, Neon tetra and Black Neon tetra demonstrate reasonably robust plastic thermal tolerance dependent on thermal acclimation, but a relatively steady climate with temperatures already near lethal thermal maximum puts both species at risk when accounting for projected temperature increases. These species occupy similar ecological niches, but it appears that Black Neon tetra possesses a more robust physiology to combat increased ambient temperatures. The metabolic responses for Neon tetra indicate that if South American habitats warm by 3 °C this century, up from 28 °C to 31 °C (Costa et al., 2015; Espírito-Santo et al., 2009), this species will incur an increased metabolic cost relative to Black Neon tetra. In addition, increases in stream temperatures above 31 °C could have more immediate physiological consequences for Neon tetra, compared to Black Neon tetra. These species, however, appear able to acclimate to increased environmental temperatures well above the lower average values recorded currently, as well as the lower end of predicted warming in the near future.

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