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Loss of behavioral stress response in blind cavefish reduces energy expenditure

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ABSTRACT

The stress response is essential for animal self-defense and survival. However, species may exhibit stress response variation depending on their specific environmental and selection pressures. Blind cavefish dwell in cave environments, which differ markedly in stressors and resource availability compared to surface aquatic environments. However, whether blind cavefish exhibit differences in stress response as an adaptation to their cave environments remains unclear. Here, we investigated differences in stress response in six closely related *Triplophysa* species, including three blind cavefish (*T. longibarbata*, *T. jiarongensis*, and *T. rosa*) and three normal-sighted river fish (*T. nasobarbatula*, *T. dongsaiensis*, and *T. bleekeri*). Results showed that blind cavefish exhibited a range of distinct behavioral responses compared to sighted river fish, including greater levels of activity, shorter duration of freezing, absence of erratic movements or thrashing behavior, and opposite behavioral trends over time. Furthermore, the cavefish species demonstrated attenuated increases in metabolic rate in response to stressors related to novel environments. Cave-dwelling *T. rosa* also exhibited lower basal hypothalamic-pituitary-inter-renal (HPI) axis-related gene expression levels and stress hormone concentrations compared to river-dwelling *T. bleekeri*. These results suggest that blind cavefish may have lost their behavioral stress response, potentially mediated by a reduction in basal activity of the HPI axis, thus enabling the conservation of energy by reducing unnecessary expenditure in energy-limited caves.

Keywords: Cavefish; Stress responses; Behavior; Metabolic rate

INTRODUCTION

Stress responses of organisms are specific or nonspecific responses induced by stressors and are highly conserved in vertebrates, enabling the maintenance of internal homeostasis and survival under nonideal conditions (Barton, 2002; Bonga, 1997; Moberg & Mench, 2000; Schulte, 2014). Multiple studies have suggested that stress responses are modulated by the neuroendocrine system and influenced by the number, intensity, and duration of environmental stressors as well as an individual's nutritional history and availability of food resources (Bonga, 1997; Heinen-Kay & Langerhans, 2013; Sokolova et al., 2012). These animal responses can be classified into two coping styles, characterized by differential proactive-reactive behavioral and physiological changes, and have important ecological and evolutionary implications at the population and species levels (Careau et al., 2008; Koolhaas et al., 1999; Réale et al., 2010). Although various studies have investigated the underlying mechanisms and environmental significance of the stress response (Bijlsma & Loeschcke, 2005; Bonga, 1997; Sanders, 1993), how ecological selection pressures drive the evolution of this animal response remains unclear.

Blind cavefish possess unique morphological, behavioral, and physiological features that distinguish them from river fish, such as degenerated eyes (Krishnan & Rohner, 2017), lower resting metabolic rates (Moran et al., 2014; Shi et al., 2018), lack of shoaling behavior (Kowalko et al., 2013), and increased exploratory tendencies (Patton et al., 2010). Consequently, these species have become models for studying the evolution of animal adaptations to extreme environments (Riddle et al., 2018; Yoshizawa, 2015), as well as ideal models to study environmental influences on the stress response of organisms. In a given environment, stressors and resource availability can shape evolution and environmental adaptation (Bijlsma & Loeschcke, 2005; Petitjean et al., 2019; Sokolova, 2013). Although multiple stressors, including physical, chemical, and biological stressors, exist in surface aquatic environments (Bonga, 1997; Schreck & Tort, 2016), this may differ in cave aquatic environments characterized by stable water temperatures, extremely low species and parasite abundance, low predation pressure, and low human disturbance (Gibert & Deharveng,

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2002; Peuß et al., 2020; Tabin et al., 2018). Thus, the stress response of cavefish may be impacted by their lack of exposure to unstable environmental conditions. Furthermore, although the absence of primary producers in cave environments may not trigger a stress response, the resulting scarcity of food resources could pose an environmental challenge that diminishes the stress response of fish (Moran et al., 2021; Sokolova, 2013). Consequently, the reduced exposure to stressors and scarcity of resources in blind cavefish may induce distinct stress responses compared to river fish.

The stress response of cavefish is predicted to be weakened. According to the neutral mutation hypothesis, the stress response of cavefish may be attenuated or even disappear as cave environments have few stressors, and a stress response is not necessary for survival or evolutionary fitness (Kimura & Ota, 1971). Conversely, according to the adaptation hypothesis, attenuation of the stress response may allow cavefish to conserve energy for functional maintenance, which is advantageous in energy and resource-limited cave environments (Brandon, 1978). In both cases, the stress response of cavefish would be weakened or even absent. Studies on *Astyanax mexicanus* have revealed that the stress response to novel environments is weaker in cave-dwelling populations than in noncave-dwelling populations, as indicated by the reduction in freezing behaviors and increase in activity in novel tanks as well as the unchanged freezing duration and cortisol response upon electric shock (Chin et al., 2018, 2020). In addition, *A. mexicanus* also exhibits robust thigmotaxis under novel environment exposure (Pierre et al., 2020). However, it remains unclear whether cavefish demonstrate particular stress response patterns over time, rather than simply “more or less stress”. Furthermore, the stress response in other cavefish species, including stress-induced changes in behavior and metabolism, as well as the underlying neurophysiological mechanisms (e.g., function of the hypothalamic-pituitary-inter-renal (HPI) axis), remain unclear.

The open field test is a well-known method to assess the behavioral stress response of animals, including fish (Gould et al., 2009; Prut & Belzung, 2003). When fish are introduced in the open field arena, they typically exhibit stress responses, such as freezing, thigmotaxis, erratic movements, and leaping above the water surface due to the novel environment and exposed central area. However, as the fish habituate to their surroundings, their stress response tends to decrease and they begin to display exploratory behavior (Matsunaga & Watanabe, 2010; Wong et al., 2010). Behavioral responses to stressors have impact on and are impacted by the neuroendocrine stress axis, including activation of the hypothalamic-pituitary-adrenal (HPA) axis (HPI axis in fish) and eventual release of glucocorticoids, and activation of the sympathetic-adreno-medullar axis and eventual release of catecholamines (sympathetic reactivity) (Bonga, 1997; Koolhaas et al., 2010; Wong et al., 2019). According to the “proactive-reactive axis” hypothesis, the temporal patterns of the stress response vary among individuals and species. Specifically, ‘proactive’ animals react to stressors with higher aggression, higher activity, rapid exploration, high sympathetic reactivity, and low HPA (or HPI) axis activity, whereas ‘reactive’ animals react to stressors with lower aggression, lower activity, slow but thorough exploration, low sympathetic reactivity, and high HPA (or HPI) axis activity (Careau et al., 2008; Koolhaas et al., 1999; Sih et al., 2004). Given the attenuated stress responses of cavefish, it is predicted that they exhibit a proactive coping style, characterized by lower HPI axis activity and higher activity and exploration. However, experimental evidence is needed to support this prediction.

The stress response is energetically costly due to both physiological and physical demands (Bonga, 1997; Schreck & Tort, 2016; Sokolova et al., 2012), leading to an increase in the metabolic rate in fish (Bonga, 1997; Schreck & Tort, 2016; Sokolova, 2013). While stress has typically been viewed as a factor to avoid or eliminate during metabolic rate measurements (Chabot et al., 2016; Killen et al., 2021), recent studies suggest that the use of a respirometry chamber as a novel environment may induce stress responses, potentially impacting the metabolic rate of fish (Careau et al., 2008; Martins et al., 2011) and providing additional data on the stress response to complement behavioral observations.. If cavefish have an attenuated stress response, it is expected that they will exhibit lower energy consumption and a smaller increase in metabolic rate upon entry into the respirometry chamber.

The genus *Triplophysa* (order Cypriniformes, family Nemacheilidae) contains many species of cave-dwelling fish (Lan et al., 2013; Rendahl, 1933). These cave-dwelling species diverged from the noncave-dwelling species approximately 15 million years ago, and most exhibit eye degradation (Yan, 2017). In this study, we investigated differences in the stress response between blind cavefish (*T. longibarbata*, *T. jiarongensis*, and *T. rosa*) and sighted river fish (*T. nasobarbatula*, *T. dongsaiensis*, and *T. bleekeri*). The open field test was used to examine behavioral responses, intermittent-flow respirometry was applied to study metabolic responses to a novel environmental stressor, and enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR) were used to assess the biochemical and molecular characteristics of the HPI axis underlying the stress response. We predicted that the blind cavefish would exhibit attenuated behavioral and metabolic responses to stress and reduced basal HPI axis activity due to the absence of environmental stressors and limited energy availability in cave habitats.

MATERIALS AND METHODS

Experimental fish

The sighted river fish *T. bleekeri* (1.5–6.4 g, 5.7–8.4 cm, *n*=17) was collected from Wuxi County in Chongqing, China, while *T. nasobarbatula* (0.3–6.1 g, 3.4–5.2 cm, *n*=12) and *T. dongsaiensis,* (1.3–2.9 g, 5.1–6.5 cm, *n*=23) were collected from Libo County in Guizhou, China. The blind cavefish *T. rosa* (0.9–2.8 g, 4.1–8.2 cm, *n*=13) was collected from Wulong County in Chongqing, China, while *T. longibarbata* (0.3–1.7 g, 3.0–5.3 cm, *n*=10) and *T. jiarongensis* (0.3–2.2 g, 2.7–6.2 cm, *n*=10) were collected from Libo County in Guizhou, China (Figure 1A, B). A phylogenetic tree of the six *Triplophysa* species constructed based on the maximum-likelihood method from mitochondrial genomic data was downloaded from the National Center for Biotechnology (NCBI) database (Figure 1C). The *T. longibarbata* and *T. jiarongensis* cavefish showed the earliest divergence from the other species, while *T. rosa* diverged more recently from the noncave species (Yan, 2017; Zhao et al., 2022).

All fish were collected using small cages, nets, and bait, and were transported by vehicle to the fish laboratory at Southwest

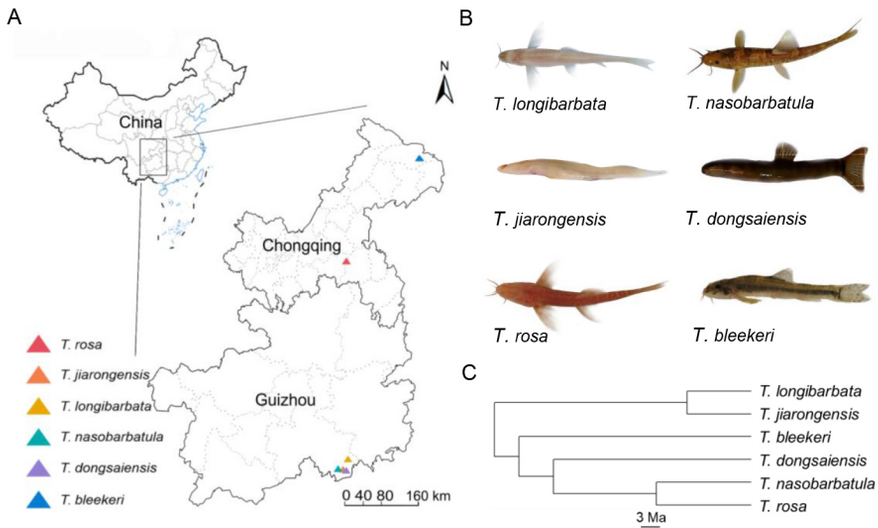

Figure 1 Experimental fish

A: Collection sites. Warm colors indicate cavefish (goldenrod: *T. longibarbata*; coral: *T. jiarongensis*; and crimson: *T. rosa*), cool colors indicate river fish (sea green: *T. nasobarbatula*; purple: *T. dongsaiensis*; and dodger blue: *T. bleekeri*). B: Snapshots. C: Phylogenetic relationship among six species of *Triplophysa* based on mitochondrial genomic data downloaded from NCBI. *T. longibarbata*, NC_058004.1; *T. jiarongensis*, MH681779.1; *T. rosa*, NC_019587.1; *T. nasobarbatula*, NC_058005.1; *T. dongsaiensis*, MH681778.1; *T. bleekeri*, NC_018774.1. Values of branch lengths are given in million years.

University in Chongqing (China). The collected fish were maintained in aquariums with aerated natural groundwater for three days before the experiment and fed to satiation daily at 1800h with bloodworms. The water temperature was maintained at 15±0.1 °C (close to the original water temperature) using a thermostat (JRB-250, Sunsun, China), and dissolved oxygen content was maintained at 99% using a continuously running air pump (BKL-902, Chongte Pet Products, China). To ensure the same rearing conditions for all experimental fish, we adopted all-dark rearing. All handling and treatment procedures were approved by the Institutional Animal Care and Use Committee of Southwest University (IACUC-20210119-01) and permitted under local fishing management provisions. The experiment was carried out 24 h after feeding to satiety (i.e., 24 h of fasting).

Behavioral stress responses

The open field test was used to determine the behavioral responses of the fish to a novel environment (Godwin et al., 2012). A cylindrical testing arena (diameter=35 cm, height= 15 cm) constructed with white frosted acrylic was placed on a blacklight table (approximately 300 Lux, Hongsheng-6629, Hongsheng Lighting, China) with a vibration isolation system (HGPT-TB456B, Henggong Instrument, China) located at the bottom of an isolation booth (constructed in-house) surrounded by acoustic panels (80 cm×80 cm×130 cm, L×W×H) (Figure 2A). A camera (30 frames/s) (Logitech C270i, Logitech, Switzerland) was fixed to a steel frame 100 cm above the water surface and connected to an external computer (ThinkPad X13, Lenovo, China).

Before behavioral testing, 7 L of aerated fresh water was poured into the testing tank to create a water depth of approximately 7 cm, effectively restricting fish movements to the horizontal plane. The fish were moved individually from the aquarium to the testing arena using a fishing net and bailer and gently released into the tank in the corner closest to the experimenter. Fish behavior was recorded for 30 min using the overhead camera to determine behavioral responses to the novel testing environment. After recording, the focal fish was placed in a tank parallel to their original aquarium and separated by independent nylon cells. The water in the testing arena was refreshed between trials to ensure consistent dissolved oxygen content and temperature and to prevent any potential effects of compounds released from previously tested fish.

The video recordings were converted to 10 frames/s using Leawo Professional Media software (Leawo, China). Over the recording period, the center of each fish was tracked offline using the Animal Tracker plugin in ImageJ (National Institutes of Health, USA). The swimming trajectories of the fish are shown in Figure 2B, with representative examples of each species over the 30 min open-field testing session. The behavioral stress response to a novel environment was assessed by calculating movement variables for each minute. Movement duration (total swimming duration, s), distance traveled (total swimming distance, body length (BL)), velocity (average swimming velocity, BL/s), freezing duration (total freezing time, s), latency to move (time from start of recording to first movement, min), latency to enter the center (LTEC) (time from start of recording to first center entry, min), and distance to center (inverse measure of thigmotaxis, cm) were calculated from the trace data. Freezing was defined as any period of inactivity equal to or longer than 2 s (Duboué et al., 2017). Erratic movements (sharp changes in direction or velocity, repeated rapid darting, or zig-zagging behaviors, time) and thrashing behaviors (forceful back-and-forth swimming against the wall of the test arena, time) were identified visually according to the methodology of Blaser & Gerlai (2006).

Metabolic stress responses

The oxygen consumption rate of the fish was determined using a multichannel intermittent-flow respirometer modified from Svendsen et al. (2016) according to the methods of Killen et al. (2021) (Figure 2C). Six acrylic respirometry chambers (46 mL) were submerged in a temperature-regulated water tank. Each chamber contained a fiberoptic oxygen probe (OEC-PSt3-NAU, Presen-Precision Sending GmbH

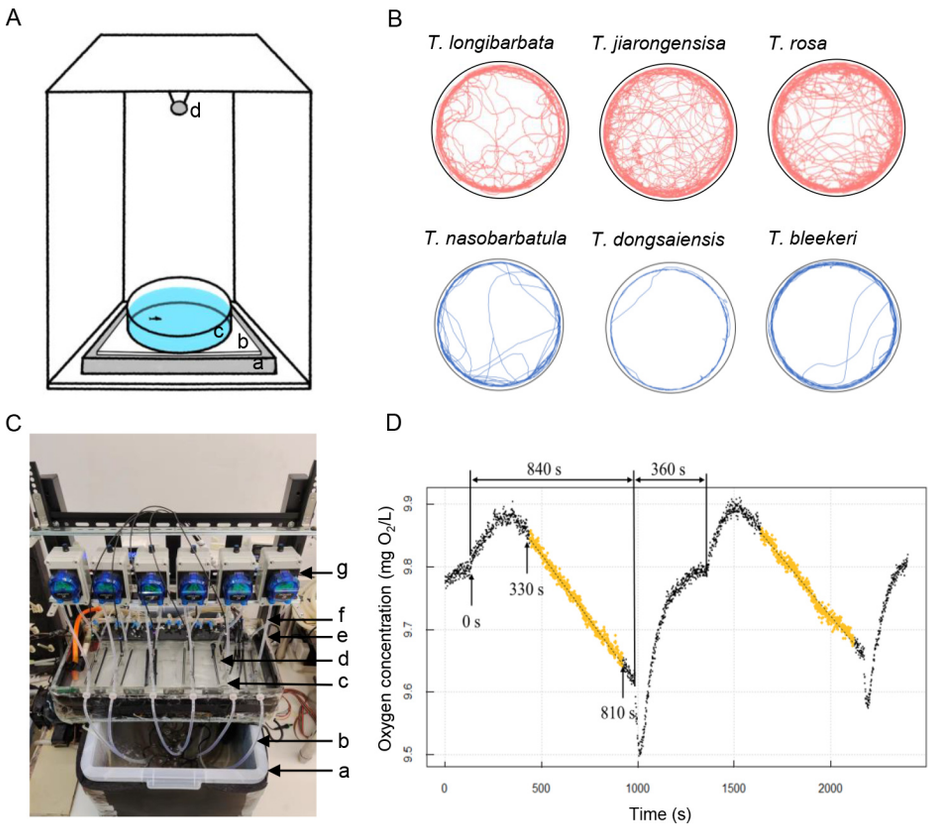

Figure 2 Experimental diagrams and devices as well as example analyses

A: Diagram of open field test, (a) vibration isolation system, (b) blacklight table, (c) testing arena, and (d) camera. B: Representative swimming trajectories of each species during 30 min assay period. Red line indicates cavefish, blue line indicates river fish, and black line indicates border of open field arena. C: Diagram of intermittent-flow respirometer, (a) temperature-regulated water tank, (b) flush tube, (c) respirometry chamber, (d) fiber-optic oxygen probe, (e) water tank, (f) recirculation tube, and (g) recirculation pump. Flush pump (not visible in diagram) was immersed in temperature-regulated water tank and connected to the breathing chamber via a flush tube. D: Examples of metabolic rate calculations. Yellow points (from 330 to 810 s, i.e., from 5.5 to 13.5 min of each assay period) were selected to calculate metabolic rate. Rate value was valid when R^2>0.85.

Regensburg, Germany), flush pump (Q500, Kamoer Fluid Tech (Shanghai), China, flow rate: 60 L/h), and recirculation pump (KHM-12B3N16, Kamoer Fluid Tech (Shanghai), China, flow rate: 20 L/h). The flush pump was used to deliver oxygenated water from a temperature-regulated water tank (60 L) into the chambers between test periods and to expel oxygen-depleted water via snorkels attached to the chambers. The recirculation pump was used to constantly mix the water within the chambers during the test periods. The dissolved oxygen content of water in each chamber was determined using a fiberoptic probe located in the center of the chamber and a multichannel dissolved oxygen meter (PreSens OXY-10 SMA, Presen-Precision Sending GmbH Regensburg, Germany), with data recorded once per second. The water temperature was controlled at 15±0.1 °C by a thermostat (QD65H, Qianjiang Refrigeration, China), and dissolved oxygen content was maintained above 99% by continuously pumping air into the water tank through an air pump (XT-502, Sunsun, China).

After behavioral testing, the fish were maintained in their original aquarium overnight, then gently transferred into individual chambers the following morning to determine their metabolic stress response to a novel chamber environment. One chamber was kept empty and used as a control for background oxygen consumption. Each 20 min measurement cycle consisted of a 14 min closed system metabolism assay period and a 6 min open system flush period. Experiments were conducted under normoxic conditions, and dissolved oxygen was maintained above 90% (at the end of the flush period) and did not fall below 80% (at the end of the assay period) to prevent fish stress due to hypoxia (Svendsen et al., 2016). Oxygen consumption measurements lasted for 12 h and were used to represent the metabolic rate of the fish.

Based on our experimental facilities and the general principles of intermittent-flow respirometry (Killen et al., 2021; Snyder et al., 2016; Svendsen et al., 2016), we selected dissolved oxygen data from minutes 5.5 to 13.5 of the measuring period, as this period ensures the authenticity and validity of most data. The slope (mg O_2/L/s) was calculated using the respR package (Harianto et al., 2019). Changes in oxygen concentration in the chamber water during the measurement cycle are illustrated in Figure 2D. The slope value was considered valid at R^2>0.85 (Svendsen et al., 2016). The following formula was used to calculate the metabolic rate of individual fish:

$$\text{Individual MR} = (V - M) \times (S_a - S_e) \times 3600 \quad (1)$$

where MR (mg O_2/h) is the metabolic rate; V (L) is the total volume of the respirometry chamber, including the circulatory tubes; M (kg) is the body mass of the experimental fish, assuming the animal is neutrally buoyant (ρ=1 kg/L); S_a is the slope in the assay chamber (mg O_2/L/s); and S_e is the slope in

the empty chamber (mg O_2/L/s).

Metabolic rate recovery duration was determined as the time taken for the metabolic rate to first decrease to the mean metabolic rate observed during the last 4 h of the measurement period plus one standard error for each fish. Excess post-stress oxygen consumption (EPOC) (mg O_2) was calculated as the magnitude of excess oxygen consumption during the recovery phase.

Basal stress hormone levels and gene expression

To explore the physiological mechanism underlying the stress response in blind cavefish, we determined the whole-body concentration and subsequent gene expression of stress-related hormones in the *T. rosa* cavefish and *T. bleekeri* river fish. The transcriptome, genome, and neuroanatomy of both species have been reported previously (Huang et al., 2013; Zhao et al., 2020, 2022), thus facilitating comparison of gene expression and hormone regulation between the two species. After metabolic stress response assessment, the experimental fish were kept in the respirometry chamber overnight. To prevent circadian rhythm interference with hormone and gene expression levels, sampling was performed in the morning on the same day (Ellis et al., 2012). Fish were transferred to beakers and anesthetized with MS-222 (0.15 g/L). Data from the experimental fish, including length and weight, were recorded. After removal of the head, tail, gonads, and digestive organs, the remaining body was used to measure hormone concentrations (Shams et al., 2017), while the brain was used to analyze gene expression levels (Tsalafouta et al., 2014). We removed the gonads and digestive organs from all fish carcasses to prevent any confounding variation due to differences in egg production in female fish and food consumption in all fish during the last feeding. Tissues were stored in liquid nitrogen. Whole-body concentrations of cortisol, epinephrine (EPI), and norepinephrine (NE) were measured using fish cortisol, epinephrine, and noradrenaline ELISA kits, respectively (Shanghai Zhenke Biotechnology, China), following the manufacturer's instructions. All samples were analyzed independently in triplicate.

Analysis of corticotropic releasing factor (*CRF*), pro-opiomelanocortin (*POMC*), and nuclear receptor subfamily 3 group C member 1 (*nr3c1*) expression was performed using qRT-PCR. Total RNA was extracted from the brain using TRIzol (Vazyme Biotech, China) and chloroform. RNA purity was determined by OD260/280 analysis using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The RNA was then reverse transcribed into cDNA using the HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech, China). Gene-specific primers used in this study were designed using the NCBI Primer-Basic Local Alignment Search tool (BLAST) (http://blast.ncbi.nih.gov/): *T. rosa*: *CRF*-forward primer, TGGTGAAAAGGCAACGAGC, *POMC*-forward primer, CGAGATCCTTGTACCGCTATCC, *nr3c1*-forward primer, CTCGAAATAAGGGATGAAAAGG; *T. bleekeri*: *CRF*-forward primer, GCTTTTGGCACGCTTAGGA, *POMC*-forward primer, ATCCTTTCACCGCTATCCCC, *nr3c1*-forward primer, CAGGAGAATGAAAGGCAGAGC; and *GAPDH*-forward primer, GTCCGCCTTGAGAAACCAG. Additionally, qRT-PCR was performed using the CFX96 Touch System (Bio-Rad, USA) in a total volume of 10 μL containing 5 μL of SYBR Green Mix (Vazyme Biotech, China), 0.5 μL of each primer (10 μmol/L), 2 μL of cDNA, and 2.5 μL of double-distilled water. The PCR conditions were as follows: 95 °C for 5 min and 40 amplification cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The expression levels of all genes analyzed were normalized to the reference gene *GAPDH*. Target gene expression was determined using the $2^{-\Delta Ct}$ method, with $2^{-\Delta\Delta Ct}$ values used to calculate significant differences (Livak & Schmittgen, 2001). All samples were analyzed independently in triplicate.

Statistical analysis

Data were analyzed using Excel 2019 (Microsoft Corporation, USA). Statistical analyses were performed using R (R Core Team, 2021). To eliminate the effect of interspecies variation in body mass, the metabolic rate was adjusted from the individual value (mg O_2/h) to a value at 1.5 g (crossover value for all species) using individual body mass (*M*, kg) and the scaling exponent of 0.75 (West et al., 1997):

$$\text{Adjusted MR} = \text{Individual MR} \times \left(\frac{1.5}{M} \times 1000\right)^{0.75} \quad (2)$$

where MR (mg O_2/h) is the metabolic rate. As the metabolic rate and behavioral data continuously decreased or increased at an ever-slower rate and eventually reached a positive asymptotic value, we fit the data of each species using an exponential model with a nonzero asymptote, with time as a fixed effect and fish ID as a random effect. The model was constructed using "nlmer" in the "lme4" package (Bates et al., 2015) with a self-starting nonlinear asymptotic regression model:

$$y_t = \text{Asym} + (R_0 - \text{Asym}) \times e^{-k \times t} \quad (3)$$

where t is time, y_t is the value of the dependent variable at time t, R_0 is the initial value of the dependent variable at time zero, Asym is the asymptotic value, and k is the rate constant. The influence of random effects on model parameters was analyzed by model comparison according to the Akaike information criterion (AIC). The model with the lowest AIC was selected as the best model. The ΔAIC<2 model (ΔAIC refers to the difference between the AIC of one model and the AIC of the best model) was considered to have similar support as the best model and was deemed interchangeable (Burnham et al., 2011; Burnham & Anderson, 2004). The potential impact of the difference in capture time on each test indicator was assessed and removed if analysis was nonsignificant. Linear mixed-effects models (LMMs), with time as a fixed effect and fish ID as a random effect, were also included in the comparison. Details on the fitted model comparisons are provided in Supplementary Table S1.

Before intraspecific and interspecific comparisons, movement duration, freezing duration, LTEC, and latency to move were log-transformed, while distance traveled, velocity, and metabolic rate were square root-transformed. Phylogenetic analysis of variance (phylANOVA in the "phytools" package) was used to determine differences in variables between the blind and river fish, and each phylANOVA model was used to conduct hypothesis tests for significant phylogenetic signals (phylosig in the "phytools" package) (Freckleton et al., 2002; Revell, 2012). This comparison incorporated phylogenetic relatedness and branch lengths (Figure 1C) assigned from the mitochondrial genomic data downloaded from NCBI. The value of λ can be used as a metric of the degree of phylogenetic correlation between traits: a maximum-likelihood value of λ equal to 1 indicates a strong phylogenetic signal, whereas a value of 0 indicates that the species data can be considered statistically independent, and

intermediate values of λ specify models in which trait evolution is phylogenetically correlated but to a lesser extent than expected under Brownian motion evolution (Freckleton et al., 2002). One-way ANOVA with Tukey's HSD *post hoc* test was used to compare differences in latency to move, LTEC, erratic movement, thrashing behavior, and metabolic index among the species. Intraspecific correlations between the metabolic rate and behavioral variables were assessed using the "corrplot" package, while interspecific correlations among the six species were assessed using phylogenetic generalized least squares (PGLS) in the "caper" package. Each PGLS model was used to conduct hypothesis testing for significant phylogenetic signals (Freckleton et al., 2002), and *P*-values were adjusted using the Benjamini-Hochberg procedure to control for the fact that small *P*-values (less than 5%) sometimes happen by chance (Benjamini & Hochberg, 1995). Student's *t*-test was used to compare differences in hormone concentrations and mRNA expression levels between *T. rosa* and *T. bleekeri*. Significance was determined at $P<0.05$. Data were visualized using the "ggplot2" package (Wickham, 2016).

RESULTS

Model selection

We employed model construction and comparison to analyze the changes in behavioral variables and metabolic rate during stress. Among the candidate models, the nonlinear asymptotic regression model with a fixed rate constant, a random initial value, and an asymptotic value (Model 2) had the lowest AIC in most cases, as well as satisfactory ΔAIC values (lower than 2) in some cases (Supplementary Tables S2, S3). Thus, Model 2 was chosen to ensure model uniformity across variables and species. However, for some variable-species pairs (specifically, movement duration of *T. rosa* and freezing duration of *T. longibarbata*), this model was not appropriate because the constants did not change with time, and thus other models were utilized. The model estimates are presented in Tables 1, 2.

The hypothesis tests conducted to determine significant phylogenetic signals indicated that for each phylANOVA and PGLS model, including the models on freezing duration, movement duration, velocity and distance traveled in the initial period of the open field test, freezing and movement duration in the final period of the open field test, latency to move, LTEC, erratic movement, thrashing behavior, initial metabolic rate, final metabolic rate, span metabolic rate (difference between maximum and minimum metabolic rates), relative metabolic rate, and relative EPOC, the maximum-likelihood value of λ was equal to 1, suggesting a strong phylogenetic signal for most behavioral and metabolic parameters.

Table 1 Changes in behavioral stress responses among experimental species

Species	Dwelling	R_0			*Asym*			*k*		
		Mean±*SE*	*t*	*P*	Mean±*SE*	*t*	*P*	Mean±*SE*	*t*	*P*
Movement duration (s)										
T. longibarbata	Cave	59.7±0.1	463.37	<0.001	59.4±0.2	290.05	<0.001	0.03±0.01	3.13	0.002
T. jiarongensis	Cave	57.5±2.4	23.88	<0.001	53.7±0.5	112.12	<0.001	1.09±0.48	2.25	0.025
T. rosa	Cave	27.7±3.4	8.11	<0.001	N/A	N/A	N/A	0.05±0.07	0.68	0.497
T. nasobarbatula	River	9.2±1.9	4.84	<0.001	30.1±1.2	25.12	<0.001	0.11±0.01	8.99	<0.001
T. dongsaiensis	River	5.3±0.6	9.21	<0.001	16.5±1.9	8.87	<0.001	0.03±0.01	5.21	<0.001
T. bleekeri	River	2.9 ±1.0	2.80	0.005	28.4±2.1	13.80	<0.001	0.05±0.01	7.86	<0.001
Freezing duration (s)										
T. longibarbata	Cave	0.0±0.0	0.529	0.602	N/A	N/A	N/A	0.00±0.00	1.28	0.203
T. jiarongensis	Cave	1.7±2.1	0.8	0.406	4.7±0.4	11.1	<0.001	0.92±0.41	2.25	0.025
T. rosa	Cave	27.4±1.3	20.95	<0.001	24.9±1.1	21.758	<0.001	0.06±0.01	9.05	<0.001
T. nasobarbatula	River	53.5±2.5	21.82	<0.001	24.0±1.4	17.21	<0.001	0.11±0.01	9.21	<0.001
T. dongsaiensis	River	51.4±0.7	70.74	<0.001	41.7±2.1	19.86	<0.001	0.02±0.00	5.71	<0.001
T. bleekeri	River	58.3±1.4	42.29	<0.001	33.0±1.4	23.83	<0.001	0.07±0.01	9.88	<0.001
Velocity (BL/s)										
T. longibarbata	Cave	0.84±0.03	26.49	<0.001	0.70±0.01	52.35	<0.001	0.17±0.03	5.23	<0.001
T. jiarongensis	Cave	1.26±0.05	24.24	<0.001	0.54±0.03	21.03	<0.001	0.17±0.03	6.22	<0.001
T. rosa	Cave	0.39±0.03	14.28	<0.001	0.34±0.02	15.63	<0.001	0.07±0.01	6.13	<0.001
T. nasobarbatula	River	0.13±0.06	2.38	0.018	0.66±0.03	26.10	<0.001	0.16±0.02	7.65	<0.001
T. dongsaiensis	River	0.17±0.02	11.39	<0.001	0.41±0.05	8.24	<0.001	0.02±0.00	5.33	<0.001
T. bleekeri	River	0.11±0.06	1.91	0.057	0.67±0.04	17.76	<0.001	0.11±0.02	6.31	<0.001
Distance traveled (BL)										
T. longibarbata	Cave	50.0±1.8	27.88	<0.001	41.0±0.9	47.49	<0.001	0.14±0.03	5.40	<0.001
T. jiarongensis	Cave	72.5±3.1	23.09	<0.001	30.6±1.6	19.55	<0.001	0.17±0.03	6.00	<0.001
T. rosa	Cave	14.3±1.3	11.44	<0.001	12.3±0.9	13.65	<0.001	0.07±0.01	8.29	<0.001
T. nasobarbatula	River	7.4±2.3	3.17	0.002	23.4±1.0	22.80	<0.001	0.16±0.02	7.53	<0.001
T. dongsaiensis	River	0.4±0.5	0.82	0.414	10.9±1.4	7.72	<0.001	0.03±0.00	6.00	<0.001
T. bleekeri	River	4.5±1.4	3.15	0.002	19.7±1.2	16.28	<0.001	0.08±0.01	8.05	<0.001

Movement duration of *T. rosa* and freezing duration of *T. longibarbata* were estimated using linear models; those of other species were estimated using self-starting nonlinear asymptotic models. In the regression model, R_0 represents initial value of the dependent variable at time zero, *Asym* is the asymptotic value, and *k* is the rate constant. N/A: Not available.

Table 2 Estimates of metabolic rate over time (mg O_2/h) from self-starting nonlinear asymptotic regression model for all species

Species	Dwelling	R_0			*Asym*			*k*		
		Mean±*SE*	*t*	*P*	Mean±*SE*	*t*	*P*	Mean±*SE*	*t*	*P*
T. longibarbata	Cave	0.175±0.012	14.467	<0.001	0.067±0.003	22.274	<0.001	1.224±0.250	4.853	<0.001
T. jiarongensis	Cave	0.097±0.007	14.139	<0.001	0.039±0.003	13.448	<0.001	0.560±0.158	3.539	0.001
T. rosa	Cave	0.314±0.015	21.240	<0.001	0.131±0.005	28.900	<0.001	0.659±0.072	9.160	<0.001
T. nasobarbatula	River	0.584±0.019	30.427	<0.001	0.105±0.003	32.434	<0.001	7.100±1.215	5.845	<0.001
T. dongsaiensis	River	0.349±0.003	102.340	<0.001	0.057±0.001	97.500	<0.001	5.783±0.252	22.910	<0.001
T. bleekeri	River	0.796±0.029	27.580	<0.001	0.170±0.006	29.810	<0.001	1.591±0.141	11.290	<0.001

In the regression model, R_0 represents initial value of the dependent variable at time zero, *Asym* is the asymptotic value, and *k* is the rate constant.

Behavioral stress responses

In the open field test, the river fish and cavefish showed marked differences in behavioral responses. After entering the open field arena, the three river fish species showed a typical behavioral stress response, with initial freezing followed by asymptotic recovery of activity. Over time, freezing behavior tended to decrease, while all activity variables (movement duration, distance traveled, and velocity) tended to increase, with larger changes in *T. nasobarbatula* and *T. bleekeri* and smaller changes in *T. dongsaiensis* (Figure 3; Table 1). In contrast, the three cavefish species did not exhibit any obvious freezing behaviors when entering the arena and maintained higher levels of activity throughout the test, especially *T. longibarbata* and *T. jiarongensis,* even exhibiting increases in swimming velocity and distance traveled at the start of the test. These differences in behavioral response between the cavefish and river fish were reflected in the asymptotic model estimates (Figure 3; Table 1).

One-way ANOVA showed that during the initial and final periods of the open field test, differences in movement duration (F=47.520, P<0.001; F=18.570, P<0.001), freezing duration (F=48.960, P<0.001; F=10.020, P<0.001), velocity (F=12.710, P<0.001; F=2.482, P=0.038), and distance traveled (F=31.640, P<0.001; F=7.022, P<0.001) were significant among species. PhylANOVA showed that during the initial period of the open field test, movement duration (F=31.00, P=0.006), velocity (F=24.45, P=0.006), and distance traveled (F=13.54, P=0.019) were higher in cavefish than in river fish, while freezing duration was shorter (F=14.25, P=0.029). During the final period of the open field test, no significant differences in distance traveled or velocity were observed between the cavefish and river fish, but movement duration was longer (F=20.07, P=0.016) and freezing duration was shorter (F=12.12, P=0.033) in the cavefish compared to the river fish, especially for *T. longibarbata* and *T. jiarongensis* (Figure 3; Table 1).

The phylANOVA results also showed that the rate constant estimates from the nonlinear model for movement duration (F=0.292, P=0.603), freezing duration (F=2.596, P=0.159), velocity (F=0.636, P=0.469), and distance traveled (F=754, P=0.403) did not differ among species. Latency to move (F=34.15, P=0.006) and LTEC (F=31.35, P=0.007) were shorter in the cavefish than in the river fish (Figure 4A, B). During the initial period of the open field test, the cavefish showed no erratic movements or thrashing behavior, except for two *T. rosa* individuals. In contrast, all river fish either froze (predominant response) or exhibited erratic movements and thrashing behavior (Figure 4C, D). Moreover, during the first minute of the open field test, all cavefish maintained a constant velocity, with no significant change in velocity over time (*T. longibarbata*: F=0.003, P=0.953; *T. jiarongensis*: F=0.600, P=0.440; and *T. rosa*: F=0.666, P=0.416); conversely, most river fish exhibited near-zero velocity, with no significant change in velocity over time (*T. nasobarbatula*: F=0.731, P=0.394; *T. dongsaiensis*: F=2.215, P=0.138; and *T. bleekeri*: F=0.053, P=0.818) (Figure 4E). However, distance to the center (inverse measure of thigmotaxis) did not significantly differ between the cavefish and river fish (F=0.163, P=0.685) (Supplementary Figure S1).

Metabolic stress responses

Immediately after placement in the respirometry chambers, almost all individuals showed maximum metabolic rates. However, the metabolic rates gradually returned to low, stable levels over time (Figure 5A), as demonstrated by the larger initial values relative to the asymptotic values estimated by the nonlinear model of the metabolic rate vs. time for each fish species (Table 2).

One-way ANOVA showed that the initial metabolic rate (F=24.034, P<0.001), final metabolic rate (F=8.734, P<0.001), relative metabolic rate (F=9.501, P<0.001), EPOC (F=7.828, P<0.001), and relative EPOC (F=2.706, P=0.027) differed significantly among species (Table 3). The phylANOVA results showed that both the initial metabolic rate (F=4.493, P=0.115) and final metabolic rate (F=0.791, P=0.466) did not differ significantly between the river fish and cavefish (Table 3). The relative metabolic rate of each species tended to gradually decrease over time (Figure 5B), although the river fish had significantly larger initial values than the cavefish (F=20.32, P=0.015) (Table 3). The rate constant estimates of the nonlinear model were significantly larger in the river fish than in the cavefish (F=9.962, P=0.023) (Table 2). However, the metabolic rate recovery time did not significantly differ between the river fish and cavefish (F=0.86, P=0.397) (Table 3). While EPOC did not significantly differ between the cavefish and river fish (F=3.46, P=0.130) (Table 3), the ratio of EPOC to basal oxygen consumption during the metabolic recovery period was significantly lower in the cavefish than in the river fish (F=75.135, P=0.003) (Table 3).

Correlations between metabolic and behavioral variables

Correlation analysis showed that freezing and movement durations in *T. dongsaiensis* during the initial period of the open field test were positively (R^2=0.27, P=0.012) and negatively correlated with the span metabolic rate (R^2=0.27, P=0.011), respectively. Intraspecific correlations between metabolic rate and behavioral variables were not significant in the other species (Supplementary Figure S2). Interspecific correlation analysis using PGLS showed that the relative metabolic rate was positively correlated with latency to move (R^2=0.94, P=0.002) and LTEC (R^2=0.81, P=0.029), and negatively correlated with movement duration during the initial period of the open field test (R^2=0.88, P=0.010) (Figure 6).

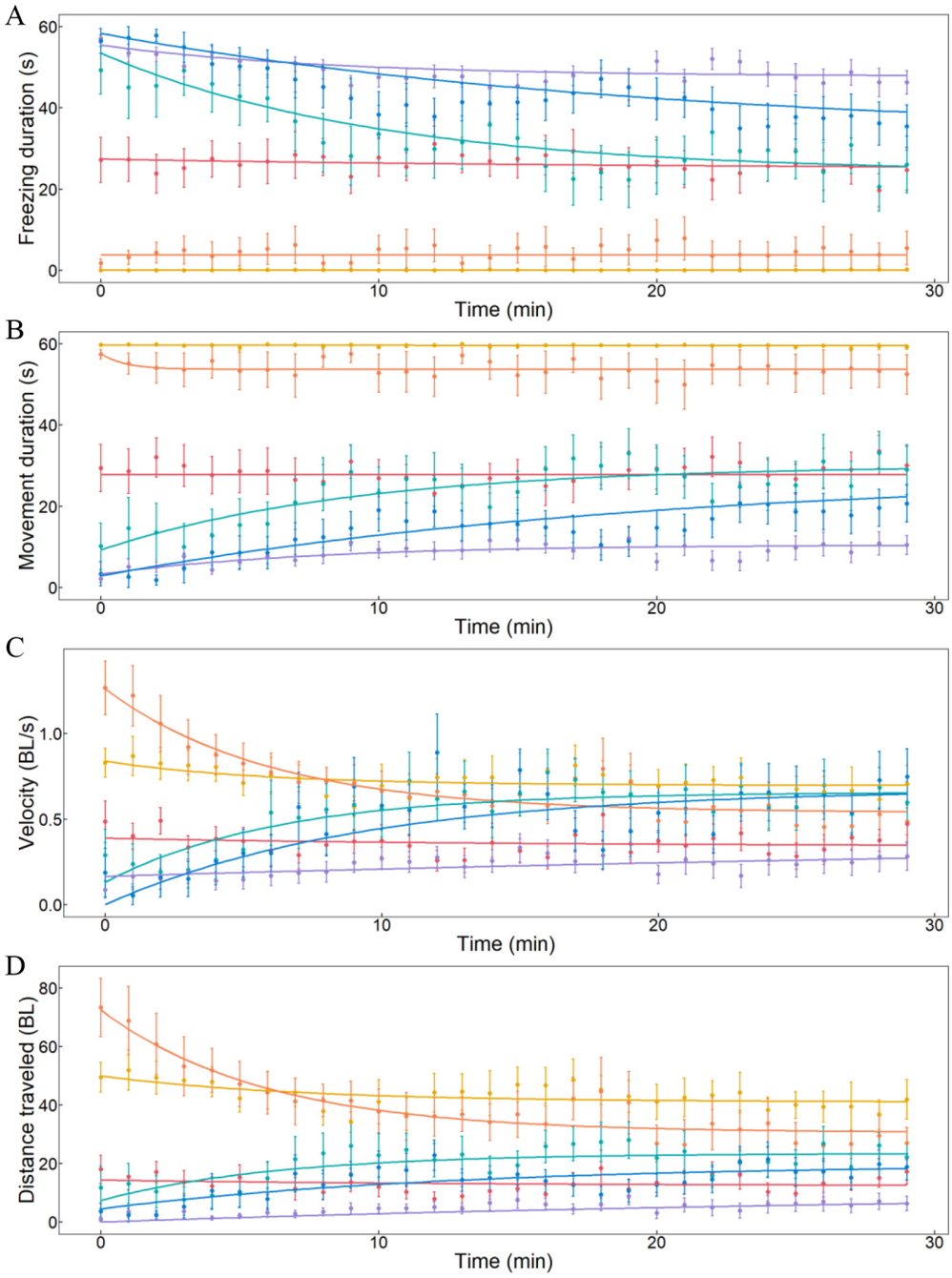

Figure 3 Temporal variations in freezing- and activity-related variables in blind cavefish and river fish upon novel environment entry

A–D: Curves represent nonlinear models of freezing duration (s) (A), movement duration (s) (B), velocity (BL/s) in 30 min open field (C), and distance traveled (BL) (D). BL: Body length. Warm colors indicate cavefish (goldenrod: *T. longibarbata*; coral: *T. jiarongensis*; and crimson: *T. rosa*), cool colors indicate river fish (sea green: *T. nasobarbatula*; purple: *T. dongsaiensis*; and dodger blue: *T. bleekeri*). All error bars represent mean±standard error (*SE*).

Table 3 Novel environment-related stress-induced metabolic changes

Species	Initial MR(mg O_2/h)	Final MR(mg O_2/h)	Relative MR	Recovery time(h)	EPOC(mg O_2)	Relative EPOC
T. longibarbata	0.191±0.023^{c}	0.032±0.006^{b}	2.8±0.4^{bc}	1.30±0.39	0.037±0.013^{b}	0.200±0.085
T. jiarongensis	0.136±0.019^{c}	0.025±0.006^{b}	2.8±0.3^{bc}	2.04±0.59	0.023±0.008^{b}	0.168±0.043
T. rosa	0.475±0.070^{b}	0.075±0.016^{ab}	2.6±0.3^{c}	1.83±0.31	0.132±0.034^{b}	0.277±0.090
T. nasobarbatula	0.366±0.041^{bc}	0.042±0.006^{b}	6.0±1.1^{a}	1.95±0.39	0.185±0.042^{ab}	0.745±0.203
T. dongsaiensis	0.343±0.022^{bc}	0.039±0.003^{b}	6.3±0.4^{a}	1.98±0.29	0.095±0.012^{b}	0.497±0.091
T. bleekeri	0.868±0.074^{a}	0.114±0.018^{a}	5.4±0.7^{ab}	1.43±0.19	0.327±0.069^{a}	0.545±0.111

MR: Metabolic rate; Relative MR: Ratio between maximum MR and minimum MR; EPOC: Excess post-stress oxygen consumption; Relative EPOC: Ratio of EPOC to basal oxygen consumption during MR recovery period. Each value represents mean±standard error (*SE*), and different superscripts (a, b, c) indicate significant interspecific differences (P<0.05).

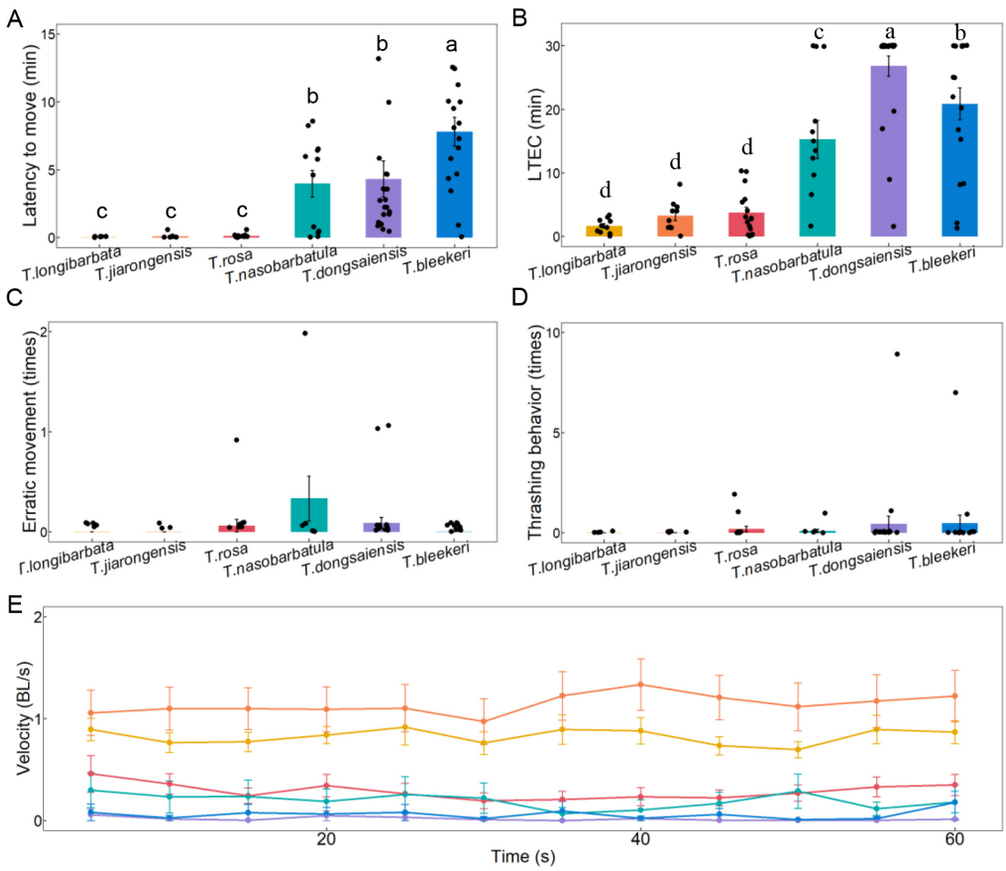

Figure 4 Latency to move and erratic movement in novel environment

A: Latency to move (min). B: Latency to enter the center (LTEC, min). C: Erratic movement duration (times). D: Thrashing behavior (times). E: Velocity (BL/s) during first minute of the open field test. Warm colors indicate cavefish (goldenrod: *T. longibarbata*; coral: *T. jiarongensis*; and crimson: *T. rosa*), cool colors indicate river fish (sea green: *T. nasobarbatula*; purple: *T. dongsaiensis*; and dodger blue: *T. bleekeri*). BL stands for body length. Differences among species were analyzed by one-way ANOVA with Tukey's honest significant difference (HSD) *post hoc* test; significant differences (P<0.05) among species are indicated by unique superscripts (a–d). All error bars represent mean±standard error (*SE*).

Basal levels of stress hormones and gene expression

The relative mRNA expression levels of *CRF* and *POMC* were higher in *T. bleekeri* than in *T. rosa* (*CRF*: t=54.06, P=0.006; *POMC*: t=14.54, P=0.007), showing 23.5-fold and 27.6-fold increases, respectively. The relative mRNA expression level of the glucocorticoid receptor (GR) *nr3c1* was also higher in *T. bleekeri* than in *T. rosa* (t=3.54, P=0.031), with a 4.1-fold increase (Figure 7A). The concentrations of cortisol and NE were significantly higher in *T. bleekeri* (2.11±0.10 ng/mg pro, 2.41±0.06 ng/mg pro) than in *T. rosa* (1.64±0.10 ng/mg pro, 2.20±0.06 ng/mg pro) (t=3.465, P=0.002; t=2.443, P=0.020), but the concentration of EPI showed no significant difference (*T. bleekeri*, 0.38±0.02 ng/mg pro; *T. rosa*, 0.37±0.01 ng/mg pro) (t=0.873, P=0.389) (Figure 7B).

DISCUSSION

Loss of behavioral stress response in blind cavefish

In this study, all blind cavefish species showed reduced freezing duration, increased activity, shorter latency to move, and shorter LTEC in the open field test compared to the river fish (Figure 3; Figure 4A, B), with *T. longibarbata*, in particular, showing almost no freezing behavior. These results suggest that blind cavefish display weaker behavioral stress responses than river fish.

Of note, our study showed that the cavefish exhibited a contrasting behavioral response pattern over time compared to the river fish. Specifically, the three river fish species showed immediate freezing behavior upon transfer to the open field arena, with a gradual increase in activity over time (Figure 3). This is considered a typical behavioral pattern in fish in response to a novel environment (i.e., a reactive coping style, characterized by stress-induced freezing, followed by exploration) (Careau et al., 2008; Koolhaas et al., 1999; Matsunaga & Watanabe, 2010; Wong et al., 2010). In contrast, freezing duration of the three cavefish species did not change with time after transfer to the open field and activity did not increase (remaining constant or decreasing) with time (Figure 3). In addition, the cavefish did not exhibit other stress-induced behavioral responses, such as leaping, erratic movement, or thrashing (Blaser & Gerlai, 2006; Kalueff et al., 2013) (Figure 4C, D). These findings suggest that when confronted with a novel environment, cavefish may not display a stress response but rather immediately commence exploration (Figure 8A). Pierre et al. (2020) also speculated that the response of *A. mexicanus* to a novel environment may not be entirely anxiety-related, but rather exploratory, suggesting that exploration prompted by a new environment may be a conserved characteristic in cavefish.

The high activity of the cavefish in the novel environment suggested they may exhibit a proactive coping style during stress (Careau et al., 2008; Koolhaas et al., 1999; Réale et al., 2010; Sih et al., 2004), which is considered an adaptive strategy for animals that live in stable environments compared

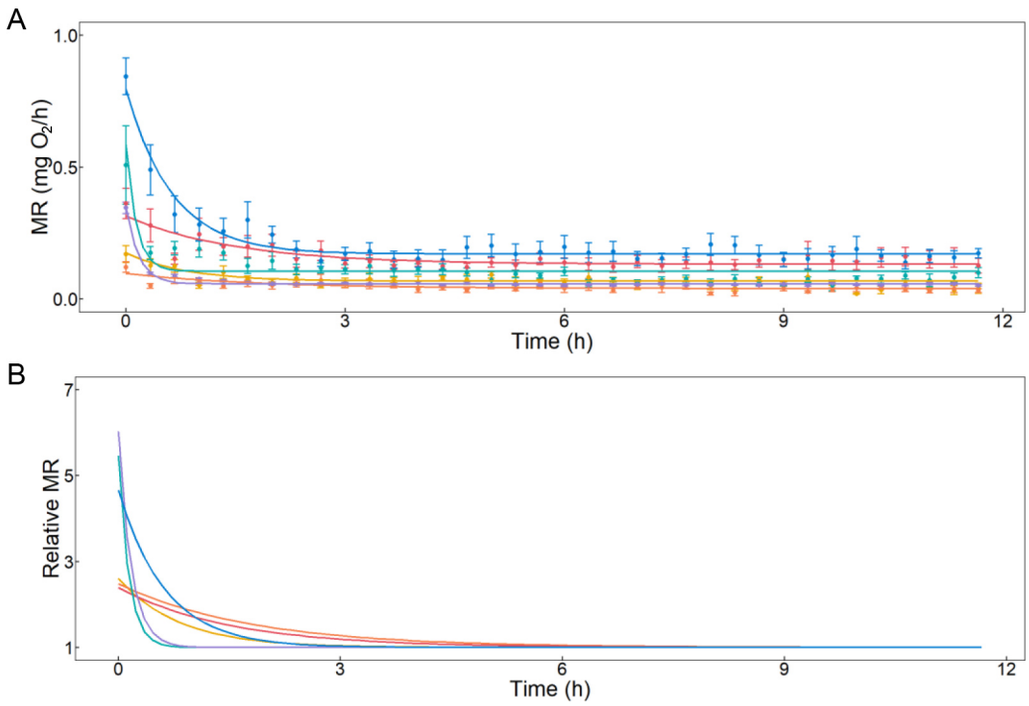

Figure 5 Novel environment-related stress-induced metabolic changes

A, B: Curves represent nonlinear fit of the metabolic rate (MR, mg O_2/h) (A) and relative MR (B) over time. Warm colors indicate cavefish (goldenrod: *T. longibarbata*; coral: *T. jiarongensis*; and crimson: *T. rosa*), and cool colors indicate river fish (sea green: *T. nasobarbatula*; purple: *T. dongsaiensis*; and dodger blue: *T. bleekeri*). Relative MR: ratio between maximum and minimum MR. All error bars represent mean±standard error (*SE*).

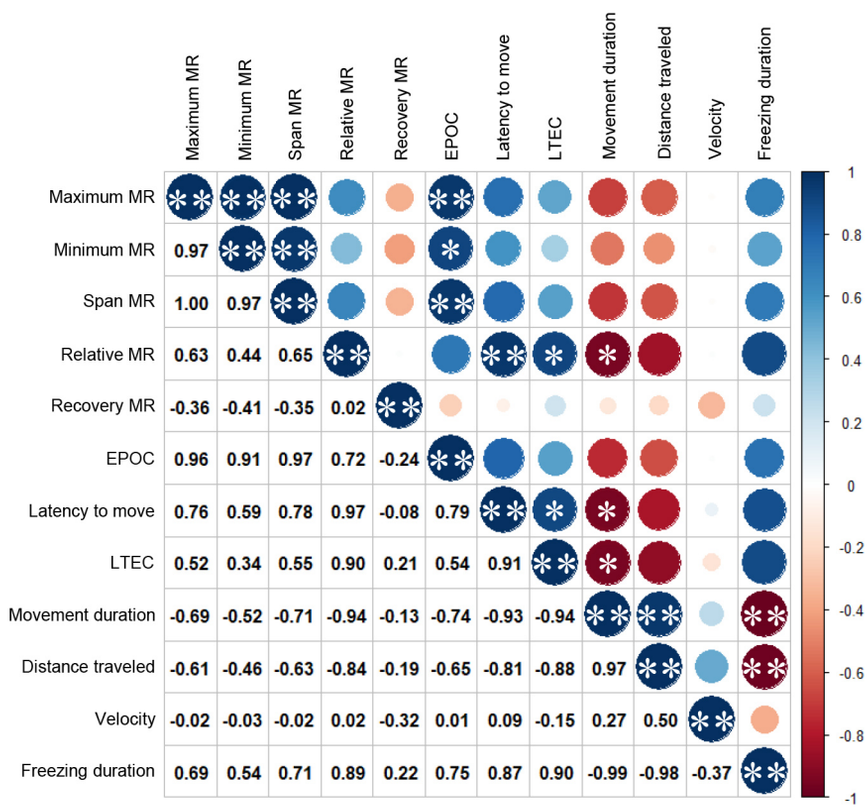

Figure 6 Interspecific correlations of metabolic rate (MR) with behavioral variables among six species of *Triplophysa*

Span MR: difference between maximum and minimum MR; relative MR: ratio between maximum and minimum MR; recovery MR: MR recovery duration; EPOC: excess post-stress oxygen consumption; LTEC: latency to enter the center. Movement duration, velocity, distance traveled, and freezing duration data are from the initial period of the open field test. Correlation analysis was conducted using PGLS. Number in the lower left corner and size of the circle in the upper right corner indicate correlation coefficients, colors in the upper right corner from blue to red indicate correlation coefficients from 1 to -1, and * indicates significance of the correlation (*: P<0.05; **: P<0.01).

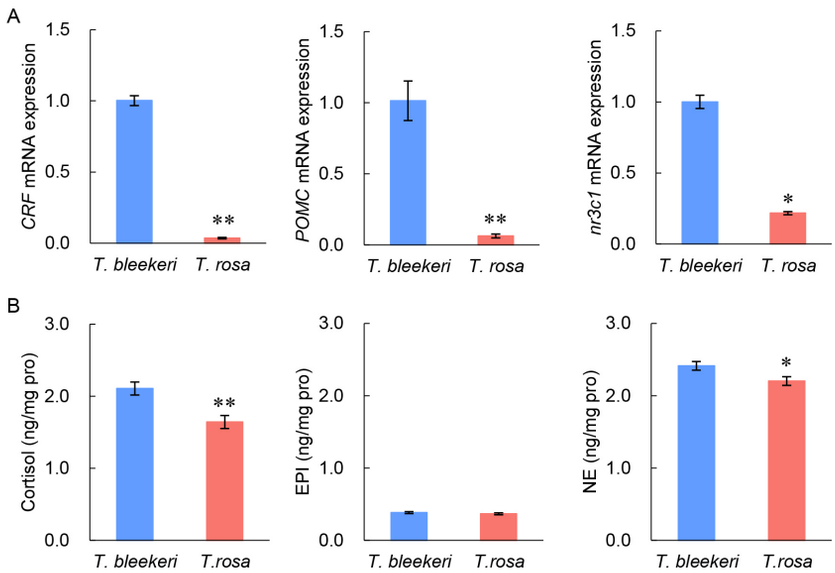

Figure 7 Comparison of basal levels of hormones between *T. rosa* and *T. bleekeri*

A: Quantification of gene expression (corticotropic releasing factor (*CRF*), pro-opiomelanocortin (*POMC*), and nuclear receptor subfamily 3 group C member 1 (*nr3c1*)) in the brain. B: Concentration of hormones (cortisol, epinephrine (EPI), and norepinephrine (NE), ng/mg pro) in the whole body. Blue indicates river fish, red indicates cavefish. Differences between river fish and cavefish were determined by Student's *t*-test: *: $P<0.05$; **: $P<0.01$. All error bars represent mean±standard error (*SE*).

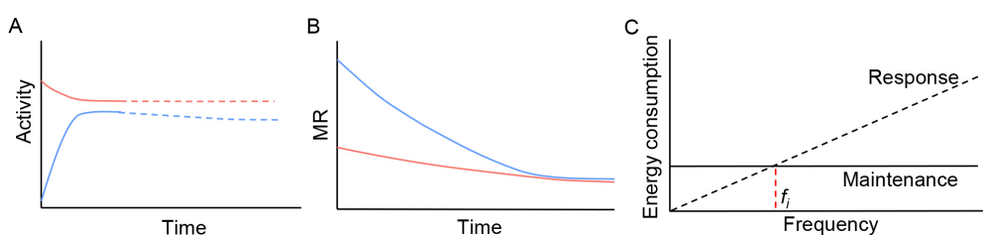

Figure 8 Schematic of differences in behavioral and metabolic responses between cavefish and river fish

A, B: Changes in activity (A) and metabolic rate (MR) (B) over time in cavefish and river fish after transfer to the novel environment. Blue line indicates river fish, red line indicates cavefish. After entering the novel environment, river fish showed freezing behavior and a sharply increased MR, while cavefish showed increased activity and a slightly increased MR. C: Model prediction of stress-related energy consumption under varying frequencies of stressors. Solid line indicates energy consumption of functional maintenance (maintenance), dotted line indicates energy consumption of the stress response (response). Frequency: Frequency of stressors. f_i: Frequency of stressors at which energy consumption of the stress response is equal to energy consumption of functional maintenance. As stressor frequency increases, energy consumption of the stress response of fish increases, while energy consumption of functional maintenance remains constant. Accordingly, the importance of stress response energy consumption in total stress-related energy consumption is higher under higher stressor frequencies.

to those that live in changing environments, in which a reactive coping style is advantageous (Careau et al., 2008; Koolhaas et al., 1999, 2010). However, the responses of the cavefish were extremely attenuated, in contrast to animals with a proactive coping strategy and heightened stress response, which tend to show panicked, erratic, and thrashing behaviors (Careau et al., 2008; Koolhaas et al., 1999; Sih et al., 2004). This discrepancy may be explained by the two-tier model of interspecific differences in stress responses, which consists of a qualitative coping style axis and a quantitative stress reactivity axis (Koolhaas et al., 2007, 2010). Based on this model, cavefish are more likely to be "bold" species, with a proactive coping style and low stress response. Given their energy-limited cave environments, "bold" cavefish that display high activity and immediate exploratory behavior have a clear adaptive advantage in foraging. While stress responses are crucial for self-defense and survival and are highly conserved in vertebrates (Barton, 2002; Moberg & Mench, 2000; Schreck & Tort, 2016), stress-induced behavioral and metabolic responses may not be necessary for the survival of cavefish that inhabit environments in which stressors are rare. Two different frameworks may explain these findings: notably, the neutral mutation hypothesis suggests that the loss of the behavioral stress response does not affect cavefish survival, while the adaptation hypothesis suggests that cavefish may conserve energy (i.e., gain an advantage) due to a lack of stress response given their energy-limited cave environment.

Our study revealed differences in the behavioral stress response among the three cavefish species. Although all three species exhibited unchanged freezing durations over time during the open field test (Figure 3A), indicating no stress response, *T. rosa* exhibited significantly lower activity levels compared to the other two cavefish (Figure 3B–D), more similar to the activity levels of river fish. This pattern may be related to the phylogenetic relationships among these fish, as *T. rosa* has a closer phylogenetic relationship to the river fish (Figure 1C). Recent comparative genomic studies have also suggested that *T. rosa* may have a low mutation rate and slow evolutionary rate (Zhao et al., 2022). Furthermore, we

observed residual eye spots in some *T. rosa* individuals, indicating variability in the adaptive evolution of cavefish. Thus, these findings suggest that cavefish species such as *T. rosa*, which is more closely related to river fish, may be used as an intermediate animal model to study the ecological and evolutionary differentiation between cavefish and river fish.

Reduced metabolic energy consumption in cavefish under stress

Fish respond to stressors by increasing their metabolic rate and mobilizing energy stores to initiate a stress response (Bonga, 1997; Schreck & Tort, 2016; Sokolova et al., 2012). Our study found that cavefish and river fish had higher metabolic rates at the beginning of stress exposure to a novel environment (respirometry chamber), but then gradually recovered (Figure 5A). The magnitude of metabolic consumption during recovery (EPOC) was lower in cavefish than in river fish, but not significantly (Table 3). However, after controlling for species-specific differences in basal metabolic rate, the cavefish showed a smaller increase in relative metabolic rate, slower metabolic recovery (Figure 5B), and smaller relative EPOC compared to river fish (Table 3). These differences in metabolic response may be attributed to their distinct behavioral responses to novel environments. Specifically, cavefish tended to continue exploring without exhibiting freezing behavior, while river fish tended to exhibit significant freezing behavior. Therefore, the immediate increase in metabolic rate after transfer to a novel environment may serve different functions in the different species. Specifically, the increased metabolic rate in cavefish may be due to the demands of moderate physical activity, rather than directed toward a neuroendocrine stress response (Hurlimann et al., 2019; Mathot et al., 2019). In contrast, the increased metabolic rate of river fish may be due to the demands of the neuroendocrine stress response related to the immediate, intense increase in anxiety and physiological activity (Careau et al., 2008; Gormally & Romero, 2020; Wong et al., 2010). These results suggest that cavefish may prevent excess energy consumption from stress-induced physiological activity but maintain energy consumption for exploration. Exploratory behavior may be more advantageous for fitness (e.g., foraging and courtship) in the life history of cavefish, which live in dark environments with limited food resources (Conrad et al., 2011; Patton et al., 2010).

In addition, the cavefish displayed a lower relative metabolic rate at the onset of novel environment exposure and a smaller EPOC relative to their basal metabolic rate compared to the river fish (Table 3). These findings suggest that the activity-related energy consumption in cavefish may be lower than the stress-related energy consumption in river fish. Similarly, the stress-induced physiological energy consumption in river fish may be markedly higher than their exploration-related energy consumption. Although stress-induced physiological energy consumption has been overlooked in many previous studies, it is a crucial component of energy allocation and consumption in fish and should be considered in the context energetics.

The different behavioral and metabolic responses to a novel environment between the cavefish and river fish are summarized in Figure 8A, B. Briefly, the cavefish initially exhibited high activity, which remained stable or gradually decreased over time, while the river fish initially froze and then gradually increased activity over time. Overall, the activity level of cavefish during the final period of novel exposure (open field test) was slightly higher than that of the river fish (Figure 8A). Regarding the metabolic response, all tested species exhibited a high initial metabolic rate, which gradually decreased over time. However, the initial and final metabolic rates, as well as the relative metabolic rate, were lower in cavefish than in river fish (Figure 8B). We hypothesize that excess oxygen consumption may support exploration in cavefish and stress-induced physiological responses in river fish.

Lost stress response is associated with decreased HPI axis activity in cavefish

Behavioral stress responses are linked to basal hormone levels, which are important indicators of physiological stress in fish (Alfonso et al., 2020; Carbillet et al., 2022; Koolhaas et al., 1999, 2010). To reveal the mechanisms underlying the loss of behavioral stress response in cavefish, we compared the basal mRNA expression levels and basal hormone levels related to the neuroendocrine stress response, including *CRF*, *POMC*, *nr3c1*, cortisol, EPI, and NE, between the *T. rosa* cavefish and *T. bleekeri* river fish. Results showed that the mRNA expression levels of HPI axis-related genes were down-regulated in *T. rosa* compared to *T. bleekeri* (Figure 7A). Additionally, the mRNA expression levels of *CRF* and *POMC* were nearly 25-fold lower in *T. rosa* than in *T. bleekeri*. Moreover, the concentrations of stress hormones (cortisol and NE) were lower in *T. rosa* than in *T. bleekeri* (Figure 7B). These results suggest that *T. rosa* has lower baseline HPI-axis activity than *T. bleekeri*, consistent with the loss of behavioral stress response in cavefish.

Stress response attenuation in cavefish may be due to negative physiological feedback from the up-regulation of GRs in the HPI axis (Bonga, 1997; Sapolsky et al., 2000) or to systemic decline in the baseline activity of the HPI axis, e.g., a decrease during ontogenesis (Faught & Vijayan, 2016; Tsalafouta et al., 2014). In the present study, down-regulation of GR mRNA expression excluded the possibility of negative feedback on the HPI axis in *T. rosa*, instead suggesting a systemic decrease in HPI-axis activity in cavefish. In contrast, cave-dwelling *A. mexicanus* larvae (9 days post fertilization (dpf)) show less freezing and up-regulated basal GR expression compared to noncave-dwelling surface populations, indicating possible negative feedback from GRs on the HPI axis and, ultimately, on the stress response (Chin et al., 2020). However, mineralocorticoid receptors rather than GRs are important for the stress response in the early stages of fish development (Alsop & Vijayan, 2008; Tsalafouta et al., 2014, 2018). Thus, the up-regulated GR expression in larvae may influence the ontogenesis of multiple organs, such as the mesoderm or muscle (Nesan et al., 2012; Pikulkaew et al., 2011), rather than providing negative feedback to the neuroendocrine stress axis. Hence, the loss of stress response in cavefish may more likely be mediated by a systemic reduction in basal HPI axis activity, whereby cavefish conserve energy consumption to maintain stress response mechanisms (Bonga, 1997; Schreck & Tort, 2016). The hypothalamus and pituitary gland are key central nervous system sites for stress response regulation in fish (Bonga, 1997; Demin et al., 2021). Neuroanatomical studies have shown that *T. rosa* has a smaller hypothalamus than *T. bleekeri* (Huang et al., 2013), suggesting that the shrinking of stress response-related neurocentral structures may lead to a reduction in basal HPI axis activity in cavefish. Studies on *A.*

mexicanus have found that the eyes of cavefish are similar to those of river fish during the first few hours of development, only beginning to degenerate after 24 h (Jeffery, 2005; Krishnan & Rohner, 2017). Therefore, whether differences in the stress response between blind cavefish and sighted river fish are temporally consistent with abnormal eye development in cavefish deserves further consideration and research.

We explored possible explanations for the attenuation of basal HPI axis activity in the cavefish from the perspective of energetics. In organisms, stress-related energy consumption encompasses both the energetic demands of functional maintenance (e.g., maintenance of baseline HPI axis activity) and the excess energetic demands of stress response processes (e.g., repair of DNA or protein damage and removal of cellular and molecular debris) (Bonga, 1997; Kalueff et al., 2013; Kültz, 2005; Sokolova, 2013). Fish inhabiting energy-limited environments may benefit from reducing energy consumption, including stress-related energy consumption, depending on the frequency of stressors. With an increase in stressor frequency, energy consumption of the stress response in fish also increases, while energy consumption for functional maintenance remains constant. Thus, under high stressor frequencies, stress response energy consumption becomes more important in overall stress-related energy consumption (Figure 8C). Therefore, high environmental stress frequency may result in selection pressure to reduce the intensity of the stress response (i.e., HPI axis reactivity) to conserve energy rather than reduce functional maintenance, as the latter is essential for survival in such environments. For example, the intensity of the stress response in Gibel carp (*Carassius auratus gibelio*) and largemouth bass (*Micropterus salmoides*) declines after 4 weeks of fasting (Jiang et al., 2017). Conversely, low environmental stress frequency may result in selection pressure to decrease functional maintenance (i.e., HPI axis activity) in nonlife-threatening situations. The may account for the loss of stress response in blind cavefish, which rarely experience environmental stressors, by reducing HPI axis activity.

Cortisol and NE can promote gluconeogenesis and hepatic glycogenolysis in organisms (Bonga, 1997; Schreck & Tort, 2016). The reduced basal concentrations of cortisol and NE in *T. rosa* suggest that this species may consume less stored energy material, thereby facilitating adaptation to the energy-limited cave environment. Notably, as NE can lower the threshold for aggressive responses to environmental stimuli (Volavka et al., 2004), the reduction in basal NE levels in *T. rosa* may be attributed to its behavioral inclination towards boldness, rather than panic, as per the two-tier model (Koolhaas et al., 2007, 2010).

Correlations between behavior and metabolism in *Triplophysa*

Differences in behavioral traits may be related to differences in basal and/or maximum metabolic rates (Careau et al., 2008; Mathot et al., 2019), which can be modulated by environmental stressors (Killen et al., 2013). In the present study, the maximum metabolic rate was the post-stress rate rather than the maximum metabolic rate of fish in general. Intraspecific analysis found almost no significant correlations between metabolic and behavioral variables, probably due to the relatively small intraspecific variation. However, interspecific analysis found that species with strong behavioral stress responses (i.e., shorter movement duration, longer latency to move, and longer LTEC) also had higher relative post-stress metabolic rates (Figure 6). These results suggest that stress-induced behavioral and metabolic responses may be correlated, providing evidence that physiological stress is energetically costly (approximately 5.9 times the minimum metabolic rate). However, correlating stress behavior in the open field test with stress metabolism in the respirometry chamber is a preliminary experimental result and such correlations may be affected by inconsistencies in size or hydrodynamics between the open field arena and the respirometry chamber, which need to be examined. In future studies, video recordings of the behavioral activity of the fish in the chambers should be conducted to provide direct evidence for the causality between the stress response and metabolic rate.

In the present study, the open field test was used as *Triplophysa* species are bottom dwellers (Breed & Moore, 2015; Feng et al., 2017). Except for thigmotaxis, behavioral stress response indices, such as freezing duration, LTEC, and distance traveled, clearly differentiated the cavefish and river fish. This suggests that the open field test is an effective method for evaluating the behavioral stress response of *Triplophysa*. Our results showed no difference in thigmotaxis between the cavefish and river fish (Supplementary Figure S1). It is possible that cavefish exhibit stronger thigmotaxis than river fish (Patton et al., 2010), which may mask any differences in stress-induced thigmotaxis. Therefore, thigmotaxis was not considered a suitable stress response index and was not further analyzed.

CONCLUSIONS

Our study revealed distinct differences in the behavioral and metabolic stress responses of blind cavefish and sighted river fish to a novel environment. Specifically, the blind cavefish showed a gradual shift from high to low activity and reduced energy consumption, while the sighted river fish showed a gradual shift from freezing behavior to higher activity and higher energy expenditure. Both groups returned to their daily activities after approximately 10 min and daily metabolic rates after 1.3–2.0 h. In addition, the basal expression of genes related to the HPI axis and stress hormone concentrations were lower in blind *T. rosa* than in sighted *T. bleekeri*. These results suggest that the loss of the behavioral stress response in blind cavefish may be mediated by a reduction in the basal activity of the HPI axis, thus favoring a reduction in stress energy consumption in these fish in energy-limited cave environments.

DATA AVAILABILITY

Data related to this paper have been deposited in Figshare: https://doi.org/10.6084/m9.figshare.20781154.v2.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.H.Z. and Y.P.L. conceived the ideas and designed the methodology; J.H.Z., R.L., Y.Y.J., P.Z., Y.X., W.X., and Y.Q.Z. collected the data; J.H.Z. analyzed the data and drafted the manuscript. Y.P.L. critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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