Acclimation to fluctuating hypoxia alters activity and escape performance, but not metabolism, in guppies

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Abstract

Organisms living in fluctuating environments must cope with constantly changing conditions. Here we investigated how acclimation to either fluctuating or constant oxygen affects behavioural and physiological responses to hypoxia in guppies (*Poecilia reticulata*). Guppies were acclimated to either fluctuating hypoxia (100% of air saturation during day to 40% at night) or constant normoxia (100% of air saturation). Their activity, escape response, and metabolic rates (standard and maximum) were quantified under three oxygen exposure scenarios: (i) normoxia: 100%, (ii) acute hypoxia: 40%, and (iii) reoxygenation: 100% after experiencing 40% of air saturation. We observed that activity decreased under acute hypoxia, but to a lesser extent in the fish acclimated to fluctuating hypoxia. Acclimation also influenced the responses after reoxygenation as normoxia-acclimated guppies fully recovered their activity levels after hypoxia, whereas the guppies acclimated to fluctuating hypoxia only partially recovered. Escape response also differed between acclimation groups, with normoxia-acclimated guppies showing decreased responsiveness during and after acute hypoxia, whereas fluctuating hypoxia-acclimated guppies were most responsive during acute hypoxia but showed similar decreased responsiveness after reoxygenation. Metabolic rates decreased under acute hypoxia, with sex-specific effects on aerobic scope, whereas acclimation had no significant effect on metabolism. Our results demonstrate that the effects of acclimation to fluctuating hypoxia on whole-organism performance in guppies are scenario- and trait-specific, but that overall, an acute exposure to hypoxia had stronger effects on performance than acclimation. Organism performance after exposure to acute hypoxia can also differ depending on the periodicity and recurrence of hypoxia. We highlight the reoxygenation period following acute hypoxia as a critical period that deserves more research to get a better understanding of the overall impact that fluctuating hypoxia has on organism performance.

Introduction

Daily fluctuations in dissolved oxygen occur naturally in many aquatic habitats (e.g. intertidal, coral reefs, freshwater pools), decreasing at night and increasing during the day, due to the combined effects of photosynthesis and respiration. When temperatures are high, or water is eutrophic, oxygen can decline to hypoxic levels during the night, and this is becoming more common and more severe with anthropogenic climate change and eutrophication (Diaz and Rosenberg, 2008; Tyler *et al.*, 2009; Duvall *et al.*, 2022). Despite fluctuating hypoxia being a situation that many aquatic species naturally face, most experimental studies looking at the effects of hypoxia on aquatic organisms have been carried out under constant hypoxia (Borowiec *et al.*, 2015). Much remains to be understood about how fluctuating oxygen regimes contribute to the evolution of physiological and behavioural strategies that enable organisms to optimise performance in environments with occasional or periodic (daily) periods of hypoxia; and how these strategies may influence the effects that increasingly recurrent and/or acute hypoxic events may have on organisms.

Fish have various behavioural and physiological responses to low oxygen levels which are crucial for survival. Behavioural responses to hypoxia include: reducing activity levels to minimize oxygen consumption, moving to areas with higher oxygen concentrations, either vertically in the water column or horizontally across the water body (Pichavant *et al.*, 2001; Diaz and Breitburg, 2009; Richards, 2009; Duvall *et al.*, 2022), or performing aquatic surface respiration (ASR) (Kramer and Mehegan, 1981; Talbot and Kramer, 1986). Whilst these strategies can be advantageous, they also impose trade-offs, for example ASR can make the fish more conspicuous and so increase predation risk, and it can be energetically costly to maintain their position at the water-air interface (Sloman, 2024). Whereas reduced activity can cause lost foraging opportunities, leading to decreased food intake and, if sustained over time, reduced growth (Breitburg, 2002). Behavioural responses may vary depending on whether a fish experiences hypoxia rarely or is regularly exposed to nocturnal hypoxic episodes. Levels of exposure, recurrence and periodicity to hypoxic episodes will therefore determine which behavioural strategies are optimal for overcoming these events in different scenarios.

Physiological responses to low oxygen levels can include respiratory or metabolic adjustments. For example, an increase in ventilation, termed the hypoxic ventilatory response (Powell *et al.*, 1998; Borowiec and Leonard, 2024), has been observed as an immediate response to short periods of hypoxia or mild hypoxia. This can help maintain oxygen delivery and sustain ATP production, but is inefficient and energetically expensive over prolonged periods, or during severe hypoxia. From the perspective of metabolic functioning, metabolic depression has been described as a strategy where oxygen demand is reduced to match the lower availability of oxygen in the water (Regan *et al.*, 2019). For example, after acclimation to constant hypoxia, compensatory changes, such as increased mitochondrial efficiency (Gnaiger *et al.*, 2000; Thoral *et al.*, 2021) can reduce the need for oxygen, reflected by a lower maximum (MMR) (Gilmore *et al.*, 2018) and standard (SMR) (Borowiec *et al.*, 2018) metabolic rate. Similar reductions in metabolic rates have been observed following acute exposure to hypoxia (Chabot and Claireaux, 2008). At the acute level, this decrease may be due to a switch to anaerobic respiration, though this is less efficient and leads to an accumulation of toxic metabolites, making it a time-limited strategy (Diaz and Rosenberg, 1995; Breitburg, 2002; Farrell and Richards, 2009; Richards, 2009). Interestingly, SMR for fish acclimated to fluctuating hypoxia does not differ with fish held under constant normoxia (Borowiec *et al.*, 2018), suggesting that responses to constant, and acute hypoxia may differ from those seen under fluctuating levels.

Hypoxia can also affect the senses of fish (Fay and Ream, 1992; Robinson *et al.*, 2013; Tigert and Porteus, 2023). One way this could occur is through impaired neurological function which may make fish slower and less agile, hindering their ability to escape from predators (Domenici *et al.*, 2007). Additionally, hypoxia can decrease fish responsiveness (Lefrançois *et al.*, 2005; Lefrançois and Domenici, 2006; Sánchez-García *et al.*, 2019), and the absence of an escape response would significantly influence the outcome of a predator-prey interaction. Acclimation to constant hypoxia has been shown to benefit escape responses in the African mormyrid (*Marcusenius victoriae)* (Ackerly *et al.*, 2017); but no study to our knowledge has investigated the effect of acclimation to fluctuating hypoxia on a fish's escape response.

Hypoxia-induced changes in behaviour, physiology and neurological function may persist even after a fish returns to oxygenated waters. For example, a reduced escape response persists upon reoxygenation in the white grunt (*Haemulon plumerieri*) (Sánchez-García *et al.*, 2019). Hypoxia can also incur detrimental effects, such as oxygen debt from anaerobic respiration causing an accumulation of lactate (Heath and Pritchard, 1965), which requires a recovery period upon reoxygenation before returning to pre-hypoxia baseline levels. Additionally, the reoxygenation period itself may lead to negative effects, such as increased oxidative damage, caused by an excess of reactive oxygen species (ROS) production in the mitochondria (Li and Jackson, 2002; Wang *et al.*, 2021). At the behavioural level, weakfish (*Cynoscion regalis*) recover swimming speed faster after hypoxia if they have been acclimated to fluctuating hypoxia than if they have been acclimated to normoxia (Brady *et al.*, 2009). However, they were overall less active and less responsive. This suggests that the physiological mechanisms that allow fish to cope with hypoxic conditions and recover during reoxygenation (e.g., faster recovery of aerobic metabolism or clearance of oxygen debt) may simultaneously limit their capacity to perform other vital behaviours, such as foraging or predator avoidance. Overall, the balances between these competing demands could vary between species, and the generality of this finding remains largely unexplored. Therefore, because acclimation to fluctuating hypoxia may alter performance upon reoxygenation, and since reoxygenation occurs daily for many fish species living in fluctuating environments, it is crucial to incorporate a reoxygenation period in experimental designs for both acclimated and non-acclimated fish to fully understand the lasting and secondary effects of hypoxia.

Our objective here is to investigate (i) whether acclimation to fluctuating hypoxia can affect the behavioural and physiological responses of fish to hypoxia, (ii) whether exposure to acute hypoxia may have lasting effects, and (iii) whether acclimation to fluctuating hypoxia may aid or hinder recovery. To do this we used guppies (*Poecilia reticulata*) as model species because they are hypoxia tolerant species that occur in habitats with fluctuating oxygen levels (Magurran, 2005). We acclimated the fish to either fluctuating hypoxia (100% of air saturation in daytime – 40% at night) or to constant normoxia (100% of air saturation) and measured both behavioural and physiological traits in three different scenarios of O_2 saturation: (i) normoxia: 100%, (ii) acute hypoxia: 40%, and (iii) reoxygenation: 100% after experiencing 40% of air saturation. Activity and escape-response were assessed for the three scenarios, while metabolism (both the maximum, MMR, and standard, SMR, metabolic rates) was measured only at the two first scenarios. Overall, this study aims to enhance our understanding of the effects of acclimation to fluctuating hypoxia on different aspects of whole-organism performance.

Materials and methods

Study animals

A total of 96 adult guppies of both sexes coming from nine experimental populations reared at the facilities of the University of Bergen were used for experiments (Diaz Pauli, 2012). Fish were dorsally tagged with two subcutaneous Visible Implant Elastomer Tags (Northwest Marine Technology, Inc.). Standard length (±0.01 mm) and weight (±0.01 g) were recorded immediately after tagging and at the experiment's conclusion, allowing for growth to be calculated, as the specific growth rate between initial and final lengths. Guppies were sedated with buffered MS222 (110 mg/L) for tagging and measuring, with handling lasting about 2 minutes, then returned to oxygenated water for recovery. Guppies were randomly assigned to groups of 8 with an even sex distribution across twelve 40 L plastic aquaria enriched with a synthetic plant and a plastic shelter. A flow-through system ensured full water exchange daily, and the temperature was maintained at 25°C using glass aquarium heaters in header tanks (Suppl.1). Fish were held on a photoperiod of 12:12 h light-dark regime (07:00 to 19:00 local time for light phase), with ramped light transitions. All experiments were performed at the fish facilities at the University of Bergen, from November-December 2022, and approved by the Norwegian Food Safety Authority (permit no. 29742 & 21812).

Acclimation to Fluctuating hypoxia

Aquaria were assigned to one of two acclimation treatments: (i) nightly fluctuating hypoxia (Acclimation: Fluctuating hypoxia), or (ii) full and constant oxygenated water (Acclimation:

Constant normoxia) as a control. The guppies were acclimated to these conditions for 11 days prior to the experiments (Fig. 1A). The hypoxia fluctuation was created by bubbling N_2 into a header tank connecting all aquaria in this treatment from 22:00 (local time), resulting in a constant decline in dissolved oxygen (DO) levels along the night to a minimum of 40% (Fig.1-B). From 06:00 (local time) compressed air instead of N_2 was bubbled to reoxygenate the water and maintain normoxia during the day (Suppl.1 for experimental setup details). DO levels were monitored in each aquarium using OxyGuard probes.

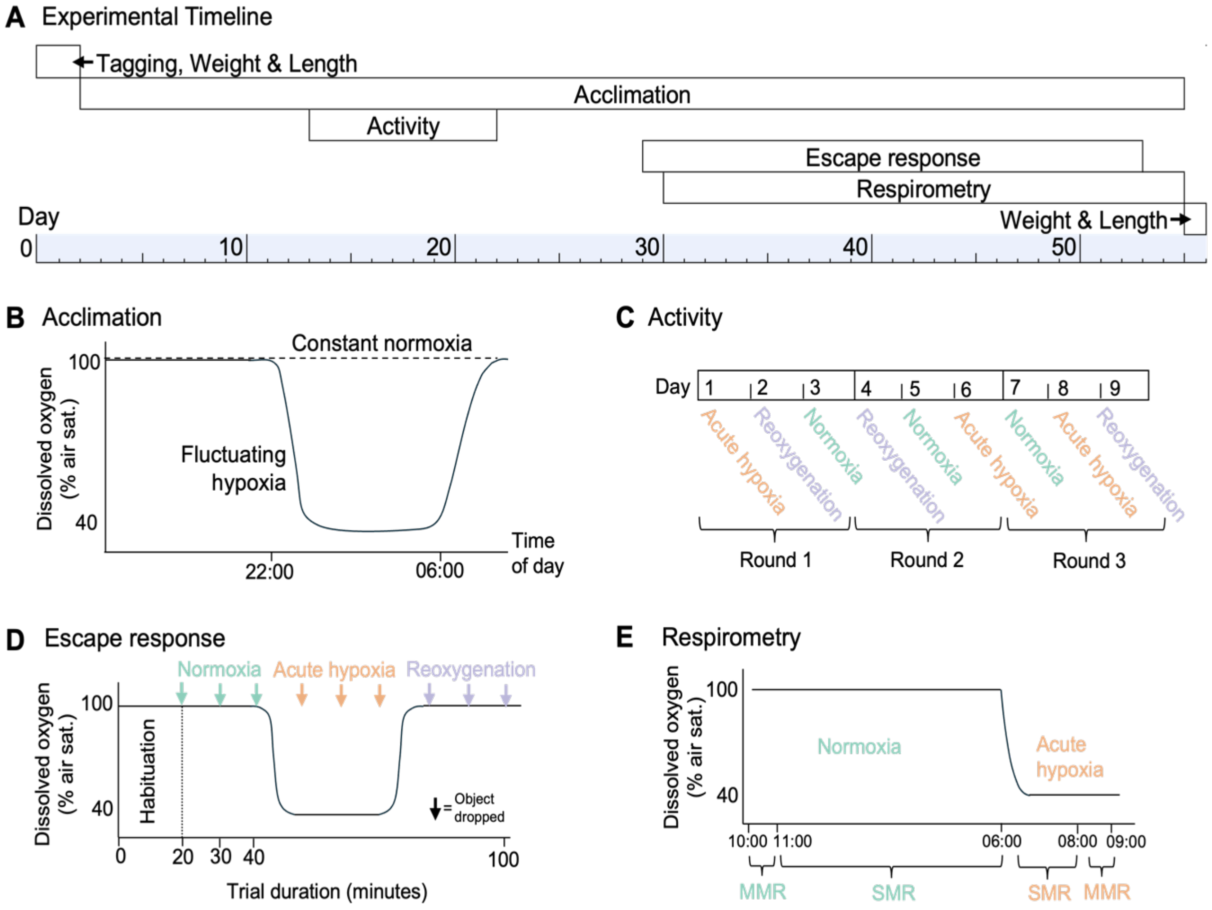

Figure 1: Overview of the whole experiment. A) Timeline for the experiment from day 0-56. B) Schematic of the acclimation treatments: Fluctuating hypoxia and Constant normoxia over a 24-hour period. C) Activity –3 trials per individual to 3 exposure scenarios: normoxia, acute hypoxia and reoxygenation, with exposure order randomised within each round. Each trial lasts 15 minutes. C) Escape response – 1 trial per individual with 3 exposure scenarios: normoxia, acute hypoxia and reoxygenation, experienced in the same order, with a trial duration of approximately 100 minutes. D) Respirometry – schematic of the 22-hour trial per individual for measuring SMR and MMR in 2 exposure scenarios: normoxia and acute hypoxia.

Regulating DO levels during experiments

During activity, escape response and respirometry experiments, DO levels were monitored using FireSting O_2 sensors (Pyroscience GmbH) and regulated by the OptoReg system (Ern and Jutfelt, 2024), which automatically controlled N_2 flow via a solenoid valve, allowing DO levels to be maintained at any set level.

Activity

The arenas for testing activity (24x20x10 cm) were arranged in 4x2 layout with two cameras positioned above to record (30 fps) simultaneously 4 arenas per camera, totalling 8 arenas per trial. Each arena contained a piece of plastic tubing as a shelter and was sanded down to prevent visibility between them, minimizing possible interactions among individuals during experiments. Activity was tested individually and quantified in three scenarios: (i) Normoxia, (ii) Acute hypoxia and (iii) Reoxygenation for all fish.

Activity trial protocol

Activity trials were conducted over nine days for all individuals. Fish groups in the holding aquaria were randomly assigned to one of the scenarios to be tested each day, ensuring that within each three-day period, every fish was exposed to all scenarios. The order of the scenarios was randomized daily whilst also accounting for potential variations in activity levels throughout the day. Over the entire nine-day period, each aquarium experienced the complete set of scenarios at different times of the day (Fig. 1C). At the onset of each behavioural trial, the arenas were filled with 700 mL of either 100% air saturated water for the Normoxia and Reoxygenation scenarios, or 40% air saturated water for the Acute hypoxia scenario. Fish tested in the Reoxygenation scenario were pre-exposed to hypoxia for 15 minutes in a 1L container filled with 40% air saturated water before being transferred to the arena. Each trial lasted for 15 minutes. To minimize disturbance, the entire setup was enclosed within a curtain. At the conclusion of each trial, the fish were returned to their holding aquaria, and arenas were emptied and refilled for the next round.

Video Data Extraction

Individual fish movement was tracked using DeepLabCut (DLC, version 2.2.2), a deep learning-based software for markerless pose estimation (Mathis et al., 2018). To train the model, 200 frames sampled from 9 different videos were manually annotated for three body parts: the head, mid-point, and tail base. The neural network architecture used was ResNet-50, based on the DeeperCut framework (Insafutdinov et al., 2016). Model training was performed using the default DLC parameters, with 200,000 training iterations. To improve accuracy, the model was retrained seven times, incorporating additional manual corrections to previously mislabelled frames. Model performance was assessed using a standard validation procedure in which the labelled dataset was randomly split into training and test sets (i.e. one shuffle). The final model achieved an average test error of 2.35 pixels and a training error of 2.36 pixels, relative to the image dimensions (282 × 336 pixels), indicating high tracking accuracy and minimal overfitting. To ensure only reliable position estimates were included in subsequent analyses, a likelihood threshold of 0.6 was applied to the model's predictions; only body part coordinates with a confidence score above this threshold were retained. The trained network was then used to analyse all experimental videos of the activity trials. Video analyses focused on a 14.5-minute trial duration, with the first 100 frames and the last 2,000 frames removed to ensure consistency in the recorded time and remove frames where the fish may be disturbed from the experimenter's presence. From the resulting tracking data, the proportion of time each fish spent moving was calculated as a measure of individual activity level. This activity metric was then used in subsequent statistical analyses.

Escape response

Escape response trials were performed following best practice guidelines (Roche *et al.*, 2023). The arena for testing the escape response consisted of a glass cylindrical container (22 x 10 cm) situated atop a transparent plexiglass plate and evenly illuminated from above. A high-speed camera (recording at 1000 fps) was aimed at a mirror positioned diagonally beneath the arena, allowing for an under-view recording of the fish without disturbing them or causing light reflections from the water. The escape response was triggered by dropping a weight from above into the arena. The weight was released through a plastic tube that ended 1 cm above the water surface, ensuring the fish could not see the weight before it hit the surface. The weight was dropped by pulling a trigger string, preventing the fish from being alerted by the experimenter's presence. A low and steady inflow and outflow of water maintained the desired oxygen level throughout the trial. To minimize disturbance, the entire setup was enclosed within a curtain.

Escape response protocol

Escape responses were quantified for each individual in three consecutive scenarios: Normoxia (100% air saturation), followed by Acute hypoxia (40 % air saturation), and finally, Reoxygenation. Within each scenario, the escape response was triggered three times (Fig. 1D). Every day, four fish from a randomly selected holding aquaria, were tested, with each individual fish undergoing only once a complete trial. After 20-minute of habituation period, the trigger was pulled to drop the weight into the arena. After each drop, the weight was repositioned, and a minimum of 10 minutes was allowed for the fish to return to calm conditions. After each completed trial, the escape response arena was fully drained, rinsed, and refilled before introducing the next fish. Escape response trials were conducted over 24 days for all individuals. Additionally, nine guppies underwent an escape response trial in constant normoxia as controls. This allowed testing whether repeated drops affected response time or responsiveness, independent of oxygen level changes (e.g., testing for exhaustion). All control fish were naïve to the escape response arena.

Video data extraction

All videos were manually analysed by counting the number of frames from the moment the weight hit the water until the onset of the fish's response. These frame counts were used to quantify two metrics for describing individual scape response: (i) time to respond (in milliseconds, ms) and (ii) responsiveness (1 = response; 0 = no response). If a fish did not exhibit a response within four seconds following the weight's impact, it was classified as non-responsive.

Metabolic rates

Metabolism was measured using intermittent-flow-through respirometry. Oxygen uptake was measured simultaneously for four fish within each trial following best practices (Clark *et al.*, 2013; Killen *et al.*, 2021). Each respirometer consisted of a glass chamber sealed with plastic caps and O-rings. The DO was measured in a 10- or 15-minute interval (adjusted for size of the fish) followed by five minutes of flushing, with time intervals controlled automatically. An oxygen sensor (Pyroscience robust oxygen probe) was connected to the outflow tubing to record the reduction of DO in each chamber and a peristaltic pump was used to ensure thorough mixing within the chambers. A separate water reservoir was used to supply the chambers with either normoxic or hypoxic water, regulated using the OptoReg system. For exhausting the fish for MMR measurements, the fish were individually swum in a customised 0.6 L cylindrical swimming chamber as in (Nilsson *et al.*, 2007; Morgan *et al.*, 2022). The chamber consisted of a raised platform with a magnetic stir bar underneath which created a water flow. A piece of plastic piping was fixed to the centre of the chamber so that the fish swam closer to the outside of the chamber. The chamber was filled with 25°C water and a DO level matching either the Normoxia or Acute hypoxia scenario they were being measured in. The chamber was placed on a stir plate and the speed was gradually increased until the fish could not maintain its position in the water column. The speed was then reduced slightly and after 10 minutes the fish were immediately transferred to individual respirometers, which were sealed (within 2 minutes of the fish being removed from the swimming chamber) allowing their MMR to be quantified.

Respirometry protocol

Respirometry trials started the day after the escape response trial. Fish were housed overnight in a separate container within their holding aquaria, to prevent any foraging opportunities before entering the respirometry trials and ensuring the guppies were fasted for 48 hours prior to respirometry. The respirometry trials were conducted over 25 days (Fig. 1A)**.** For each trial, every morning at 10:00 local time, four guppies were placed individually in a cylindrical swimming chamber filled with 25°C water at 100% air sat. and exercised to exhaust the fish. After 10 minutes, fish were transferred to individual respirometers and their MMR quantified in Normoxia. Then, fish remained undisturbed in the chambers for 18 hours, and SMR was quantified during night. Next morning at 06:00 local time, the DO in the water reservoir was decreased to 40% air sat. The SMR of the fish was then recorded under Acute hypoxia (40 % air saturation) for two hours. Last, the fish were removed from their respirometry chambers and transferred to the swimming chamber with water at 40% air saturation. After 10 minutes of forced swimming, they were returned to their individual respirometers, and MMR was quantified for 15 minutes during Acute hypoxia (Fig. 1E). The total duration for the full trial was

22 hours. The guppies were then removed and weighed, while the background respiration was recorded for 15 minutes with closed chambers (no flushing) after the trial. In some trials the background was recorded with an empty chamber along the full trial. Between trials, the system was rinsed with 30 ml of bleach to 25.5 L of water. All sensors were calibrated in 100 % air sat. at 25°C before each trial.

Metabolic rate data extraction

SMR was defined as the mean of the lowest 10th percentile of all extracted oxygen consumption rates, which were calculated using the R-package “respR” (Harianto *et al.*, 2019). MMR was extracted using a rolling regression that determines the highest rate over 15 or 20% of the data. Background data were recorded straight after each trial, and a single rate from each chamber was calculated to adjust the respective metabolic rates. Trials missing background data were adjusted for by two blank runs at the end of the entire experiment. The mean value of the rate calculated in the blank run was used to adjust those metabolic rates. Starting from the power scaling of metabolic rate with body mass $MR_i(w) = C_i\, w^b$, where C_i is the individual-specific scaling coefficient (intercept) and *b* is the scaling exponent (slope) for metabolic rate (MR) with weight (Norin and Gamperl, 2016), the individual metabolic rates were standardized to the mean weight of all fish following eq.1:

$$MR_i(\overline{w}) = MR_i(w_i) \times \left(\frac{\overline{w}}{w_i}\right)^b \qquad \text{eq.1}$$

where $MR_i(\overline{w})$ is the standardized MR for the individual fish *i* to the average body weight of all fish, $MR_i(w_i)$ is the measured metabolic rate for the individual *i* as a function of its measured weight, w_i is the measured weight of the individual fish *i* at the end of the metabolism trial and $\overline{w}$ is the mean body weight of all fish at the end of the metabolism trial (0.345 g). The scaling exponent *b* was calculated from a least squares linear regression analysis on the log-log transformed data for all measured metabolic rates against all measured body weights for all fish (Suppl. 2) and was estimated as 0.71 for SMR and 0.87 for the MMR. The aerobic scope (AS) was calculated as the difference between $MMR_i(\overline{w})$ and $SMR_i(\overline{w})$ (Clark *et al.*, 2013).

Statistical analysis

The effects of acclimation treatments (Constant normoxia and Fluctuating hypoxia) and exposure scenarios (Normoxia, Acute hypoxia and Reoxygenation) on Activity, SMR, MMR and AS (each tested separated) were tested using linear mixed models. Activity (proportion of time spent moving) was square root transformed for normalisation. All models were fit with a gaussian distribution using the “lme4” R-package (Bates *et al.*, 2015). Sex, length and growth were included as co-variants if they significantly improved the model. Interactions between acclimation treatment, exposure scenario and sex were also tested in each combination and retained depending on the AIC value. AIC values were used for model selection, with the model that had the lowest AIC value selected. Individual fish ID and the holding aquarium were included in all models as random effects. The residuals of the models were tested using the

"DHARMAa" package (Hartig *et al.*, 2024) and pairwise differences between treatments and scenarios and estimated marginal means were extracted using the R-package "emmeans" (Lenth *et al.*, 2024).

The repeatability of activity across trials and the corresponding 95% confidence intervals were estimated using the R-package "rptR" (Stoffel *et al.*, 2019). Trial was included as a fixed effect and individual fish ID was included as a random effect. The repeatability within each combination of acclimation and scenario was estimated in separate models.

The escape response was analysed using a Cox proportional hazards model with mixed effects (R-package "coxme"; Therneau, 2024). This model treats the fish's response time (in milliseconds) as a time-to-event variable, allowing it to simultaneously account for both how quickly a fish responded and whether it responded at all, effectively combining response time and responsiveness into a single survival outcome. Individuals that did not respond within the maximum observed time (300 ms) were treated as censored in the model. Acclimation, scenario, and their interaction were included in the model as fixed effects, with fish length as a covariate. Individual fish ID nested within aquarium was included as a random effect.

Finally, we explored the correlations among all measured traits (SMR, MMR, aerobic scope, growth, Activity, Response time, Responsiveness) within each acclimation group and across scenarios using Pearson´s correlation coefficients.

All data visualisation and analysis were done using the statistical program R version 4.2.2 (R Core Team, 2023) and effect sizes with a $p < 0.05$ were considered statistically significant.

Results

Activity: Time spent moving

Acute hypoxia decreased activity regardless of acclimation, yet this decrease was larger in the Constant normoxia acclimated fish (Fig. 2A, Table 1). These fish also showed similar activity levels in the Reoxygenation scenario as those in the Normoxia scenario (Fig. 2A, Table 1); while the fish acclimated to Fluctuating hypoxia showed levels of activity not significantly different from either the Normoxia or Hypoxia scenarios in the Reoxygenation scenario (Fig. 2A, Table 1). There was no significant effect of sex on activity levels (Fig. 2A, Table 1).

Activity levels showed high repeatability within each exposure scenario for fish acclimated to Constant normoxia (Fig. 2B), and activity levels were highly correlated between scenarios ($r = 0.71 - 0.77$; $p < 0.001$, Suppl. 5). Contrary, activity levels for fish acclimated to Fluctuating hypoxia did not show significant repeatability (Fig. 2B), and weaker, albeit still significant, correlations between scenarios ($r = 0.43 - 0.47$; $p < 0.01$, Suppl. 5).

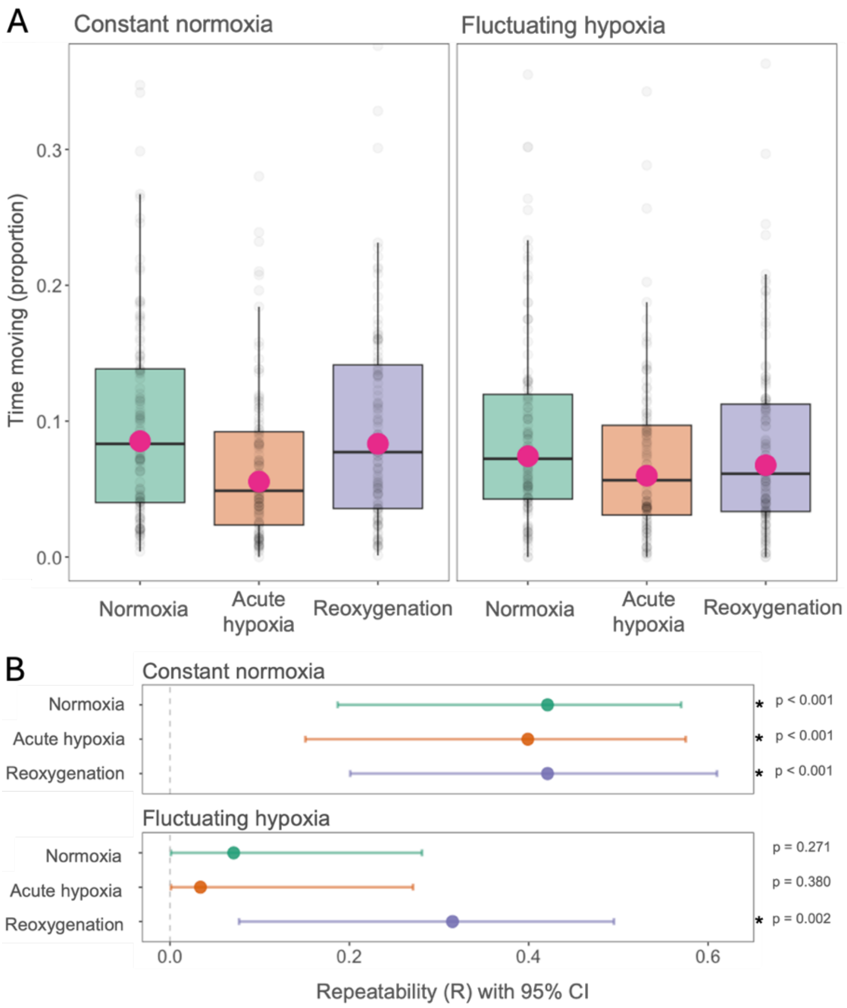

Figure 2: A - Boxplots for activity (Proportion of time moving) for fish acclimated to Constant normoxia (first panel) or Fluctuating hypoxia (second panel) and exposed to Normoxia (green; 100% air saturation), Acute hypoxia (orange; 40% air saturation) and Reoxygenation (purple; 100% air saturation after hypoxia. Pink dots indicate the modelled estimated marginal means (see Suppl. 3) whereas the box represents the 25% and 75% quartiles and the median (solid line). B - Individual repeatability of activity in each scenario (R) with 95% confidence intervals and significance level (see Suppl. 4).

Escape response

During the escape response trial, fish acclimated to Constant normoxia had a decreased likelihood of responding in Acute hypoxia relative to Normoxia (Fig. 3, Normoxia-Hypoxia, z = 2.679, p = 0.007). This decreased responsiveness remains upon returning to normoxia in the Reoxygenation scenario (Fig 3; Hypoxia-Reoxygenation, z = 1.675, p = 0.094; Normoxia-Reoxygenation, z = 4.275, p < 0.001). Fish acclimated to Fluctuating hypoxia had a similar responsiveness during both the Normoxia and Acute hypoxia scenarios (Fig 3; Normoxia-Hypoxia, z = - 1.58, p = 0.114) and the Normoxia and Reoxygenation scenario (Fig 3; Normoxia-Reoxygenation, z = 0.508, p = 0.612) but when comparing between Acute hypoxia and Reoxygenation the responsiveness decreased (Fig 3; Hypoxia-Reoxygenation, z = 2.057, p = 0.040). Fish showed higher responsiveness in the scenario that was similar to their acclimation conditions, i.e., fish acclimated to Normoxia showed higher responsiveness in normoxia while fish acclimated to Fluctuating hypoxia showed higher responsiveness when exposed to acute hypoxia. Control fish (i.e., those exposed only to Normoxia for nine repeated drops) did not differ in their responsiveness (Suppl.6; Start-End, i.e. drops 1-3 vs drops 7-9, z=-0.541, p=0.589).

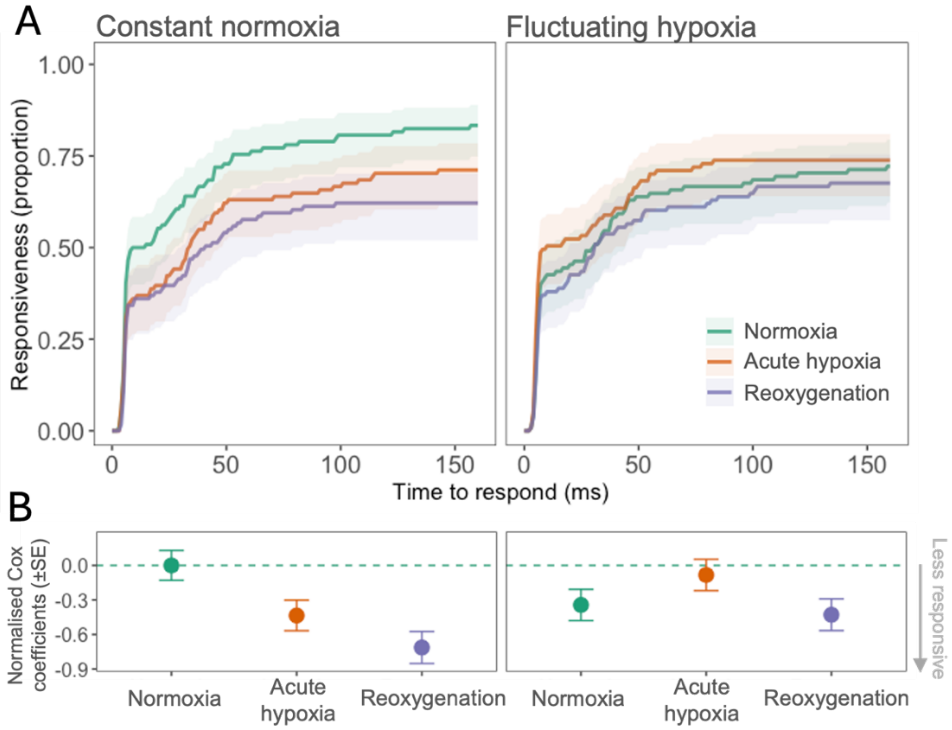

Figure 3: Escape response visualised in panel A with a Kaplan-Meier Survival curve showing likelihood of responding to a stimulus (object dropped) over time (ms) for fish acclimated to Constant normoxia and Fluctuating hypoxia and then exposed to Normoxia (green), Acute hypoxia (orange; 40% air saturation) and Reoxygenation after hypoxia (purple). Panel B shows cox coefficients ±SE from the Cox proportional hazards model and normalised to Constant normoxia acclimation, and Normoxia scenario.

Responsiveness showed a positive correlation between scenarios within each acclimation treatment (r = 0.39-0.48, $p < 0.01$, for Constant normoxia-acclimated fish and r = 0.48-0.5, $p < 0.01$ for Fluctuating hypoxia-acclimated fish, Suppl. 5). This indicates that individuals who exhibited higher responsiveness when exposed to Normoxia also demonstrated higher responsiveness under Acute hypoxia and Reoxygenation, regardless of their acclimation treatment. Additionally, time to respond was positively correlated in the Normoxia and Reoxygenation scenarios for both acclimation treatments (Constant normoxia, r= 0.34, p=0.046; Fluctuating hypoxia, r = 0.5, p=0.003) but not with Acute hypoxia (Suppl. 5).

Metabolic rates

Acclimation had no significant effect on either SMR or MMR (Fig. 4; Table 1). Exposure to Acute hypoxia did however cause a significant decrease in both metabolic rates (Fig 4; Table 1). Albeit non-significantly, the decrease of SMR under Acute hypoxia was greater for males than for females, while the opposite happened for MMR (Fig 5a, b), leading to an overall reduced aerobic scope (AS), which was significantly lower for females (Fig 5c).

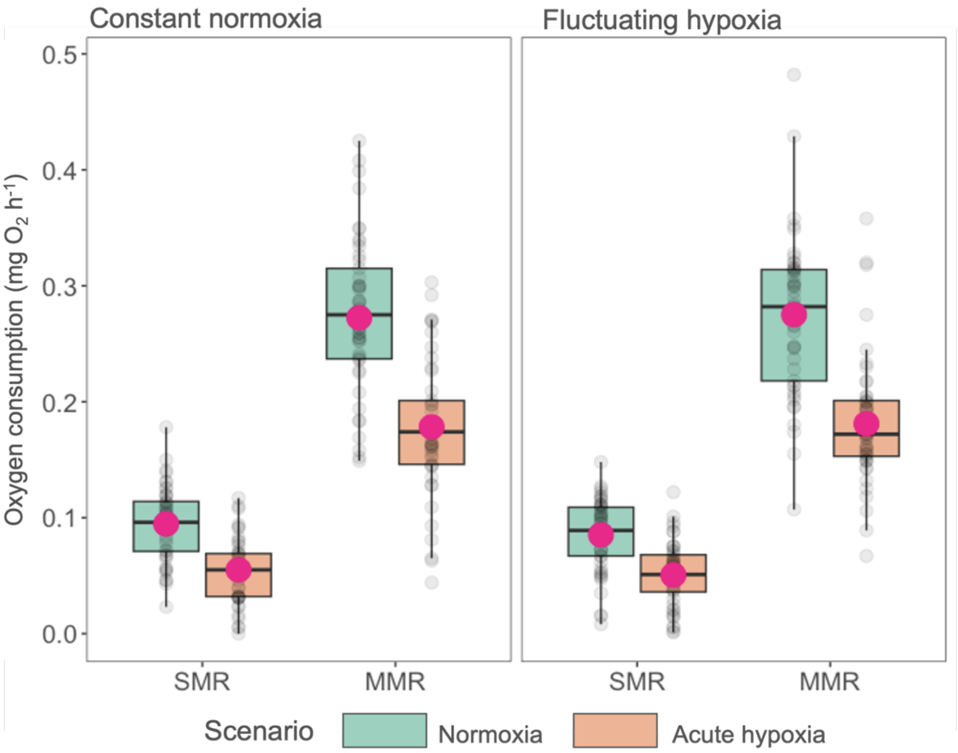

Figure 4: The standard (SMR) and maximum (MMR) metabolic rates for fish acclimated to either Constant normoxia or Fluctuating hypoxia and exposed to Normoxia (green) and Acute hypoxia (orange; 40% air saturation). Values for the metabolic rates are standardised to a common body mass of 0.345g (mean of all fish). The pink dots represent the estimated marginal means (derived from best-fit models, see Suppl. 3), whereas the box represents the 25% and 75% quartiles and the median (solid line).

MMR under Acute hypoxia was positively correlated with both SMR and MMR under Normoxia, indicating that individuals with higher metabolic rates in normoxic conditions also tended to maintain higher MMR in hypoxic conditions, regardless of acclimation treatment (r = 0.45 – 0.73, $p < 0.001$; Suppl. 5). Only weak correlations however were observed between SMR and MMR within each scenario (Suppl. 5). Similarly, metabolic rates did not correlate with activity levels across scenarios for any of the acclimation treatments (Suppl. 5).

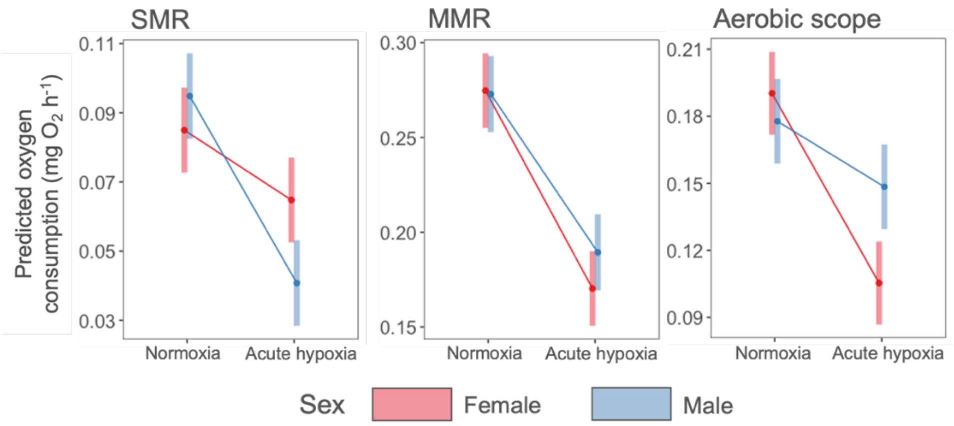

Figure 5: Sex effect under Acute hypoxia on metabolic rates (SMR, MMR and AS). Data shown are estimated marginal means ± confidence intervals (Suppl. 3). Acclimation had no significant effect on metabolism so is excluded.

Table 1: Summary of model-derived contrasts for activity (square-root transformed), standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic scope (AS), based on best-fit linear mixed-effects models. Models include fixed effects of scenario (Normoxia, Acute hypoxia, Reoxygenation), acclimation (Constant normoxia, Fluctuating hypoxia) and sex (Male, M and Female F), where applicable, and random intercepts for each individual fish (individual) and aquarium. For estimated marginal means and standard errors, see Suppl. 3.

	Contrast	**Estimate**	**SE**	**df**	**t-ratio**	**p-value**
Activity: ***Model = lmer (sqrt (Activity) ~ Scenario * Acclimation + Sex + (1 | Individual) + (1 | Aquarium)***						
Constant normoxia	Normoxia - Acute hypoxia	0.056	0.013	577	4.258	**<0.001**
	Normoxia - Reoxygenation	0.003	0.013	575	0.248	0.804
	Acute hypoxia – Reoxygenation	-0.053	0.014	578	-3.941	**<0.001**
Fluct. hypox.	Normoxia – Acute hypoxia	0.028	0.014	581	2.019	**0.044**
	Normoxia - Reoxygenation	0.012	0.013	579	0.934	0.351
	Acute hypoxia - Reoxygenation	-0.016	0.013	576	-1.165	0.245
Normoxia	Constant normoxia – Fluct. hypox.	0.020	0.023	16	0.865	0.399
Acute Hypoxia	Constant normoxia – Fluct. hypox.	-0.009	0.023	17	-0.390	0.701
Reoxygenation	Constant normoxia – Fluct. hypox.	0.029	0.023	16	1.267	0.223
Sex	Female – Male	-0.025	0.137	83	-1.780	0.079
SMR***: Model = lmer (SMR ~ Scenario * Acclimation * Sex + (1 | Individual) + (1 | Aquarium)***						
Constant normoxia	Normoxia – Acute hypoxia	0.040	0.004	86	9.358	**<0.001**
Fluct. hypox.	Normoxia – Acute hypoxia	0.035	0.004	86	8.169	**<0.001**
Normoxia	Constant normoxia – Fluct. hypox.	0.009	0.011	12	0.900	0.386
Acute hypoxia	Constant normoxia – Fluct. hypox.	0.004	0.011	12	0.426	0.678
Sex F	Normoxia – Acute hypoxia	0.020	0.004	86	4.870	**<0.001**
Sex M	Normoxia – Acute hypoxia	0.054	0.004	86	12.621	**<0.001**
MMR***: Model = lmer (MMR ~ Scenario + Acclimation + (1 | Individual) + (1 | Aquarium)***						
-	Normoxia – Acute hypoxia	0.094	0.007	88	14.000	**<0.001**
-	Constant normoxia – Fluct. hypox.	-0.003	0.013	10	-0.204	0.842
Aerobic Scope: ***Model = lmer (AS ~ Scenario * Sex + (1 | Individual) + (1 | Aquarium)***						
Constant normoxia	Female - Male	0.013	0.013	146	0.940	0.349
Acute hypoxia	Female - Male	-0.043	0.013	146	-3.234	**0.002**
Sex F	Normoxia – Acute hypoxia	0.085	0.011	88	7.835	**<0.001**
Sex M	Normoxia – Acute hypoxia	0.029	0.011	88	2.649	0.0096

Discussion

Our results show that acute exposure to hypoxia significantly decreased activity and metabolism in guppies. Prior acclimation to fluctuating hypoxia did not affect metabolism, but it lessened the decrease in activity, reduced its repeatability, and improved escape performance. Following reoxygenation, normoxia-acclimated fish fully recovered their activity but showed reduced escape performance, while hypoxia-acclimated fish only partially recovered activity but maintained escape performance at normoxia levels, despite an overall reduction in responsiveness. Altogether, our findings demonstrate that acclimation to fluctuating hypoxia modulates performance in acute hypoxia and upon reoxygenation in a trait-specific and context-dependent manner.

Escape responses in guppies were influenced by both acclimation and scenario. In Constant normoxia-acclimated fish, acute hypoxia lowered escape performance, as has been shown in other fish species previously (Domenici *et al.*, 2007; Gotanda *et al.*, 2012; Milinkovitch *et al.*, 2019). Low oxygen has been hypothesised to impair the neurons within the lateral line which control the escape response, reducing the sensitivity to stimuli and thereby making fish less responsive (Lefrançois and Domenici, 2006). Similar hypoxia induced impairments have been documented in the inner ear ciliated cells of amphibia, which are like the sensory cells in the lateral line of fish (Sitko and Honrubia, 1986; Schellart and Wubbels, 1998; Lefrançois and Domenici, 2006). In addition, escape responses, which are anaerobic in nature, may cause accumulation of lactate, when occurring during hypoxia, leading to muscle fatigue and incurring an oxygen debt which may carry through to the reoxygenation phase explaining the lower escape performance observed. While the same explanation could be given for the decreased performance upon reoxygenation in the hypoxia-acclimated fish, their performance prior to hypoxia was comparable, and these fish had their highest escape performance during acute hypoxia. Studies from other animal models have shown that repeated hypoxic exposures can cause upregulation of genes related to neuroprotection which could then enhance neuron function during hypoxia (Baillieul *et al.*, 2017). A similar mechanism may have played a role here, potentially allowing for an increased responsiveness in hypoxic conditions, though remains untested.

In both acclimation groups, an acute exposure to hypoxia decreased activity. A decreased activity under hypoxia has been shown in various species such as Atlantic cod *(Gadus morhua)* (Herbert and Steffensen, 2005*;* Chabot and Claireaux*, 2008),* weakfish *(Cynoscion regalis)* (Brady *et al.*, 2009) and African mormyrid fish (Ackerly *et al.* 2017). A decrease in activity has been suggested to be a strategy for reducing oxygen demands in response to diminished aerobic metabolic capacity under hypoxic conditions (Congleton, 1980; Suthers and Gee, 1986). This could be the mechanism explaining our observations, as indeed we did measure decreased metabolic rates and aerobic scope under hypoxia. In the wild this could mean that when oxygen levels in the streams become hypoxic the guppies will have a reduced level of activity and a lower available aerobic scope, notably a lower maximum aerobic capacity. This can be both advantageous and disadvantageous: reduced activity makes the fish less conspicuous to predators and saves energy, but a lower maximum capacity hinders their ability to sustain a high swimming speed to escape predators. Yet, predatory fish species may also become less active during hypoxia, and feed less frequently, reducing the predation risk for smaller fish such as guppies (Chapman and Mckenzie, 2009; Diaz and Breitburg, 2009). Additionally, lower oxygen levels may deter certain predator species that require higher oxygen concentrations for survival, further decreasing the risk of predation. Predator-prey dynamics are therefore also influenced not only by how the guppy responds to hypoxia but also by their predator's response.

Whilst acute hypoxia decreases both activity and metabolism there is no correlation within a scenario between these two traits, meaning that individuals with the lowest activity do not necessarily have the lowest aerobic scope when measured in the same oxygen conditions. If different individuals within the same acclimation group adopt different behavioural and metabolic strategies this could explain the low correlation. For example, when exposed to acute hypoxia some fish species have been shown to respond with an increase, rather than a decrease in activity, to increase the likelihood of finding more oxygenated water (Bushnell and Brill, 1991; Herbert and Steffenson, 2006; Weltzien *et al.,* 1999). Some individuals with a low aerobic scope could therefore respond by increasing activity, a short-term energetic cost that may have a long-term benefit if they find more oxygenated water, whereas others with low aerobic scope decrease activity to conserve energy in low oxygenated water. If multiple strategies occur simultaneously this could reflect underlying heterogeneity at the population level and this variation may buffer populations during oxygen fluctuations.

Another interesting result was the difference in aerobic scope between males and females under acute hypoxia, while both sexes had a lower aerobic scope than in normoxia, females were more strongly affected. Pregnancy might explain this as it has been shown to increase metabolic costs in live-bearing fish (Webb and Brett, 1972; Boehlert *et al.*, 1991). In addition, pregnant sailfin mollies (*Poecilia latipinna*) not only had elevated metabolism in normoxia but also spent more time than non-pregnant females performing ASR when exposed to hypoxia (Timmerman and Chapman, 2003). However, some studies show no pregnancy-related metabolic cost, including in guppies (Svendsen *et al.*, 2013; Callaghan *et al.*, 2021), and we found no difference between sexes in normoxia, leaving the role of pregnancy in reducing aerobic scope under acute hypoxia unclear.

Although there were not large differences in activity levels between acclimation treatments, the fluctuating hypoxia-acclimated fish exhibited lower repeatability in their behaviour, indicating greater variability and less consistency. Behavioural repeatability is context specific and can be altered by environmental stress (Briffa *et al.*, 2013; Killen *et al.*, 2016). Fluctuating hypoxia could have increased variation in the physiological state of the fish (e.g., in hormone levels or hunger), leading to less repeatable behaviour at the individual level. Unpredictable behaviour could represent an adaptive response to an unpredictable environment, decreasing susceptibility to predation (Brembs, 2011). However, reduced repeatability may mask stable behavioural traits upon which natural selection operates, with potential broader evolutionary effects (Killen *et al.* 2013).

In fluctuating hypoxia, we observed a reduced change in activity across scenarios, suggesting either more consistent behaviour or a decreased sensitivity to changes in dissolved oxygen. This could be due to two possible reasons. First, lower behavioural repeatability in fluctuating hypoxia-acclimated fish could mask clear activity responses, as individuals react in more varied and unpredictable ways (Killen *et al.*, 2013). Second, acclimation to fluctuating oxygen levels may minimise the need for frequent behavioural changes, leading a more stable performance across conditions. In such environments, maintaining behavioural consistency might allow individuals to conserve energy for other processes, such as recovering from previous stress or allowing greater flexibility to respond to unpredictable situations (Holt and Jørgensen, 2015), such as another hypoxic events. In this way, behavioural consistency may serve as a compensatory strategy that buffers against environmental unpredictability and could indicate an alternate strategy that enhances long-term survival in fluctuating environments.

In our study, SMR decreased significantly during acute hypoxia in both normoxia- and fluctuating hypoxia-acclimated fish, indicating no effect of acclimation. This contrasts with other studies (Borowiec et al. 2018, killifish, *Fundulus heteroclitus*; Ducros et al. 2024, Arctic charr, *Salvelinus alpinus*) which found that fish acclimated to fluctuating hypoxia maintained SMR under acute hypoxia, similar to normoxia-acclimated fish in normoxia. However, Ducros et al. (2024) did find RMR (i.e. routine metabolic rate) decreased in fluctuating hypoxia acclimated fish when measured under acute hypoxia, similar to our results, which they attributed to reduced activity. While we measured SMR (i.e. minimum metabolic rate, in the absence of activity) our estimates may reflect metabolism incorporating some activity. Whilst this may partly explain the decrease in metabolism under acute hypoxia in our study, our behavioural data shows only a modest reduction in activity under acute hypoxia and therefore seems insufficient to fully explain our results. Another study which also showed similar results to ours measured RMR in normoxia (Borowiec et al. 2015) and reported no significant difference between fish acclimated to fluctuating hypoxia (minimum 22% air saturation) and those held in constant normoxia. Yet, a separate group which they exposed to more severe fluctuations, (minimum 9% air saturation), did show significant reductions in RMR, suggesting the severity of the hypoxic fluctuations, plays an important role in driving metabolic changes. In our study, fish were exposed to fluctuating hypoxia with a moderate oxygen minimum (40% air saturation at night) over 28 days. While the acclimation duration was similar to Borowiec et al. (2015, 2018), our oxygen minimum was higher and so may not have been low enough to trigger compensatory physiological responses such as increased haematocrit or shifts in haemoglobin isomorphs (Tun & Houston, 1986; Petersen & Petersen, 1990).

While aquatic hypoxia is often characterised by levels of dissolved oxygen below 2 - 3 mg L^{-1} (Diaz and Rosenberg, 2008), which is lower than what the guppies were exposed to in this study, the specific oxygen threshold can vary between species (Richards, 2009; Blewett *et al.*, 2022). Aquatic hypoxia can therefore be more precisely defined as any decrease in dissolved oxygen that leads to a compensatory response in an organism (Blewett *et al.*, 2022). Since the acute exposures showed responses in all measured traits, this suggests that an oxygen level of 40% does lie within the hypoxic threshold for guppies, however it could be that the moderate decrease in oxygen in the night in the fluctuating hypoxia group combined with the daytime normoxia periods may have allowed sufficient recovery, reducing the need for full acclimation to low oxygen conditions. Alternatively, the fish might have used aquatic surface respiration (ASR) at the air-water interface, a known hypoxia-coping strategy in guppies (Weber and Kramer, 1983), which could have increased the oxygen levels they experienced, reducing the severity of their hypoxic exposure. Yet even if fluctuations reaching 40% were insufficient to trigger metabolic adjustments, both activity and escape response were significantly affected by acclimation, suggesting that some traits are more sensitive to oxygen changes than others.

We could also hypothesise that metabolic changes did occur at the molecular level in our fluctuating hypoxia acclimated fish but were not captured in our whole-organism metabolic rate measurements. For example, acute hypoxia exposure may have led to an increased use of anaerobic metabolism in both acclimation groups, as is previously shown for killifish (Robertson *et al.*, 2015; Borowiec *et al.*, 2018). Yet fish acclimated to fluctuating hypoxia may have had a greater capacity to use and recover from anaerobic metabolism, e.g. increased capacity for metabolising and clearing lactate (Borowiec *et al.*, 2015). This would mean the metabolic rates in normoxia, and hypoxia could appear the same, but there may have been differences in the reoxygenation period after hypoxia. Unfortunately, we did not measure the metabolic rates during reoxygenation, or changes in enzyme activity, so cannot test this hypothesis here. The variety of responses between studies does however suggest that fish could use a range of strategies and metabolic pathways to cope with fluctuating hypoxia.

Overall, we show that acclimation to fluctuating hypoxia leads to changes in guppy performance that are both context and trait specific. While acute hypoxia reduced activity and metabolism regardless of acclimation history, escape performance was optimised to match each group's acclimation environment: hypoxia-acclimated fish performed better in hypoxia, while normoxia-acclimated fish had their highest performance in normoxia. Notably, both groups exhibited a lower escape performance upon reoxygenation, highlighting that understanding responses to hypoxia requires consideration of both the immediate and lasting effects. Finally, we suggest how changes in one trait, such as sustained low activity, which may seem detrimental, can be part of a broader strategy that aids long-term survival. In summary, our results demonstrate that performance under hypoxia is shaped not only by current oxygen conditions, but also by prior acclimation history and the specific traits being measured. This highlights the complexity of interpreting responses to fluctuating environments and suggests that a deeper understanding of acclimation processes is necessary for predicting organismal performance in dynamic habitats.

Acknowledgements

We are grateful to Heikki Savolainen and Frank Midtøy at the University of Bergen for logistical support when setting up the lab, Christian Jørgensen for helpful discussions and Mikko Heino for financial support to ED & MF during the writing phase.

Authors contributions

ED & MF: investigation, formal analysis, methodology, writing-initial draft; ACC: conceptualisation, supervision, writing – reviewing and editing; BDP: conceptualisation, formal analysis, resources, writing – reviewing and editing; RM: conceptualisation, supervision, methodology, resources, formal analysis, writing – final draft.

Conflicts of Interest

The authors declare there are no conflicts of interest

Funding

The Research Council of Norway funded RM and ACC (project number 338973) and BDP, MF and ED (project number 275125)

Data Availability

All data and code are available at https://github.com/RachaelLMorgan/Data-and-code-for-Doddema-et-al.-Fluctuating-hypoxia-guppies

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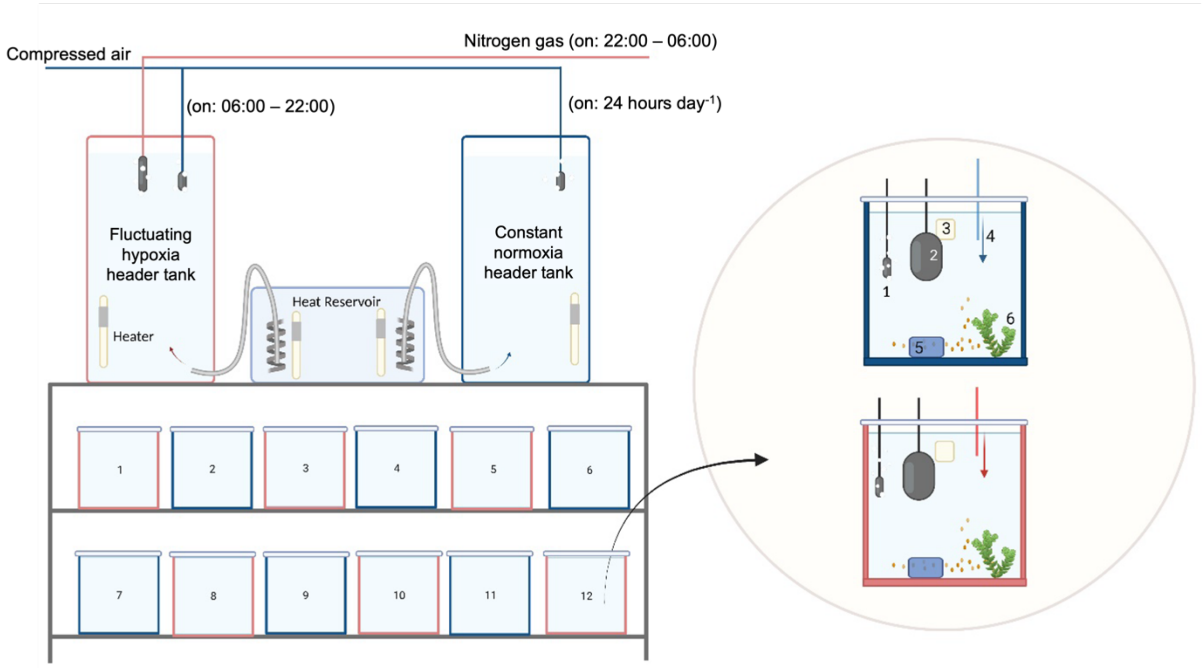
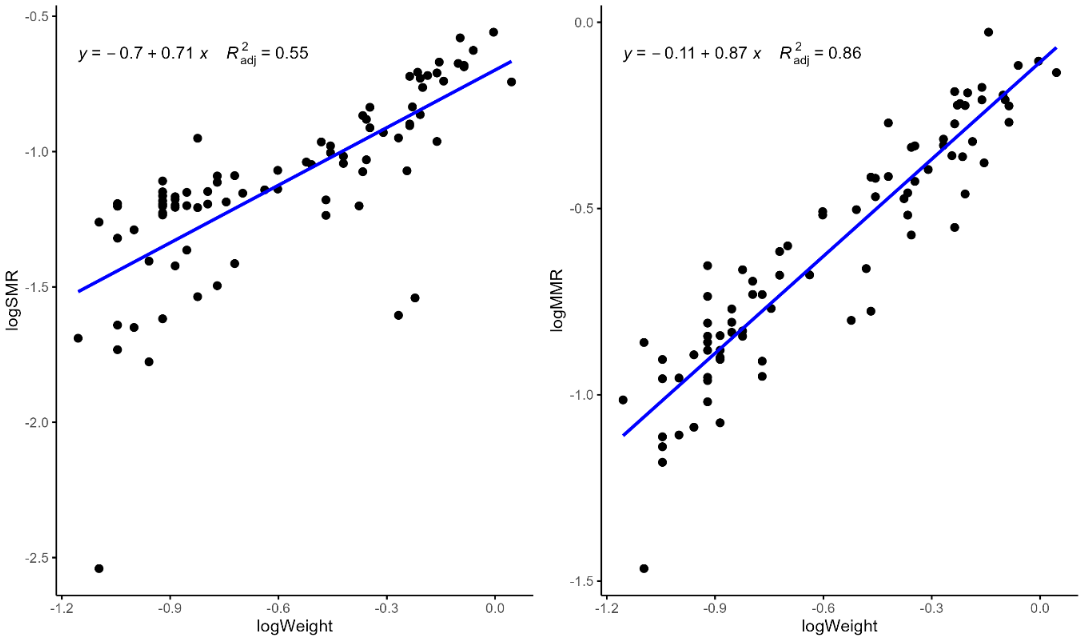
Supplementary Information



Supplementary Information 1: Overview of the aquarium set-up for acclimation to either constant normoxia (blue) or fluctuating hypoxia (red). Timers were used to open and close the nitrogen and compressed air lines to create the Fluctuating hypoxia acclimation treatment (40% air sat at night and 100% air sat during day). Compressed air was continuously bubbled to the Constant normoxia tank to maintain fully aerated water (100% air sat). Water was preheated prior to entering the aquaria. Within each aquarium was 1) air stone (on from 06:00-22:00 and off from 22:00-06:00 in all aquaria) 2) oxygen sensor 3) overflow tubing 4) wate inflow tubing 5) plastic shelter 6) artificial plant.



Supplementary Information 2: Log-log scaling plot of absolute metabolic rates (SMR: left panel, MMR: right panel) and weight (g) of guppies from which the scaling exponent could be extracted and used to normalised metabolic rates to a mean weight.

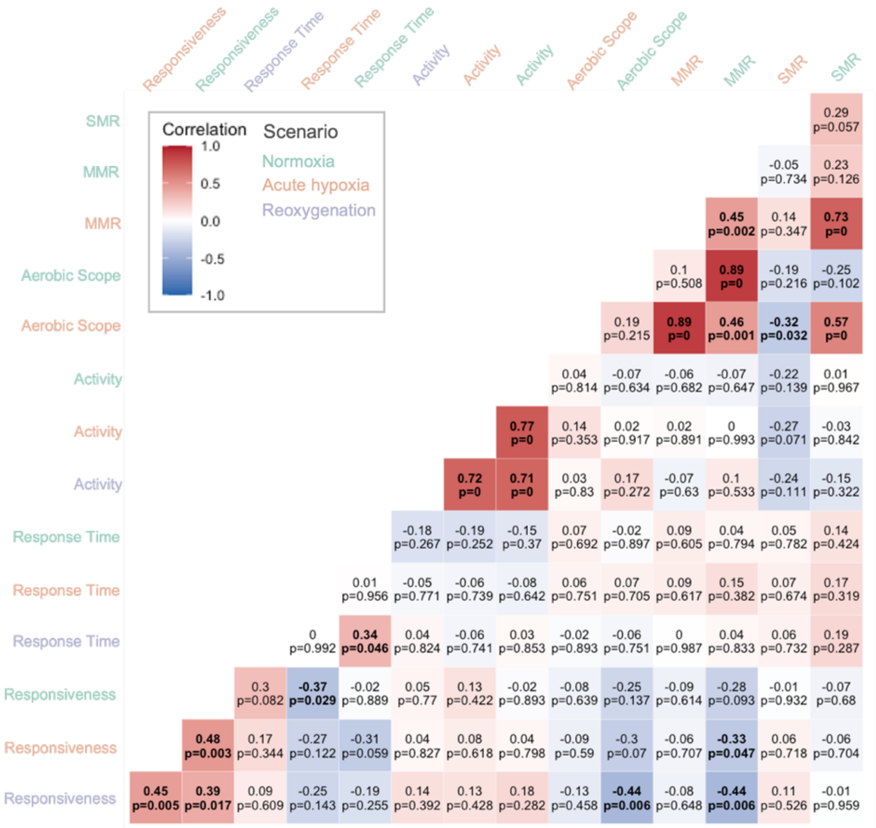
Supplementary Information 3: The final linear mixed effect models for activity and metabolic rates (SMR, MMR, aerobic scope), showing estimated marginal means and standard errors (SE). See Table 1 for contrasts between levels and significance. Activity estimates are back-transformed for ease of interpretation, SMR and MMR estimates are averaged over sex.

	Model	Estimate	SE
***Activity:* lmer (sqrt (Activity) ~ Acclimation * Scenario + Sex + (1 | Individual) + (1 | Aquarium)**			
Constant normoxia	Normoxia	0.085	0.009
	Acute Hypoxia	0.056	0.008
	Reoxygenation	0.083	0.009
Fluctuating hypoxia	Normoxia	0.074	0.009
	Acute Hypoxia	0.060	0.008
	Reoxygenation	0.070	0.008
***SMR*: lmer (SMR ~ Acclimation * Scenario * Sex + (1 | Individual) + (1 | Aquarium)**			
Constant normoxia	Normoxia	0.095	0.007
	Acute Hypoxia	0.055	0.007
Fluctuating hypoxia	Normoxia	0.085	0.007
	Acute Hypoxia	0.051	0.007
***MMR*: lmer (MMR ~ Acclimation + Scenario + (1 | Individual) + (1 | Aquarium)**			
Constant normoxia	Normoxia	0.272	0.010
	Acute Hypoxia	0.178	0.010
Fluctuating hypoxia	Normoxia	0.275	0.010
	Acute Hypoxia	0.181	0.010
***Aerobic scope*: lmer (AS ~ Sex * Scenario + (1 | Individual) + (1 | Aquarium)**			
Sex: Female	Normoxia	0.190	0.009
	Acute Hypoxia	0.105	0.009
Sex: Male	Normoxia	0.178	0.010
	Acute Hypoxia	0.148	0.010

Supplementary Information 4: Individual repeatability of Activity (Time spent moving), including 95% confidence intervals (CI) and p-values.

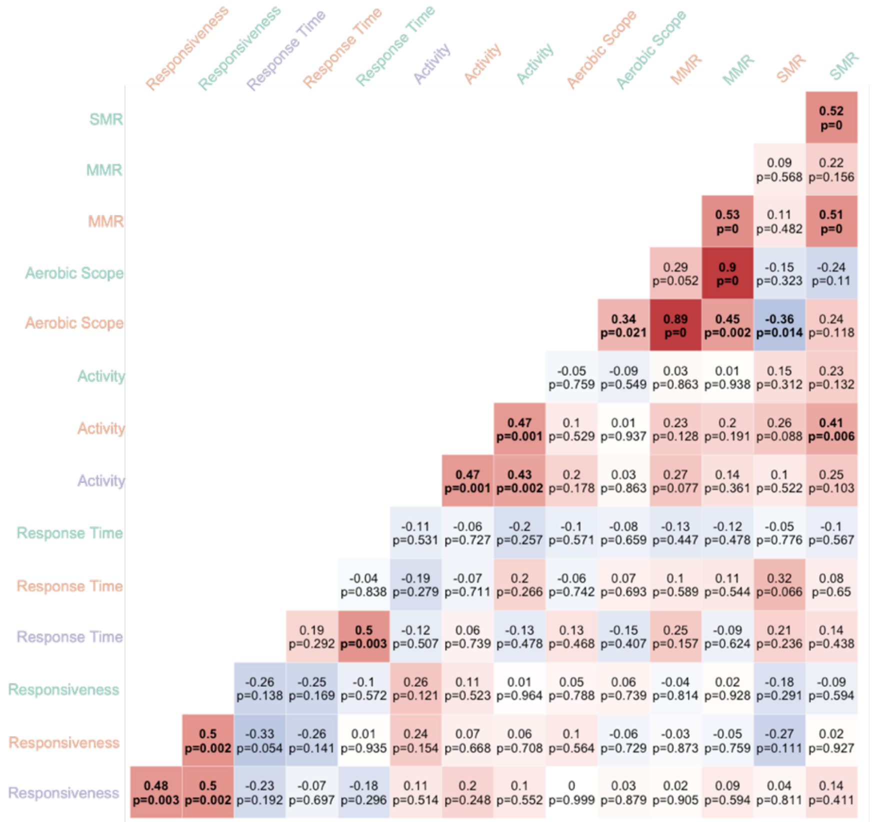
Acclimation	**Scenario**	**R**	**CI**		**p-value**
Constant normoxia	Normoxia	0.421	0.203	0.603	<0.001
	Acute hypoxia	0.366	0.117	0.561	<0.001
	Reoxygenation	0.421	0.189	0.599	<0.001
Fluctuating hypoxia	Normoxia	0.071	0.000	0.085	0.271
	Acute hypoxia	0.038	0.000	0.274	0.380
	Reoxygenation	0.315	0.074	0.508	0.002

Constant normoxia



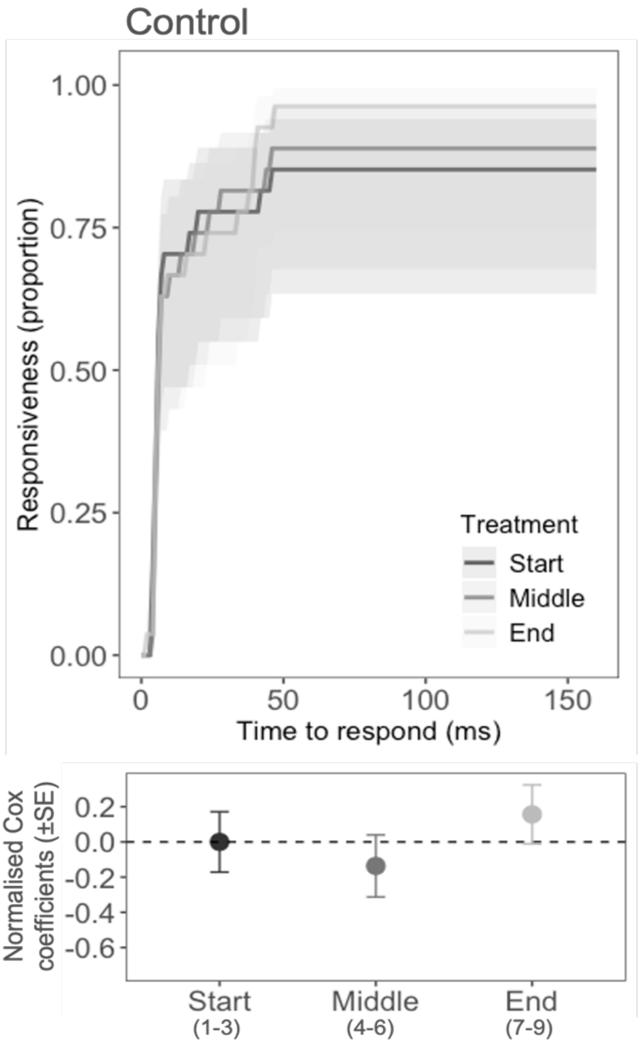
	Responsiveness	Responsiveness	Response Time	Response Time	Response Time	Activity	Activity	Activity	Aerobic Scope	Aerobic Scope	MMR	MMR	SMR	SMR
SMR														0.29 p=0.057
MMR													-0.05 p=0.734	0.23 p=0.126
MMR												**0.45** **p=0.002**	0.14 p=0.347	**0.73** **p=0**
Aerobic Scope											0.1 p=0.508	**0.89** **p=0**	-0.19 p=0.216	-0.25 p=0.102
Aerobic Scope										0.19 p=0.215	**0.89** **p=0**	**0.46** **p=0.001**	**-0.32** **p=0.032**	**0.57** **p=0**
Activity									0.04 p=0.814	-0.07 p=0.634	-0.06 p=0.682	-0.07 p=0.647	-0.22 p=0.139	0.01 p=0.967
Activity								**0.77** **p=0**	0.14 p=0.353	0.02 p=0.917	0.02 p=0.891	0 p=0.993	-0.27 p=0.071	-0.03 p=0.842
Activity							**0.72** **p=0**	**0.71** **p=0**	0.03 p=0.83	0.17 p=0.272	-0.07 p=0.63	0.1 p=0.533	-0.24 p=0.111	-0.15 p=0.322
Response Time						-0.18 p=0.267	-0.19 p=0.252	-0.15 p=0.37	0.07 p=0.692	-0.02 p=0.897	0.09 p=0.605	0.04 p=0.794	0.05 p=0.782	0.14 p=0.424
Response Time					0.01 p=0.956	-0.05 p=0.771	-0.06 p=0.739	-0.08 p=0.642	0.06 p=0.751	0.07 p=0.705	0.09 p=0.617	0.15 p=0.382	0.07 p=0.674	0.17 p=0.319
Response Time				0 p=0.992	**0.34** **p=0.046**	0.04 p=0.824	-0.06 p=0.741	0.03 p=0.853	-0.02 p=0.893	-0.06 p=0.751	0 p=0.987	0.04 p=0.833	0.06 p=0.732	0.19 p=0.287
Responsiveness			0.3 p=0.082	**-0.37** **p=0.029**	-0.02 p=0.889	0.05 p=0.77	0.13 p=0.422	-0.02 p=0.893	-0.08 p=0.639	-0.25 p=0.137	-0.09 p=0.614	-0.28 p=0.093	-0.01 p=0.932	-0.07 p=0.68
Responsiveness		**0.48** **p=0.003**	0.17 p=0.344	-0.27 p=0.122	-0.31 p=0.059	0.04 p=0.827	0.08 p=0.618	0.04 p=0.798	-0.09 p=0.59	-0.3 p=0.07	-0.06 p=0.707	**-0.33** **p=0.047**	0.06 p=0.718	-0.06 p=0.704
Responsiveness	**0.45** **p=0.005**	**0.39** **p=0.017**	0.09 p=0.609	-0.25 p=0.143	-0.19 p=0.255	0.14 p=0.392	0.13 p=0.428	0.18 p=0.282	-0.13 p=0.458	**-0.44** **p=0.006**	-0.08 p=0.648	**-0.44** **p=0.006**	0.11 p=0.526	-0.01 p=0.959

Fluctuating hypoxia



	Responsiveness	Responsiveness	Response Time	Response Time	Response Time	Activity	Activity	Activity	Aerobic Scope	Aerobic Scope	MMR	MMR	SMR	SMR
SMR														**0.52** **p=0**
MMR													0.09 p=0.568	0.22 p=0.156
MMR												**0.53** **p=0**	0.11 p=0.482	**0.51** **p=0**
Aerobic Scope											0.29 p=0.052	**0.9** **p=0**	-0.15 p=0.323	-0.24 p=0.11
Aerobic Scope										**0.34** **p=0.021**	**0.89** **p=0**	**0.45** **p=0.002**	**-0.36** **p=0.014**	0.24 p=0.118
Activity									-0.05 p=0.759	-0.09 p=0.549	0.03 p=0.863	0.01 p=0.938	0.15 p=0.312	0.23 p=0.132
Activity								**0.47** **p=0.001**	0.1 p=0.529	0.01 p=0.937	0.23 p=0.128	0.2 p=0.191	0.26 p=0.088	**0.41** **p=0.006**
Activity							**0.47** **p=0.001**	**0.43** **p=0.002**	0.2 p=0.178	0.03 p=0.863	0.27 p=0.077	0.14 p=0.361	0.1 p=0.522	0.25 p=0.103
Response Time						-0.11 p=0.531	-0.06 p=0.727	-0.2 p=0.257	-0.1 p=0.571	-0.08 p=0.659	-0.13 p=0.447	-0.12 p=0.478	-0.05 p=0.776	-0.1 p=0.567
Response Time					-0.04 p=0.838	-0.19 p=0.279	-0.07 p=0.711	0.2 p=0.266	-0.06 p=0.742	0.07 p=0.693	0.1 p=0.589	0.11 p=0.544	0.32 p=0.066	0.08 p=0.65
Response Time				0.19 p=0.292	**0.5** **p=0.003**	-0.12 p=0.507	0.06 p=0.739	-0.13 p=0.478	0.13 p=0.468	-0.15 p=0.407	0.25 p=0.157	-0.09 p=0.624	0.21 p=0.236	0.14 p=0.438
Responsiveness			-0.26 p=0.138	-0.25 p=0.169	-0.1 p=0.572	0.26 p=0.121	0.11 p=0.523	0.01 p=0.964	0.05 p=0.788	0.06 p=0.739	-0.04 p=0.814	0.02 p=0.928	-0.18 p=0.291	-0.09 p=0.594
Responsiveness		**0.5** **p=0.002**	-0.33 p=0.054	-0.26 p=0.141	0.01 p=0.935	0.24 p=0.154	0.07 p=0.668	0.06 p=0.708	0.1 p=0.564	-0.06 p=0.729	-0.03 p=0.873	-0.05 p=0.759	-0.27 p=0.111	0.02 p=0.927
Responsiveness	**0.48** **p=0.003**	**0.5** **p=0.002**	-0.23 p=0.192	-0.07 p=0.697	-0.18 p=0.296	0.11 p=0.514	0.2 p=0.248	0.1 p=0.552	0 p=0.999	0.03 p=0.879	0.02 p=0.905	0.09 p=0.594	0.04 p=0.811	0.14 p=0.411

Supplementary Information 5: Correlation matrices for Constant Normoxia acclimation (top panel) and Fluctuating hypoxia (bottom panel). Fill colour within each cell represents strength of correlation and is shown with the Pearsons correlation coefficient (R) within each cell and the p-value showing the significance of the correlation. Colour of the variable on the outside of the matrix indicates the scenario with green = Normoxia, orange = Acute hypoxia and purple = Reoxygenation.



Supplementary Information 6: Escape response of control fish (i.e. naïve fish which experienced 9 consecutive drops. Drops have been grouped into three time points: drop 1-3 = Start, drops 4-6 = Middle and drops 7-9 = End. The top panel shows the likelihood of responding to a stimulus (object dropped) over time (ms). The bottom panel shows cox coefficients ±SE from the Cox proportional hazards model and normalised to “Start”.