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Hypoxia but not shy-bold phenotype mediates thermal preferences in a threatened freshwater fish, *Notropis percobromus*

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ABSTRACT

For ectothermic animals, ambient temperature strongly influences developmental growth rate and individual fitness. While many ectotherms live in environments that are spatially hetero-thermal, the coupling between behavioural phenotypes (e.g., shy or bold behaviour) and thermal preferences remains uncertain. Relative to shy counterparts, bolder phenotypes may exert higher preference for ambient temperatures that are closer to their thermal optimum, thereby accelerating development. In addition, ectotherms should select colder temperatures in low oxygen conditions (hypoxia) according to the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis. Using wild caught carmine shiner (*Notropis percobromus*), this study examined thermoregulatory behaviour in individuals exhibiting consistent behavioural phenotypes along the shy-bold continuum and between ecologically relevant normal oxygen concentration (normoxic) and hypoxic treatments. Furthermore, the behaviour observed in the laboratory was compared to environmental data from the natal stream. Results demonstrated that individual shy-bold behavioural phenotype was consistent before and after a simulated aerial predator attack, indicating consistency of behaviour across situations. Individual preferred and avoidance temperatures varied substantially, but were unrelated to shy-bold behavioural phenotypes. In contrast, individual preferred and maximum avoidance temperatures were significantly reduced in hypoxia, consistent with the OCLTT hypothesis. These findings might indicate suppressed development rates in hypoxia, not only by the limited oxygen for aerobic metabolism, but also by the preference for colder water in hypoxia. Furthermore, the tolerated thermal ranges were reduced in hypoxia. Using test conditions confirmed by field data, our study demonstrates the strong influence of oxygen availability on thermoregulatory behaviours and preferences in aquatic environments.

1. Introduction

Ambient temperature strongly influences developmental growth rate and individual fitness in ectothermic animals (Guevara-Fletcher et al., 2016). For example, most ectotherms grow faster as ambient temperatures approach their thermal optimum (Elliott and Hurley, 2000). In hetero-thermal environments, mobile ectotherms may therefore control development rates by actively selecting ambient temperatures. This has been observed in many ectothermic animals, including squamate reptiles (Autumn and De Nardo, 1995), anurans (Lillywhite et al., 1973), and teleosts (Joblin, 1981). However, selecting warmer temperatures has important energetic implications because energy metabolism may increase between 1.5- to 3.0-fold for each 10 °C increase in temperature (Rosewarne et al., 2016). This means that individual ectotherms selecting warmer temperatures not only grow faster (unless they select a temperature, beyond their thermal optimum), they will also need to forage more to counter the elevated energy consumption (Pink et al., 2016). In many animals, foraging is associated with increased risk taking, partly because active vigilance is reduced while searching for food and because food may be more abundant away from the safe locations (Kie, 1999). Variation in behavioural phenotypes, including risk taking and boldness to increase foraging, could therefore be related to preferred temperature in individual ectotherms. It would be consistent with existing theory (Biro and Stamps, 2010; Réale et al., 2010) if bolder phenotypes would accelerate developmental rates by selecting higher temperatures; however, this prediction remains uncertain.

It is increasingly recognized that shy-bold behavioural phenotypes are temporally consistent within individuals. Bell et al. (2009) reviewed data across invertebrates and vertebrates and found that roughly 35%

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of the variation among individuals in behaviour is attributed to individual differences. In particular, many studies have revealed a continuum in behavioural phenotypes where some individuals within a population are consistently bolder and more risk-taking, while other individuals are consistently shyer and avoid risk (Watanabe et al., 2012; Massen et al., 2013; Andersen et al., 2015). Importantly, elevated boldness is associated with increased foraging success in many animals (Short and Petren, 2008; Carter et al., 2010, 2013; Biro et al., 2014). For example, in the three-spined stickleback (*Gasterosteus aculeatus*), a bold behavioural phenotype often means better access to food and faster growth rates compared to a shy phenotype (Ward et al., 2004).

Behavioural phenotypes may not only be repeatable across time, but may also be repeatable across different contexts (Bell, 2007; Mazué et al., 2015). Such behavioural syndromes (Andersen et al., 2015) may preclude optimal behaviours (Conrad et al., 2011) and constrain evolutionary responses (Dochtermann and Dingemanse, 2013). However, after losing fights, bold individuals may adjust their behaviour and exhibit less bold traits, as observed in rainbow trout (*Oncorhynchus mykiss*) (Frost et al., 2007). Similarly, habitat variables and risk of predation may affect shy-bold behavioural phenotypes in killifish (*Poecilia vivipara*) (Sommer-Trembo et al., 2017) suggesting that some individual fish are displaying behavioural plasticity by adjusting behaviours to match the surrounding environment (Wong and Candolin, 2015). Whether bold fish remain bold following a predator attack has been the subject of few studies (Pellegrini et al., 2010; Brown et al., 2013).

Behaviours may be coupled with physiological traits in individual animals (Carter et al., 2013). For example, intraspecific variation in schooling (Killen et al., 2012a,b), migration (Poulsen et al., 2010; Boel et al., 2014), and foraging (Breau et al., 2011) behaviours may be explained by physiological traits. However, a coupling between physiological traits and behavioural phenotypes is not always obvious and may only be revealed when animals are exposed to an environmental stressor, including hypoxia (Killen et al., 2013; Svendsen et al., 2014; Behrens et al., 2018). For example, using European seabass (*Dicentrarchus labrax*), Killen et al. (2012a,b) demonstrated links between physiological traits and behavioural phenotypes in individuals, but only when the environmental oxygen saturation was reduced to 20% air saturation (O_{2sat}).

Variation in environmental oxygen content is an important determinant of aerobic scope in most aquatic ectotherms (Svendsen et al., 2014; Rosewarne et al., 2016). The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis posits that thermal tolerance is determined by the aerobic scope (Pörtner, 2010; Pörtner and Peck, 2010). Thus, a decline in aerobic scope is correlated with a decline in thermal tolerance, suggesting that thermal tolerance is reduced in ectotherms subjected to environmental hypoxia (Pörtner, 2010; Pörtner et al., 2017). In relation to climate change, the OCLTT hypothesis may explain responses of aquatic ectotherms to variation in temperature. For example, Pörtner and Knust (2007) revealed correlations between laboratory measurements of aerobic performance, thermal tolerance, growth rate, and local abundance of a wild population of the marine eelpout *Zoarces viviparous*. Yet, the OCLTT hypothesis remains controversial and has been questioned by several recent studies (Clark et al., 2013; Jutfelt et al., 2018). Jutfelt et al. (2018) highlighted a range of outstanding issues with the hypothesis and outlined difficulties associated with tests of the hypothesis.

Here, we study thermoregulatory behaviour of a small cyprinid, the carmine shiner *Notropis percobromus* (Cope 1871), a species listed as Threatened under the Canadian *Species at Risk Act* (SARA; Fisheries, 2018). Given the protection status, this study makes an important contribution to addressing the species' recovery. The carmine shiner occur in the Birch River in Eastern Manitoba, Canada. Previous studies have indicated that the oxygen saturation of the river may drop to low levels (0.7 mg l^{-1}; equivalent to 5% O_{2sat}), particularly during summer, presumably affecting fish movements (Poulsen et al., 2011). We tested the following hypotheses: (1) individual shy-bold behavioural phenotype is consistent across time as indicated by consistent rating along the shy-bold continuum; (2) diversity in behavioural phenotype co-varies with thermoregulatory behaviour, such that shy-bold phenotype predicts thermoregulatory behaviour; and (3) individuals exposed to hypoxia would exhibit lower avoidance temperatures and reduced preferred temperatures relative to individuals in normoxia, consistent with the OCLTT hypothesis. The hypoxic level was selected according to measures of oxygen content in the field.

2. Methods

2.1. Environmental variables in the field

Water temperature (°C) and oxygen saturation (O_{2sat}) were logged hourly in the Birch River, Manitoba, Canada where carmine shiner (*Notropis percobromus*) occur. These environmental variables were measured in the field to select a relevant hypoxic level for the laboratory study and collect data in an ecologically relevant context. Temperature was measured over one year, while O_{2sat} was measured over two weeks in September. Temperature was logged using HOBO sensors (model U20-001-01; to nearest 0.01 °C; Onset, Bourne, MA, USA), whereas O_{2sat} was logged using Oxyguard sensors (model DO-1050; accuracy: ± 1% O_{2sat}; RBR Ltd., Kanata, ON, Canada).

Water temperature and O_{2sat} were logged at four and three different river locations and covered 52 and 40 river km, respectively. At each location, loggers were placed in the thalweg (i.e., lowest points along the length of the river bed) and shielded from direct sunlight.

2.2. Study animals and housing conditions

Carmine shiner (total length: 64.1 ± 0.8 mmm; body mass: 1.5 ± 0.1 g (mean ± SE)) were captured in the Birch River in Eastern Manitoba, Canada (N49.797117, W95.853357) using a beach seine (Carr et al., 2015) in August of 2011.

The fish were transferred to Fisheries and Oceans Canada's Freshwater Institute in Winnipeg, Manitoba where they were kept in 80 l tanks in a temperature controlled room (20 ± 0.2 °C) and fed daily with chironomid larvae *ad libitum*. Water temperatures at the collection sites on the Birch River ranged between 24 and 26 °C. Temperature was reduced by 1 °C day^{-1} from the capture temperature to the holding temperature (20 ± 0.2 °C). Fish were acclimated to laboratory conditions for one month prior to experimentation. The photoperiod was 16 h light: 8 h dark during holding and throughout the experiment. Air stones ensured normoxic conditions ($>$ 95% O_{2sat}). Fish were fasted for 24 h prior to experimentation to ensure a post-absorptive state that maximized the energy available for performance. For all transfers between tanks, fish were guided into a container (300 ml) and transported without air exposure (Poulsen et al., 2010).

Carmine shiner were collected from their natural environment under the provincial Scientific Collection Permit #41-11 and the Species at Risk Permit #CandA11-023. The endangered carmine shiner was studied to obtain valuable information on the species to provide science advice for the Recovery Potential Assessment and the Carmine Shiner Recovery Strategy. All procedures were reviewed and approved by the Animal Care Committee at the Freshwater Institute, Fisheries and Oceans Canada (Approval IDs: FWI-ACC-2010-012, FWI-ACC-2011-009, and FWI-ACC-2012-001) under the guidelines of the Canadian Council of Animal Care. All efforts were taken to circumvent animal suffering and undue stress, and there was no mortality during any of the tests.

2.3. Experimental setup

Shy-bold behavioural phenotypes were determined using established protocols (Killen et al., 2012a,b). A circular arena tank (diameter: 50 cm; water depth: 10 cm) was video recorded from above using a camera (uEye RE 1640; Loligo Systems, Tjele, Denmark) and the recordings were transferred to a PC where the software LoliTrack (Loligo Systems) tracked individual fish. A crescent shaped cover (80 cm^2; 12 cm long), made of black plastic (1 mm thick), attached to the tank wall was situated 1 cm above the water surface, and the arena bottom was darkened below the cover to create a hide with dim light. Preliminary trials revealed that carmine shiner preferred the hide over the area outside of the hide. Fish were not tracked directly after entering the hide, precluding any estimates of activity patterns when fish were situated inside the hide (i.e., under the black plastic cover). Fish tracking proceeded immediately when fish left the hide. Water temperature was adjusted to 20 °C (range: 19.9–20.1 °C) using a temperature controlling instrument (TMP-REG; Loligo Systems). Oxygen saturation of the arena water remained > 95% O_{2sat} throughout the experiment.

An aerial predator attack was simulated by dropping a metal object (58.1 g) through an opaque vertical pipe (61 cm long; 5.8 cm in inner diameter) that ended 1 cm above the water surface. The pipe ensured that the fish could not see the approaching object until it was close to the water surface and entered the water. The metal object was stringed such that it broke the water surface but did not hit the bottom of the arena. The object was removed from the arena immediately after breaking the surface. The set-up minimized any confounding influence of individual variation in aerial detection distance and perception of objects hitting the bottom in addition to the water surface.

The arena and the camera were shrouded in an opaque plastic blind, while the upper pipe ending and the PC were outside of the plastic blind to allow the experimenter to carry out the study with minimal disturbance of the fish. Tracking data from the arena tank were used for behavioural phenotyping based on hourly estimates of four variables that are related to shy-bold behaviours: (1) total distance swum (equivalent to swimming speed) - this variable was included because shy-bold behavioural phenotyping is related to activity levels in fish according to previous studies (Wilson and Godin, 2009; Killen et al., 2012a,b); (2) total time spent outside of the hide; (3) total time spent more than 3 cm from the tank wall; and (4) an index of exploration tendency based on crossings of a fictive line that divided the arena into two equal parts and situated furthest away from the hide.

2.4. Measuring thermal preference

Behavioural thermoregulation in a hetero-thermal environment was examined using established protocols (Petersen and Steffensen, 2003; Killen, 2014; Cooper et al., 2018). A shuttle box (Loligo Systems) was used to quantify temperature preference and avoidance in individual fish. The shuttle box consisted of two separate circular chambers (diameter: 50 cm; water depth 10 cm) connected via a corridor (8 cm long and 5 cm wide). Fish could pass freely between the two chambers using the corridor (Fig. 1). The shuttle box was shielded from external disturbance and direct lighting by an opaque plastic blind.

Water temperature within each of the circular chambers was continuously monitored using inline temperature sensors; each connected to a computerized temperature controller and data acquisition system (DAQ-M instrument; Loligo Systems). The software ShuttleSoft (Loligo Systems, Tjele, Denmark) and heating and cooling baths were employed to control the water temperature in each chamber. This was accomplished using external buffer tanks connected to the shuttle box. A temperature difference of 2 °C between the two chambers was maintained throughout trials (Petersen and Steffensen, 2003), such that one of the chambers constantly was 2 °C colder than the other chamber.

In addition to temperature control, the buffer tanks were employed to control water oxygenation. Normoxic (≥95% O_{2sat}) and hypoxic (20% O_{2sat}; range: 19.9–20.1% O_{2sat}) conditions were maintained by exposing water in the buffer tanks to streams of either air or nitrogen (N_2) bubbles. Air pumps were running continually during normoxic trials, while the supply of N_2 for the hypoxic trials (20% O_{2sat}) was controlled by an oxygen controlling instrument (OXY-REG; Loligo Systems). Both chambers were adjusted to 20% O_{2sat} during the hypoxic trials.

A camera (uEye RE 1640; Loligo Systems, Tjele, Denmark) was mounted above the shuttle box and was used by the ShuttleSoft software to detect the position of the fish. The temperature controlling unit of the software was adjusted to be either in a static or dynamic mode. When in the static mode, there was a constant temperature in each chamber, but a 2 °C differential between them. Individual fish were transferred in the shuttle box in the early evening, allowing a 16 h acclimation period, and at 10 h in the morning, the shuttle box system was adjusted to the dynamic mode. The dynamic mode allows the temperature to change within the shuttle box depending on the position of the fish, while maintaining the 2 °C differential between the warm and cold chambers. Thus, when a fish moved into the warm chamber, the temperature increased in both sides of the shuttle box (4 °C h^{-1}), but the cold chamber was always 2 °C colder than the warm chamber (Petersen and Steffensen, 2003). Conversely, when a fish moved into the cold chamber, the temperature in both sides of the shuttle box began to decrease (4 °C h^{-1}) (Petersen and Steffensen, 2003). Thus, by alternating between the warm and cold chambers of the shuttle box in response to changing temperatures, an individual could regulate the ambient temperature. This methodology to determine preferred temperatures was selected because it provides estimates that are consistent in individual fish (Killen, 2014) and not a consequence of random movements, as indicated by mock simulation trials (Nay et al., 2015).

2.5. Experimental protocol

For each of the 46 trials, an individual fish was transferred from the holding tank to the arena tank for behavioural phenotyping. Video recording and fish tracking started immediately after release and continued for 6 h in total. After 5 h, the simulated aerial predator attack was conducted, and was followed by 1 h of continued fish tracking. Similar to Killen et al. (2012a,b), the protocol provided 4 h of acclimation to the arena, 1 h of tracking before the predator attack and 1 h of tracking after the predator attack. Fish (n = 46) were transferred directly to the shuttle box when the 6 h of fish tracking was complete. All fish were introduced to the centre of the shuttle box (i.e., in the corridor between the two compartments). Five fish did not move out of the centre of the shuttle box and are consequently not included in the analysis related to temperature.

For fish acclimation to the shuttle box (16 h), temperature was adjusted to 19 °C in the cold chamber and 21 °C in the warm chamber (static mode). Oxygen saturation was adjusted to either normoxic (≥95% O_{2sat}) or hypoxic (20% O_{2sat}) during the acclimation period and throughout the subsequent data collection. Oxygen saturation for each trial was chosen randomly. After the acclimation period, software settings were changed to the dynamic mode and the fish started thermoregulating by selecting the cold or warm chamber in response to the gradually changing environmental conditions. Over the next 10 h, the fish was left undisturbed in the shuttle box and revealed its preferred temperature by avoiding lower and upper temperatures. An individual fish was tested under either normoxia or hypoxia. A total of 18 fish were tested under normoxic conditions and 23 fish under hypoxic conditions, respectively. The trial was terminated by measuring fish total length (to nearest 0.5 mm) and body mass (to nearest 0.01 g). After each trial, the arena tank and the shuttle box were cleaned and water was replaced.

2.6. Data acquisition and statistical analyses

Principal component analysis (PCA) was used to aggregate the four behavioural variables (i.e., (1) total distance swum; (2) total time spent outside of the hide; (3) total time spent more than 3 cm from the tank

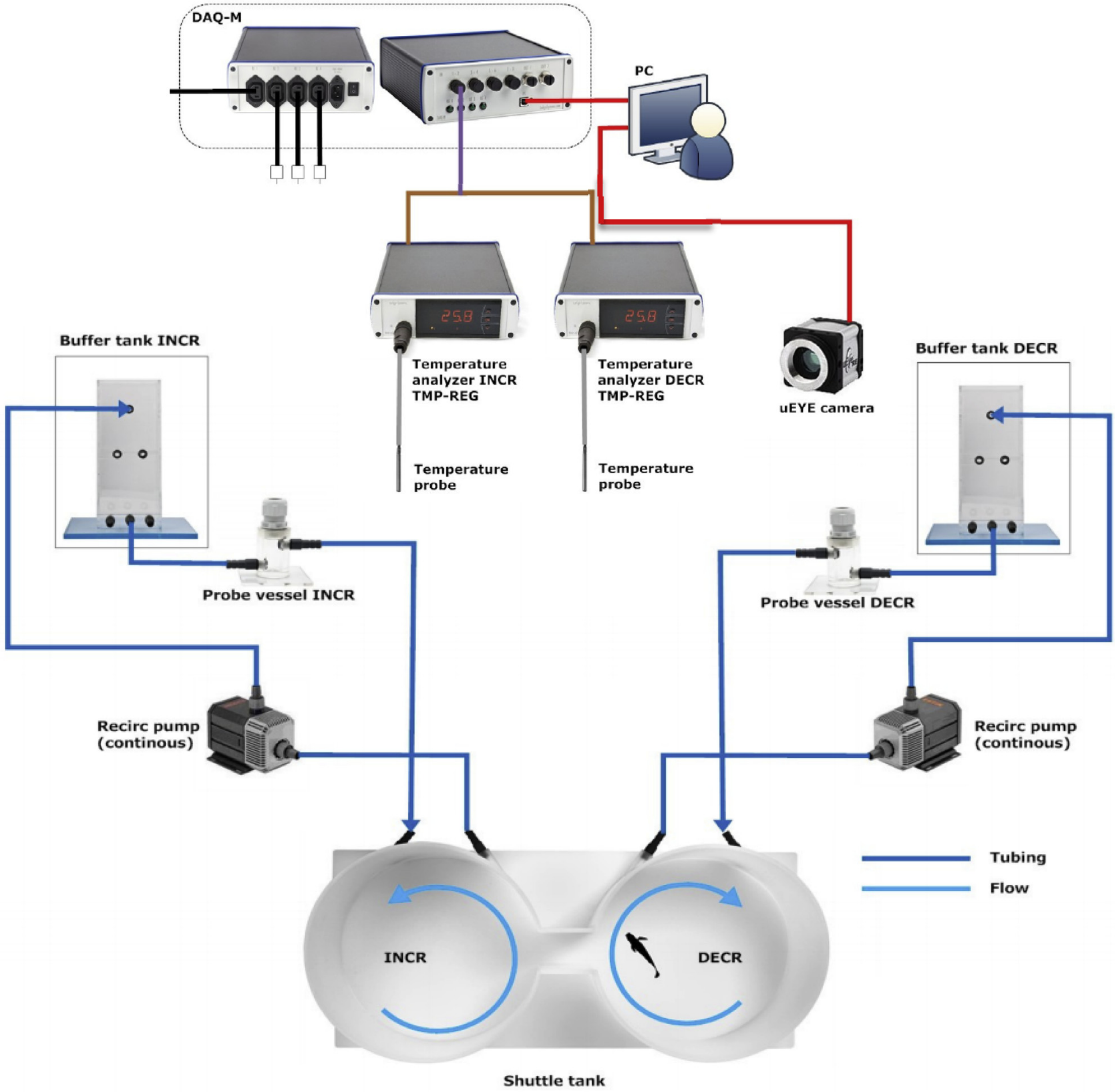

Fig. 1. Experimental setup of the shuttle box trials. Hypoxic conditions (20% air saturation) were achieved by bubbling nitrogen in both buffer tanks and monitored by galvanic oxygen sensors inside the buffer tanks and an instrument controlling the supply of nitrogen (Oxy-Reg, Loligo Systems, Tjele, Denmark).

wall; and (4) an index of exploration tendency) that were measured during six separate time periods (each 1 h over the duration of the experiment) into one estimate of shy-bold behavioural phenotype for each fish (Wilson and Godin, 2009; Killen et al., 2012a,b). Thus, for each hour spent in the arena tank, the PCA provided one estimate of the fish's behavioural phenotype. Negative and positive PCA values corresponded to shy and bold individuals, respectively; and the variable is hereafter referred to as 'risk score'. The risk scores immediately before and immediately after the simulated predator attack were then related to each other. Consistent with previous studies (Wilson and Godin, 2009; Killen et al., 2012a,b), the PCA approach was applied to minimize any risk of spurious correlations resulting from multiple tests of behavioural traits (e.g., distance swum). The PCA approach eliminated the need for multiple tests corrections (i.e., the Bonferroni adjustments). Finally, the study was concerned with shy-bold behavioural phenotypes and not with related behavioural traits such as the distance swum. The intra-class correlation coefficient (Lessells and Boag, 1987; Wolak et al., 2012) also referred to as repeatability index (R) (Roche et al., 2016) was used to examine the consistency of the fish's behavioural phenotype across the 6 h of tracking. In particular the data were used to test the hypothesis that behavioural phenotype is consistently expressed before and after a predator attack. The analysis involved Spearman's rank correlation coefficient (ρ) and hourly personality values (from the PCA) gathered immediately before and after the predator attack. Specifically, a significant positive correlation would indicate that an individual is relatively bold (or shy) both in a relaxed and disturbed state (i.e., before and after the simulated predator attack). All data collected in the arena tank involved normoxic water.

Thermal preference was estimated as the mean recorded temperature during the last 5 h of data collection (i.e., after 5 h of acclimation to changing temperatures in the shuttle box). Over the same time span, minimum and maximum thermal avoidance temperatures were estimated as the lower and upper recorded temperature (Schurmann and Steffensen, 1992). At the individual level, the thermal range was determined as the difference between the minimum and maximum avoidance temperatures. Least square linear regressions were used to examine correlations between shy-bold behavioural phenotypes (i.e., risk scores derived using the PCA, see supplementary data S1) and preferred temperatures, avoidance temperatures and thermal ranges. An analysis of covariance (ANCOVA) was conducted to test the effect of oxygen saturation and risk score on the preferred temperature, where hypoxia/normoxia was used as factor, preferred temperature as dependent variable, and the risk score (boldness) as covariate. Independently of shy-bold behavioural phenotype, the preferred and avoidance temperatures from normoxic and hypoxic trials were compared using a Welch Two Sample T-Test. Data analyses were carried out using the statistical software R (R Development Core Team, 2015). Results were considered significant at $P < 0.05$. All values are reported as means ± s.e.m. unless otherwise noted.

3. Results

3.1. Environmental variables measured in the field to support ecological relevance

Over the one year sampling period, water temperatures in the Birch River, Manitoba, Canada varied between 0 and 30 °C. Temperatures reached 20 °C in late June and fluctuated between 20 and 30 °C until late August, when temperatures started declining. Diel temperature changes peaked at 6–7 °C. Dissolved oxygen concentrations fluctuated consistently over the diel period (Fig. 2). Depending on river location and time, river oxygen saturation varied between 18.9 and 63.9% O_{2sat} (Fig. 2).

3.2. Shy-bold behavioural phenotype

Shy-bold behavioural phenotypes varied widely between individual carmine shiner (Fig. 3). Individuals with a higher risk score, i.e., higher PCA value, (1) swam a longer distance per hour; (2) spent longer time outside of the hide; (3) spent longer time more than 3 cm away from the tank wall; and (4) exhibited elevated exploration tendency (Table 1). The first and second principal component (PC1 and PC2) explained 72.9% and 21.1% of the variation (Fig. 4). In all cases, the four behavioural traits correlated significantly with the risk score ($n = 41$; $\rho > 0.62$–0.97; all $P < 0.0001$), suggesting that the risk score aggregated the behavioural variation that existed between individuals.

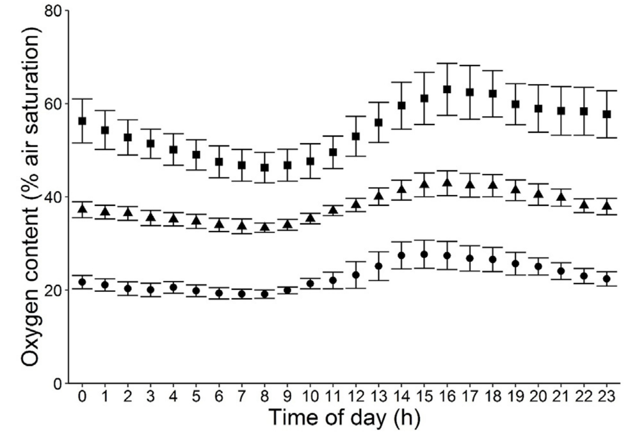

Fig. 2. Average and confidence intervals of dissolved oxygen content (% oxygen saturation (O_{2sat})) over 24 h in three different locations in the Birch River, Manitoba, Canada: river location N49.79770 W95.85489 by ●, N49.67943 W95.78884 indicated by ■, and N49.62990 W95.70113 by ▲, showing a large variation in O_{2sat} between sites. Average dissolved oxygen saturation varied between 18.9 and 63.9% O_{2sat} and revealed a clear diel pattern. The dissolved oxygen saturation reached the upper peak in the afternoon (15–16 h) and the lower peak in the morning (7–8 h). The diel amplitude varied between 9.6 and 18.7% O_{2sat}.

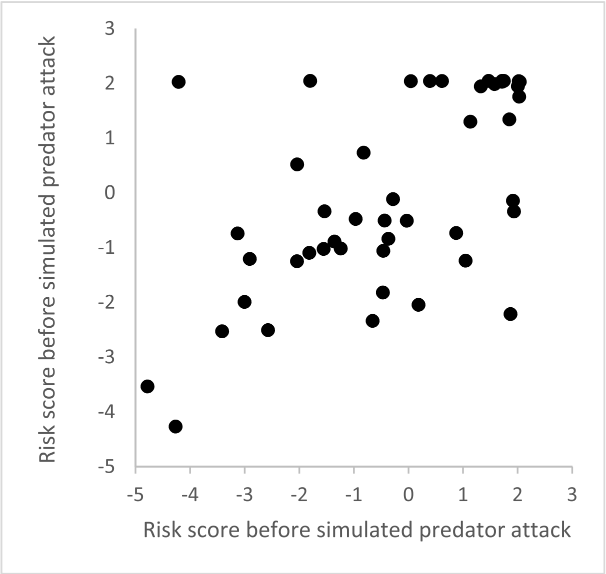

Fig. 3. Risk score derived using PCA before (x-axis) and after (y-axis) a simulated predator attack. The risk score is an index of boldness with higher values indicating bolder individuals ($n = 46$). The two data sets are positively correlated indicating that a bold fish generally remains bold after a predator attack ($n = 46$; $\rho = 0.69$; $P < 0.0001$).

Table 1
Factor loadings for the four behavioural variables tested on the first principal component (PC1) and second principal component (PC2) of the principal component analysis.

Behavioural phenotyping variable	PC1	PC2
Total distance swum	0.534	0.309
Total time spent outside of the hide	0.553	- 0.258
Total time spent > 3 cm from the tank wall	0.442	- 0.685
Index of exploration tendency	0.462	0.607

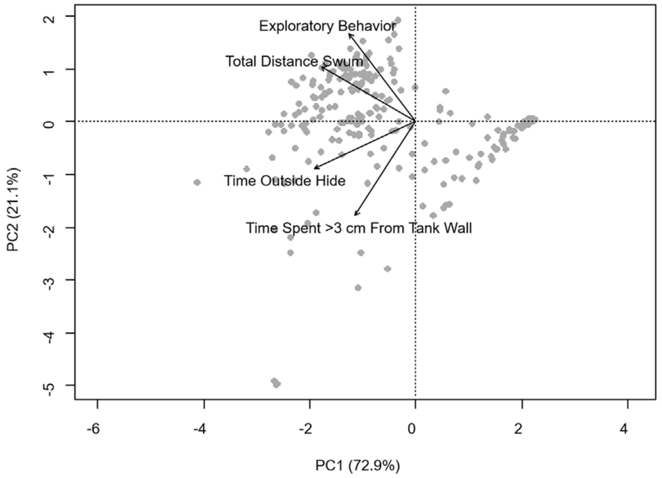

Fig. 4. Results from the principal component analysis used to aggregate the four variables into one estimate of shy-bold behavioural phenotype, i.e., (1) total distance swum, (2) total time spent outside of the hide; (3) total time spent more than 3 cm from the tank wall; and (4) an index of exploration tendency per fish.

Our analysis revealed that some fish were consistently bolder whereas others were consistently shyer. This was revealed by the

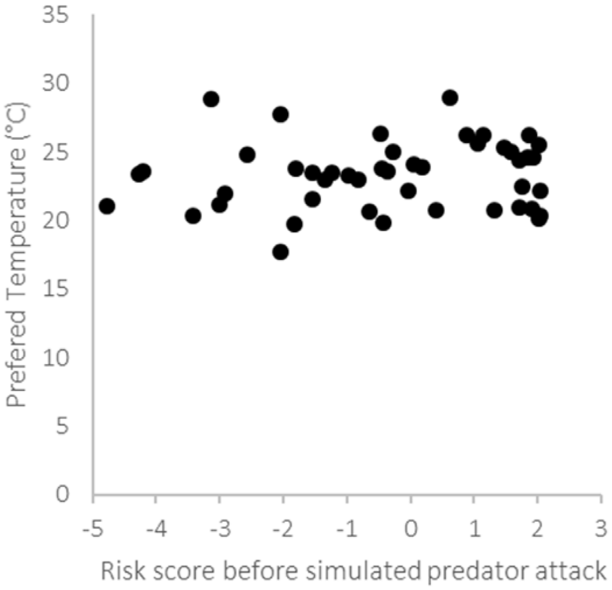

Fig. 5. Relationship between bold-shy behaviour estimated as risk score before a simulated predator attack and the temperature preference of a given fish.

significant intra-class correlation coefficient of the risk score ($n = 46$; Repeatability Index = 0.62, Confidence Interval = 0.5–0.74). These findings support our first hypothesis that shy-bold behavioural phenotypes can be consistent across time. Importantly, the risk score immediately before the simulated predator attack correlated to some extent with the risk score immediately after the simulated predator attack ($\rho = 0.69$; $P < 0.0001$; Fig. 3).

The correlation of risk scores before and after the simulated predator attack was positive, indicating that individual fish exhibited similar shy-bold behavioural phenotype despite a predator attack. Thus, across situations, individual fish tended to maintain their behavioural risk score.

3.3. Correlations of shy-bold behavioural phenotypes with preferred temperature, avoidance temperatures, and thermal range

Analyses revealed no significant correlations between the shy-bold behavioural phenotypes and thermoregulatory behaviour (Fig. 5). For example, risk scores recorded immediately before the simulated predator attack did not correlate with preferred temperatures in either normoxia or hypoxia (Fig. 5, $r^2 < 0.03$; $P > 0.45$). Likewise, risk scores did not correlate with maximum or minimum avoidance temperatures ($r^2 < 0.18$; $P > 0.09$) or thermal range calculated as the maximum avoidance temperature minus the minimum avoidance temperature ($r^2 < 0.09$; $P > 0.19$). Collectively, the findings suggest that shy-bold behavioural phenotypes, assessed using risk scores, are poor predictors of thermoregulatory behaviours.

3.4. Temperature preference, avoidance temperatures, and thermal tolerance range: effects of hypoxia

Thermoregulatory behaviour was influenced by oxygen saturation. Oxygen saturation had a significant effect on the preferred temperature (ANCOVA, $F_{1,45} = 13.57$; $P < 0.001$) but shy-bold behavioural phenotypes, assessed using risk scores had no effect on the preferred temperature (ANCOVA, $F_{1,45} = 0.04$; $P = 0.85$). In hypoxia, the preferred temperature was significantly ($t = -4.01$; $df = 34.92$; $P < 0.001$) lower than in normoxia (Fig. 6). Likewise, the maximum avoidance temperature in hypoxia was significantly ($t = -3.93$; $df = 31.75$; $P < 0.001$) lower than in normoxia (Fig. 6) indicating that fish with reduced aerobic performance selected lower temperatures. However, this pattern was not the case in terms of the minimum avoidance temperature (Fig. 6). While the minimum avoidance temperature was lower in hypoxia compared to normoxia, the difference was not statistically significant ($t = -1.74$; $df = 35.72$; $P > 0.09$).

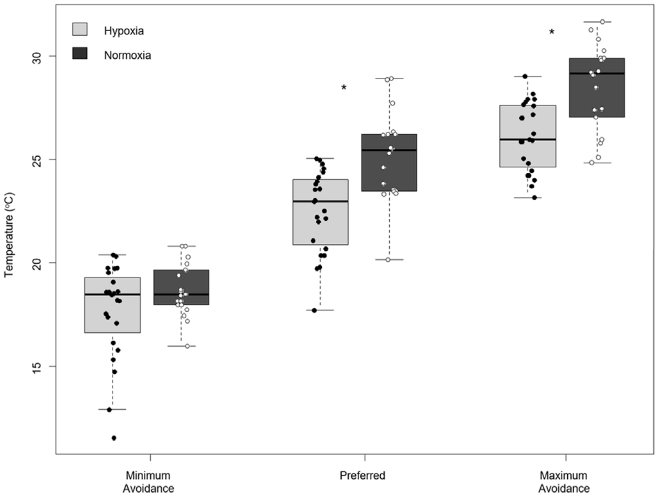

Fig. 6. Minimum avoidance, preferred and maximum avoidance temperature in normoxia (n = 18) and hypoxia (n = 23). Preferred and maximum temperature are significantly ($p < 0.001$) lower in hypoxia. In contrast, minimum avoidance temperatures in normoxia and hypoxia are not statistically different ($p > 0.44$).

4. Discussion

Using the endangered carmine shiner, this study provides evidence of consistent behavioural phenotypes, forming individual phenotypes along the shy-bold continuum. We observed inter-individual variations in behavioural phenotypes with shyer individuals swimming shorter distances, spending longer time inside the hide and near the walls as well as exhibiting reduced exploratory tendency. As predicted, shy-bold behavioural phenotypes were consistent across situations. Importantly, behavioural phenotypes were consistent before and after a predator attack. The persistence of the shy-bold behavioural phenotypes after a predator attack indicates that individuals maintain their shy-bold behavioural phenotype, independently of changes in the environment. Correspondingly, selection may act on behavioural phenotypes and favour phenotypes that are adapted behaviourally to co-exist with abundant predators (Lapiedra et al., 2018; Travis and Reznick, 2018). Carmine shiner are thought to occur in loosely associated small groups, consequently having tested the behaviour and temperature preferences individually might have affected our results as the presence of a conspecific may have altered their behaviour and potentially the thermal preference between bold and shy individuals.

In fish, bolder individuals often experience elevated mortality (Hulthén et al., 2017), however, bolder individuals might be able to alleviate the consequences of their behavioural phenotype using greater behavioural plasticity after a predator attack. Compared to shyer conspecifics, bolder individuals could exhibit wider behavioural plasticity and a safer response to predation by becoming excessively quiescent and/or sheltering after a predator attack (Andersen et al., 2015). This hypothesis would suggest no correlation or even a negative correlation between risk scores before and after a predator attack. In contrast, our data revealed a positive correlation between risk scores before and after a simulated predator attack, suggesting that bolder individuals maintained their shy-bold behavioural phenotype, even in the presence of a simulated predator. Consequently, our findings indicate that animals exhibit limited flexibility in their behavioural repertoire across different situations. Our data adds to the mechanistic basis of the elevated predation cost experienced by bolder phenotypes in the wild (Hulthén et al., 2017) and suggest that behavioural diversity is maintained by parallel selection on other co-varying traits (e.g., physiological traits). Further study is warranted to clarify why bold individuals apparently do not adjust their boldness, even in the presence of immediate predation risk.

In comparisons between individuals, various traits may co-vary within individuals (Biro and Stamps, 2010; Killen et al., 2012a,b). This is the basis of the pace-of-life syndrome (POLS) where a range of behavioural phenotypes are predicted to co-vary with other traits, including life history and physiology (Réale et al., 2010). Studies have revealed that shy-bold behavioural phenotypes may co-vary with body length (Polverino et al., 2016) and metabolic rates (Killen et al., 2012a,b; Binder et al., 2016), providing support for the POLS hypothesis. Temperature is the most influential environmental variable and determines development rates in ectothermic animals. Specifically, energy metabolism typically increases exponentially with water temperature (Rosewarne et al., 2016), highlighting the importance of thermoregulatory behaviours in ectothermic animals. Although thermoregulatory behaviour was not considered by Réale et al. (2010) when they described the POLS hypothesis, we examined relationships between behavioural phenotypes and thermoregulatory behaviours. Indeed, recent studies have revealed the large impacts of variation in thermoregulatory behaviours (Killen, 2014; Cooper et al., 2018). In aquatic environments, temperature may vary both horizontally and vertically, but thermal preference by different behavioural phenotypes has received limited attention (Villegas-Ríos et al., 2018). In the present study, we hypothesized that bolder individuals show a preference for higher temperatures, relative to shy counterparts. As ambient temperature deviates from the thermal optimum in either direction (i.e., increasing or decreasing water temperature), foraging and growth rates are expected to decline. This behaviour would elevate energy metabolism and food requirements in the bolder fish, consistent with the POLS hypothesis (Réale et al., 2010). However, data revealed no support for the hypothesis, suggesting that traits different from shy-bold behaviour drive intraspecific variation in thermoregulatory behaviour.

In contrast, oxygen saturation seems to influence the thermoregulatory behaviours. Specifically, for ectothermic animals, preferred water temperature, maximum avoidance temperature, and thermal tolerance range were suppressed significantly in hypoxia. Consistent with the OCLTT hypothesis (Pörtner, 2010; Pörtner et al., 2017), these findings indicate that fish selected lower temperatures and tolerated a narrower temperature amplitude in response to reduced aerobic performance. At a global scale, hypoxia is an increasing problem in many aquatic areas (Schmidtko et al., 2017; Breitburg et al., 2018). While oxygen thresholds for physiological performances (Rogers et al., 2016) and hypoxia avoidance behaviours (Schurmann et al., 1998; Chabot and Claireaux, 2008; Poulsen et al., 2011) have been examined, thermoregulatory behaviours in hypoxia have received limited attention. Consistent with previous studies (Schurmann and Steffensen, 1992), our data suggest that thermoregulatory behaviours are susceptible to changes in oxygen saturation. Specifically, hypoxia reduced preferred and maximum avoidance temperatures as well as the thermal tolerance range. Tank experiments have revealed that fish growth is also suppressed by hypoxia. For example, Chabot and Dutil (1999) reported reduced growth in Atlantic cod (*Gadus morhua*) in moderate levels of hypoxia at 10 °C. Using direct comparisons between normoxia and hypoxia at a common temperature, such studies reveal the distinct effects of hypoxia on growth, but the studies rarely account for the concomitant lower preferred temperatures in hypoxia that fish may exhibit in the field. Direct hypoxia effects combined with a preference for lower temperatures likely modifies the effects of hypoxia on growth. Therefore, studies examining growth rates at different oxygen levels should account for concomitant variation in the preferred temperature presumably displayed in the field. In the present study, field sampling revealed that carmine shiner is exposed to hypoxia levels similar to the levels tested in the lab, at least during parts of diel cycle. These findings suggest that carmine shiner growth may be constrained by hypoxia in the Birch River, partly because aerobic performance is suppressed, partly because the fish likely prefer temperatures that are suboptimal for growth compared to normoxic conditions.

Although the OCLTT hypothesis is attractive, temperature and hypoxia effects on fish are complex and various examples exist that do not comply with the OCLTT hypothesis (Clark et al., 2013; Jutfelt et al., 2018). In a heterothermal environment, fish control their temperature by avoiding unfavourable temperatures and seeking more favourable temperatures as tested in our laboratory experiment, in which fish were allowed to choose their temperature by shuttling back and forth in the experimental setup. The present study illustrates that carmine shiner can thermoregulate behaviourally and that their thermoregulatory behaviour is influenced by oxygen saturation. In normoxia, fish preferred higher temperatures (25.2 °C) than in hypoxia (22.6 °C). Similarly, the maximum avoidance temperature in normoxia (28.6 °C) was higher than in hypoxia (26.1 °C) suggesting that fish with reduced aerobic performance selected lower water temperatures. The wide range of minimum and maximum temperatures, ranging from 18.4 to 28.6 °C in normoxia and 17.6–26.1 °C in hypoxia, suggests that carmine shiner is a warm water species that tolerates a range of water temperatures (i.e., eurytherm species). The temperature and oxygen saturation ranges correspond largely to the summer temperatures and oxygen saturations that were observed in the natural habitat of carmine shiner. We have transferred fish in temperature ranging from 22 to 25 °C back to our laboratory facility before encountering mortalities. However, temperatures in the field reached 30 °C during the summer, which exceeded both the maximum avoidance temperature (28.6 °C) and the preferred temperatures (25.2 °C and 22.6 °C in normoxia and hypoxia, respectively), indicating that carmine shiners are exposed to suboptimal temperatures in the Birch River. With warming summers in Canada as a consequence of climate change (Schindler et al., 1996), this suboptimal thermal exposure could be exacerbated.

Predictions of future climate change suggest large and deleterious effects on freshwater fish species with narrow dispersal abilities due to limited hydrological connectivity (Dudgeon, 2010). In turn, this creates the potential for population isolation in thermally unsuitable habitats, leading to physiological stress, species declines or possible extirpation. Carmine shiner currently occupy habitats spanning a wide range of climatic conditions. July mean temperature and temperature seasonality explain carmine shiner's current distribution (Pandit et al., 2017), indicating that temperature plays a dominant role in determining the spatial extent of occupancy. Consequently, our results are timely to understand the thermal preference of carmine shiner, which will help to guide effective management and conservation actions for this species. The field measurements and the experiments were conducted under summer conditions, consequently we can only speculate if carmine shiner experience hypoxia events during the winter. Due to the fact that there are several higher gradient sections in the Birch River that stay open over the winter albeit the general harsh winter condition, the water in the Birch River may actually be very well oxygenated and consequently hypoxia might be less of an issue for carmine shiner during winter months.

Water temperature and dissolved oxygen concentrations are linked in natural environment and organisms may have to trade-off between their thermal and dissolved oxygen requirements according to the thermal niche-dissolved oxygen hypothesis (Coutant, 1985). In our experimental setup, we purposely decoupled temperature from the dissolved oxygen concentration. One interpretation of why carmine shiner prefer colder water in hypoxic conditions may be a thermoregulatory compensatory mechanism. Specifically, for ectothermic fish, warmer water increases metabolic demand, requiring higher levels of oxygen intake. However, if the partial pressure of oxygen that determines the oxygen availability for fish is not available, then the required oxygen demand cannot be met; which may result in reduced growth and fitness. To avoid this scenario, individuals might seek out cooler water temperatures in their natural environment, where the combination of lower metabolic demand and higher dissolved oxygen concentrations is more likely to meet the metabolic demand. However, this mechanism could be further influenced by food availability due to temperature effects on food conversion efficiency.

In conclusion, our study provides support for behavioural consistency where animals exhibit limited flexibility in their behavioural repertoire across different situations, precluding reduced boldness after a predator attack and possibly constraining evolutionary responses. We further hypothesized that bolder individuals would select higher temperatures to increase development rates consistent with the POLS hypothesis. The data did not, however, provide support for the hypothesis. Finally, our results suggest that fish select lower temperature in hypoxia consistent with the OCLTT hypothesis. Further research will be required to understand the impact of bold-shy behavioural types play in an ecosystem context.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.08.001.

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