RESEARCH ARTICLE

Pupil size variation as a response to stress in European catfish and its application for social stress detection in albino conspecifics

Ondřej Slavík [1]*, **Pavel Horký**[1], **Josef Velíšek**[2], **Tereza Valchářová**[1]

1 Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Prague, Czech Republic, **2** South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

* oslavik@af.czu.cz

Abstract

Hormonal changes such as increased cortisol level in blood plasma in response to stress and social environmental stimuli are common among vertebrates including humans and typically accompanied by other physiological processes, such as changes in body pigmentation and/or pupil dilatation. The role of pupil size variation (PSV) as a response to stress have yet to be investigated in fish. We exposed albino and pigmented European catfish to short-term stress and measured changes in pupil size and cortisol level. Albinos showed lower pupil dilatation and higher cortisol levels than did pigmented conspecifics. A clear positive relationship between pupil dilatation and cortisol concentrations was observed for both pigmented and albino specimens, suggesting that PSV can be used as a stress indicator in fish, irrespective of albino's inability to express social communication by coloring. During the follow-up, we investigated whether a penultimate contest between albino individuals would impact contestants' social stress during subsequent contact. We observed PSV during the contact of unfamiliar albino catfish with different penultimate experiences (winner (W) and/ or loser (L)). Then, the following treatment combinations were tested: WW, WL and LL. Twenty-four-hour contact of two unfamiliar catfish resulted in higher pupil dilatation among individuals with previous winner experience. Among treatment combinations, a WL contest displayed the highest pupil dilatation for winners. PSV reflected socially induced stress in individuals that was accompanied by the "winner" experience and dominance in albinos. To conclude, the present study validates pupil dilatation as a non-invasive method to evaluate stress level in pigmented as well as albino fish in various contexts.

Introduction

In fish, physiological responses to changes in the social environment involve changes in hormonal activity, including cortisol level, that are accompanied by other changes, such as changes in body pigmentation and eye darkening. Cortisol production in fish is reportedly

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be considered as a potential conflict of interest.

associated with negative stimuli and stress [1–3]. Cortisol level reflects the status of an individual in the social hierarchy [4, 5] and tends to be higher in subordinate individuals and individuals under chronic stress [5, 6]. More aggressive individuals display lower responsiveness to stress and produce less cortisol than do less aggressive individuals [7]. Cortisol concentrations in blood plasma are associated with levels of body pigmentation. In salmonids, more pigmented dominant individuals exhibit more intense and frequent spots than do subordinate individuals and they also display lower responsiveness to stress and lower cortisol levels [8, 9]. However, in the Arctic charr *Salvelinus alpinus*, more stressed individuals have more carotenoid spots, whilst cortisol levels in blood plasma are lower in dominant charr being positively correlated with frequency of aggressive attacks by dominant individuals [10].

However, albinos cannot express social communication by variability in coloring. Albinism is generally the result of combinations of homozygous recessive mutations from pigmented parents, and in particular, albinos are often unable to synthesize tyrosine and melatonin hormones. This disability is associated not only with red irises and light skin coloring (oculocutaneous albinism, OCA; [11]) but also with physiological, behavioral and social alterations. For example, compared to pigmented conspecifics, albinos are less active and aggressive [12–14] and albinos are socially ostracized [15], which can explain why they are presented as losers in competition for resources [16]. Hence, for detecting the stress-induced status of an albino individual accompanying its social interactions, it is not possible to use the methods based on variability in pigmentation and/or eye darkening reported for salmons [17], tropical cichlids and cyprinids [18–21]. Presumably, stress in albinos can be alternatively detected by pupil size variation (PSV). While pupil modulation occurs in response to illumination, a process that is well understood [22], it can also occur in association with various cognitive processes [23]. The diameter of the pupil is primarily controlled by the iris sphincter muscle, which constricts the pupil, and by the dilatory pupillary muscle, which promotes pupil dilatation [24]. Although both muscles are under the control of the parasympathetic and sympathetic nervous systems, sympathetic pathways primarily impend dilatation in response to arousal and not luminescence [25]. Pupil dilatation is associated with danger perception, stress [26, 27] and discomfort, such as pain [28, 29], sleep deprivation [30] and negative visual stimuli [31, 32], and has widely been used as an indicator of emotional arousal in humans [33, 34]. Furthermore, several recent animal studies have revealed that non-luminance-induced changes in pupil size can be used to track the fluctuation of cortical arousal and cognitive factors (for a review, see [35]), suggesting that the correlation between pupil size and behavioral state is a universal phenomenon across mammalian species [36]. Fluctuating arousal state has been shown to modulate pupil changes in association with perceptual performance and reaction time in rats [36], and pupil-size changes in monkeys have been shown to be evoked by both the social environment and arousal [37].

The cortisol concentration in blood plasma is often used as a physiological stress indicator [38], and an increased level is related to stress-induced pupil dilatation [39]. As reported for humans and some other mammal species, pupil size can be associated with socially induced stress [37] and cortisol levels in blood plasma [39]. These relationships have yet to be investigated in fish. The aims of our study were I) to test if there is a relationship between PSV and cortisol levels in pigmented as well as albino fish under a standardized acute stress exposure [40, 41] in a methodological experiment and II) to test the application of the PSV methodology under more complex socially induced stress [42–44] in albinos that have several physiological, behavioral and social alterations when compared to pigmented conspecifics [11–16]. In experiment II, we used the winner-loser test in which the previous winning experience of an individual strengthens its will to contest and increases the probability of its subsequent victory [45–47]. We observed a difference between socially induced stress of a winner and/or loser of

albino fish based exclusively on noninvasive PSV that is more appropriate with regard to animal welfare then blood sampling.

We used albino and pigmented European catfish *Silurus glanis* (Linnaeus, 1758), a freshwater predator achieving a size of 2.7 m and a weight up to 130 kg with high invasion potential in areas of nonnative occurrence [48], as experimental animals. Catfish are commonly hatchery-reared species [49], and juveniles are often used in laboratory experiments focusing on social and diurnal behavior, food intake, and/or energy consumption [50, 51]. Juveniles compete for food and space, and when shelters are offered, individuals fight for them [52]. Albino individuals occur in both wild and aquaculture environments [15, 53–55].

Materials and methods

Ethics statement

All of the laboratory experimental procedures complied with valid legislative regulations (Law no. 246/1992, § 19, art. 1, letter c), which derived from the Directive 2010/63/EU; the permit was subjected to O. Slavík, qualified according to according to Law no. 246/1992, § 17, art. 1; permit no. CZ00167. All laboratory sampling including PIT implantation was carried out with the relevant permission from the Departmental Expert Committee for authorization experimental project of the Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT– 1972/2016-5). The maintenance staff has been trained by law in animal care to maintain the high quality of the experiment. The number of experimental animals and all methods used comply with the reduction, replacement, and refinement of animal experimentation. The study did not involve endangered species.

Experimental animals

Two groups of juvenile European catfish from the Znojmo hatchery, Czech Republic, were transported into the laboratory one month prior to each experiment. Both groups, the group of pigmented individuals (200 individuals) and the group of albinos (200 individuals), were from different pigmented parents. In the laboratory, experimental animals were further divided into eight groups (4 albino and 4 pigmented groups), containing fifty individuals each, that were kept in separate tanks (each tank had a volume of 380 l) for 1 month. All fish were PIT tagged. To facilitate visual orientation among storage tanks, we also marked individuals using visible implant elastomer (VIE) tags (Northwest Marine Technology, USA). Fish were maintained under standard conditions with the aim to maximize their welfare [56]. Water was refined using biological filters with an integrated UV sterilizer (Pressure-Flo 5000, Rolf C. Hagen Inc., www.lagunaponds.com). The water temperature was controlled automatically using external air conditioning and held at an average of 20˚C. Light was controlled under a 12-h day/12-h night regime. The fish were fed *ad libitum* with Biomar pellets (Czech Republic) once a day. Shelters (one shelter per fish; plastic tubes; diameter 5 cm; length adjusted to the size of the fish) were installed in each tank [52]. All experimental fish survived. After the experiment, the fish were released under the control of the Fish Management Authorities into fishponds with extensive production management.

Experimental design

The aim of our study was to validate PSV as a general stress response in fish. Therefore, we performed validation using individual pigmented and albino fish subjected to a clearly defined stressor as the first step to define the basic PSV/cortisol relationship (Experiment I). Subsequently, we aimed to test whether PSV can be used as an indicator of more complicated social

stress conditions in albinos (Experiment II), for which methods based on variability in pigmentation and/or eye darkening are inapplicable [e.g. 10]. The experimental design illustrated in Fig 1.

Experiment I: PSV and cortisol concentration

Nineteen albinos (mean weight 140 g, range 87–220 g; mean standard length 255 mm, range 208–306 mm) and nineteen pigmented (mean weight 175 g, range 93–438 g; mean standard length 270 mm, range 226–376 mm) randomly selected juvenile catfish were used to investigate the relationship between PSV and concentration of cortisol in blood plasma. To induce a stress response, we exposed fish to a combination of air exposure and novel environmental stressors. Catfish were individually netted and maintained out of water for 2 min [40] and subsequently introduced to a novel environment for 5 min (aquarium; footprint 40 x 60 cm; water level 10 cm; no shelters; based on Barreto and Volpato [41]). A set of approximately 10 photos of fish eyes was taken through the glass of the aquarium after this procedure using a Canon EOS 650D camera (18 Mpx CMOS hybrid sensor) with a macro lens (Canon Inc., Japan). The photos were taken without the flash to prevent disturbing and the camera was positioned behind the longer side glass of the aquarium. The distance of camera from the glass of the aquarium varied depending on the position of the fish; generally, the distance was in tens of centimeters. The fish showed no signs of fear or abnormal behavior such as an escape or rapid changes of movement during photography. Individuals used in the experiment were well acclimatized in the laboratory and were used to people movements behind the glass of the aquarium during daily care and feeding, so people with camera behind the glass was not that different from what they were used to on a daily basis. We obtained a blood sample from each tested individual to determine cortisol concentration immediately after taking photos. Blood was drawn from the *vena caudalis* using an 18G 1½ in syringe with heparin as anticoagulant (Heparin inj., Léčiva, Czech Republic), and samples were stabilized with 40 IU of sodium heparin per 1 ml of blood. The procedure was based on unified methods for the hematological examination of fish [57, 58]. For biochemical plasma analyses, blood was separated via centrifugation at 12,000 x g for 10 min at 4˚C, and plasma samples were stored at -80˚C until analysis. Cortisol concentrations were measured using a VETTEST 8008 analyzer with snap reader (IDEXX Laboratories Inc., Westbrook, ME, USA; [59, 60]) The analyzer uses dry chemical and colorimetric analysis techniques. The aquarium was thoroughly rinsed and filled with clean aged tap water before insertion of a new specimen.

Experiment II: PSV and social stress in albinos

A total of eighty-eight albinos (mean weight, 14 g; range, 9–22 g; mean standard length 111 mm, range 93–133 mm) were used to investigate whether a penultimate experience (prior fighting/social experience according to Hsu and Wolf [46] from a contest would be reflected by a stress response during the subsequent contact. Four randomly selected familiar individuals from one storage tank were relocated into an empty storage tank of identical size. The same physical conditions, including lighting, were simulated; however, only one shelter of an identical size and shape as described for the storage tanks was included to accelerate competition among individuals. Fish were left in this tank for the following 24 hours to establish a social hierarchy. Subsequently, each group of four specimens were relocated to a novel environment (aquarium; footprint 40 x 60 cm; water level 10 cm) with only one shelter present (the same as that in the storage tank). All specimens from each group were introduced into the tank at the same time and, after the procedure described below was completed, the tank was prepared for a new group by thoroughly rinsing and filling it with clean, aged tap water. The first individual
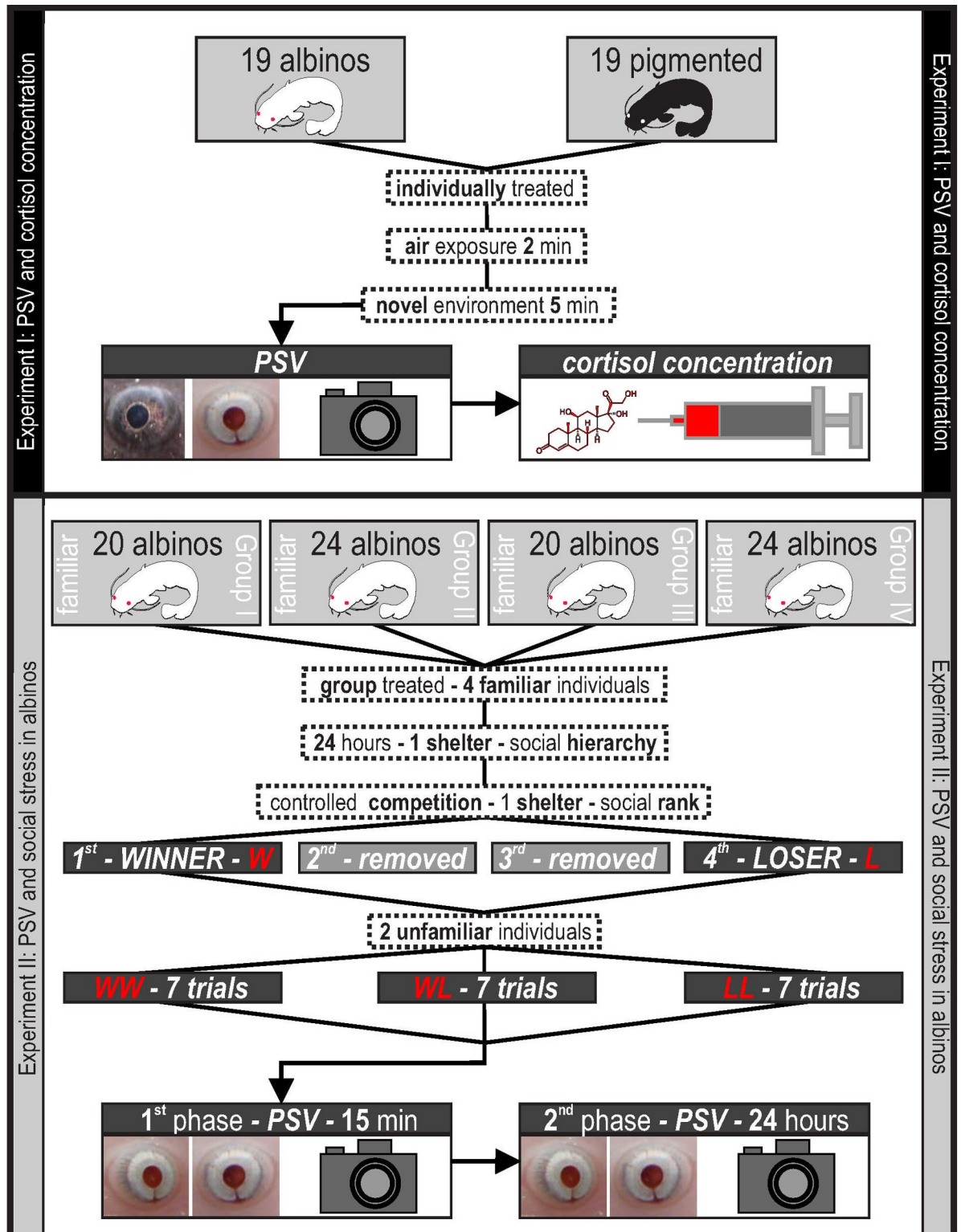
Fig 1. Illustrative figure of the experimental design.

that occupied a shelter was removed from the aquarium and identified as a winner (W); the second and third occupants were removed successively, and the last, fourth individual was identified as a loser (L). Each individual was removed together with the shelter (fish was inside the shelter) using a net and shelter was then returned to the aquarium. The fish were separated individually in order to facilitate their identification and had a recovery period of 300 s before the follow-up treatment to maintain their previous winning experience [61]. Unfamiliar winners and losers from different storage tanks were subsequently placed into a set of experimental aquaria (footprint 30 x 15 cm, depth 20 cm) without shelters to form experimental pairs in the following treatment combinations: winner-winner (WW), loser-loser (LL), and winner-loser (WL). Because unfamiliar individuals were marked with different VIE colors, we were able to visually distinguish individuals in the experimental aquaria. This marking also allowed us to identify individuals in the photos of eyes taken after 15 min of occupancy in the experimental aquarium (referred to as 'phase one'). A set of approximately 10 photos of fish eyes was taken using the same equipment used in the previous experiment. Next, the experimental pairs were left in aquaria for the following 24 h, and their eyes were photographed again (referred to as 'phase two'). Every treatment combination (WW, LL, and WL) was repeated seven times, resulting in the use of 42 individuals for the subsequent analysis of PSV.

Analysis of PSV

PSV was determined using digital photos that were analyzed with the help of ImageJ software [62]. The number of pixels depicting the pupil was compared to the number of pixels depicting the iris, and its ratio was used to determine the percentage of pupil dilatation. For every fish, 4 photos were analyzed from every sampling occasion. All four obtained pupil dilatation values per individual were used in further analyses to account for variability in pupil dilatation determination.

Statistical analyses

Statistical analyses were performed using the SAS software package (SAS Institute Inc., version 9.4, www.sas.com). Mixed models with random factors were used to account for the repeated measures collected for the same experimental units (individual fish). A separate model was fitted for data from experiments I and II. PROC MIXED was used to analyze pupil dilatation in relation to cortisol blood concentration, weight, length and pigmentation (pigmented vs. albino; experiment I; model I) and in relation to the experimental phase, weight, length, previous experience and treatment category (experiment II; model II). Variables were log transformed to meet normality requirements when needed. The significance of explanatory variables was assessed using an F-test. Least-squares means (henceforth referred to as 'adjusted means') were subsequently computed for particular classes. The differences between classes were tested with t-tests, and a Tukey-Kramer adjustment was used for multiple comparisons. Degrees of freedom were calculated using the Kenward-Roger method [63].

Results

Experiment I: PSV and cortisol concentration

The mean and standard deviation of pupil dilatation of individual fish during the first experiment were 7.9% and 0.77 respectively and there was no significant relationship between pupil dilatation and body size (weight $F_{2, 152} = 0.05$, $P > 0.82$; length $F_{2, 152} = 0.05$, $P > 0.83$). No significant size differences were detected between the pigmented and albino catfish (weight $P > 0.1$, n = 38; standard length $P > 0.16$, n = 38). Albinos showed lower pupil dilatation ($F_{1, 152}$

= 68.81, P < 0.0001; Fig 2A) and higher cortisol values ($F_{1, 38} = 117.46$, P < 0.0001; Fig 2B) than did pigmented conspecifics. Mean raw cortisol value of albino catfish was 174.91 ng / ml (range 120.36–263.97 ng / ml), while mean raw cortisol value of pigmented catfish was 85.89 ng / ml (range 50.26–120.35) ng / ml.

Our results proved that PSV could be used as a stress indicator in fish, as after exposure to a standardized stressor, catfish showed a clear positive relationship between pupil dilatation and cortisol concentrations ($F_{2, 152} = 36.32$, P < 0.0001). This relationship was observed for both pigmented (Fig 3A) and albino (Fig 3B) specimens, suggesting its stability irrespective of individual ability to express social communication by coloring.

Experiment II: PSV and social stress in albinos

The follow-up experiment, in which the mean and standard deviation of pupil dilatation of individual fish were 16.8% and 2.76 respectively, showed that albino catfish adjusted their pupil dilatation according to the result of their previous contest as well as their subsequent contact. Pupil dilatation was generally increased after 24 h contact of two unfamiliar catfish specimens in a novel environment ($F_{1, 211} = 7.15$, P < 0.0081; Fig 4A), and higher pupil dilatation was observed in winners than in losers of the previous contest across both experimental phases ($F_{1, 275} = 8.60$,
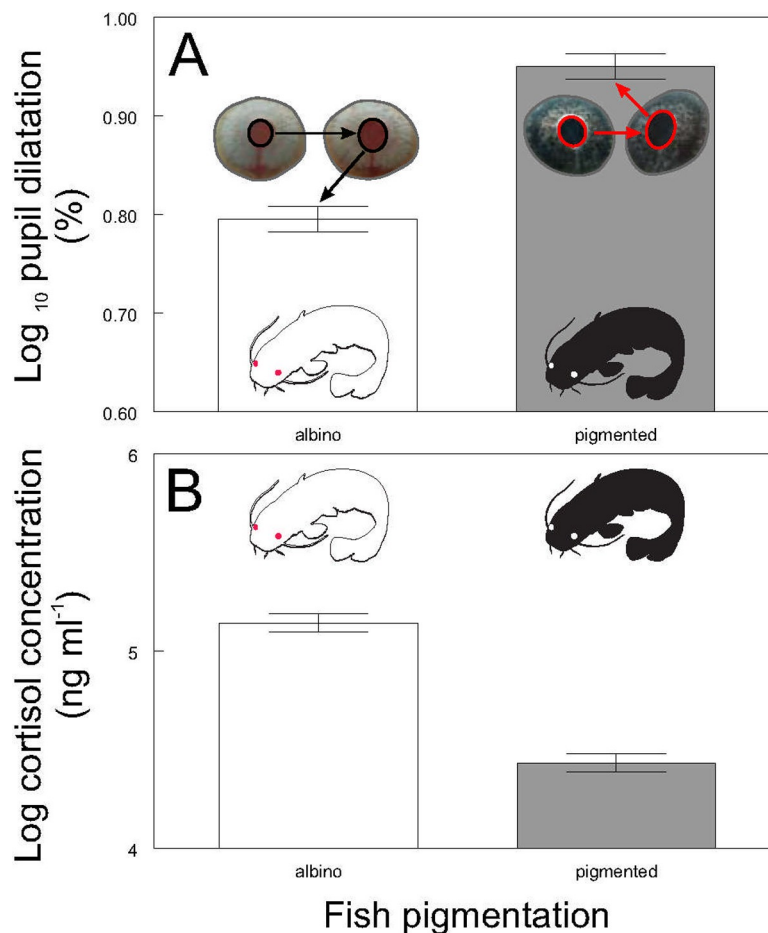

Fig 2. Pupil dilatation (**A**) and cortisol blood concentration (**B**) according to the fish pigmentation. Values are adjusted means +/- S.E. Different colored bars within one part of the figure indicate significant differences (Adj. P < 0.05). Part A includes illustrative picture of pupil dilatation before and after acute stress.
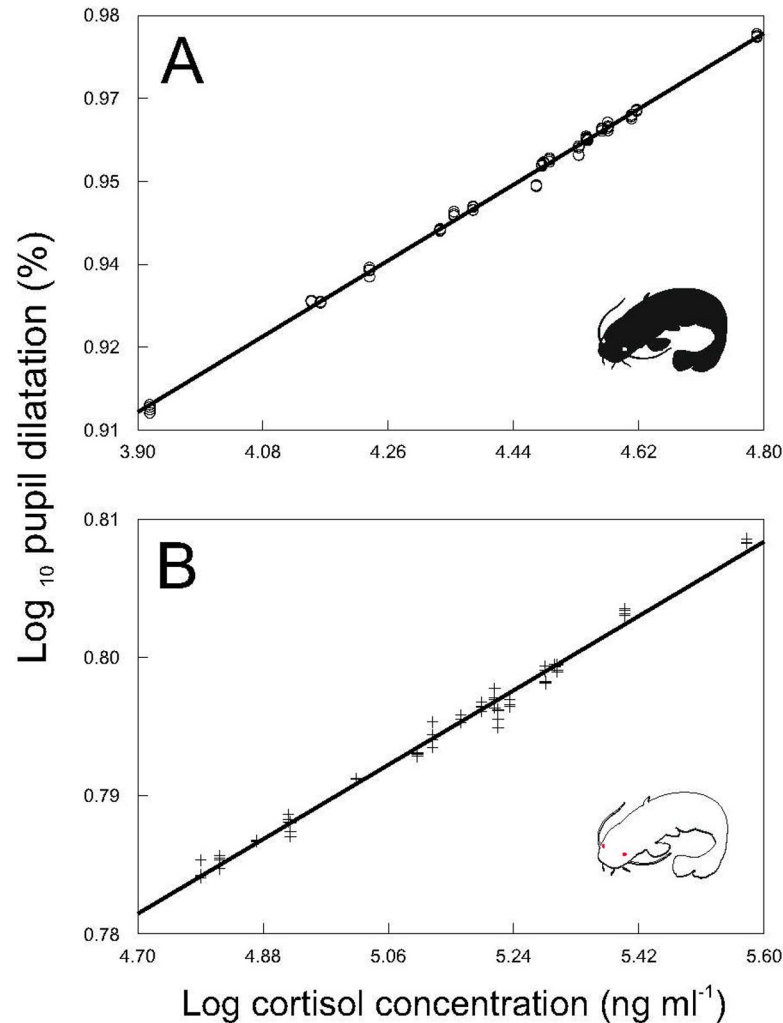
Fig 3. Relationship between pupil dilatation and cortisol blood concentrations. Values in pigmented (**A**; $y = 0.6359 + 0.0711x$; $r^2 = 0.99$; $P < 0.0001$) and albino (**B**; $y = 0.641 + 0.0299x$; $r^2 = 0.98$; $P < 0.0001$) catfish. Predicted values are from the mixed model analyses.

$P < 0.0036$; Fig 4B) with no significant relationship observed between pupil dilatation and body size (weight $F_{1, 99.5} = 0.06$, $P > 0.8$; length $_{F1, 104} = 2.17$, $P > 0.14$). Furthermore, the highest pupil dilatation among participants and treatment combinations was observed for the winners of WL contests, which was significantly higher than that observed in all other treatment combinations ($F_{2, 312} = 4.59$, $P < 0.0109$; Fig 4C; Adj. $P < 0.05$). The second highest pupil dilation was found for the winner in a WW contest, followed by the loser in an LL and the loser in a WL contest; nevertheless, differences among these treatments were nonsignificant (Adj. $P > 0.05$).

Discussion

Many methodological approaches have been used to observe stress in animals, although non-invasive methods that limit the manipulation of tested individuals are becoming increasingly appreciated. The observation of PSV associated with stress and arousal in humans and mammals has been extended to stress observation in fish. The method proposed in the present study can be considered for use in laboratory experiments and/or aquaculture production
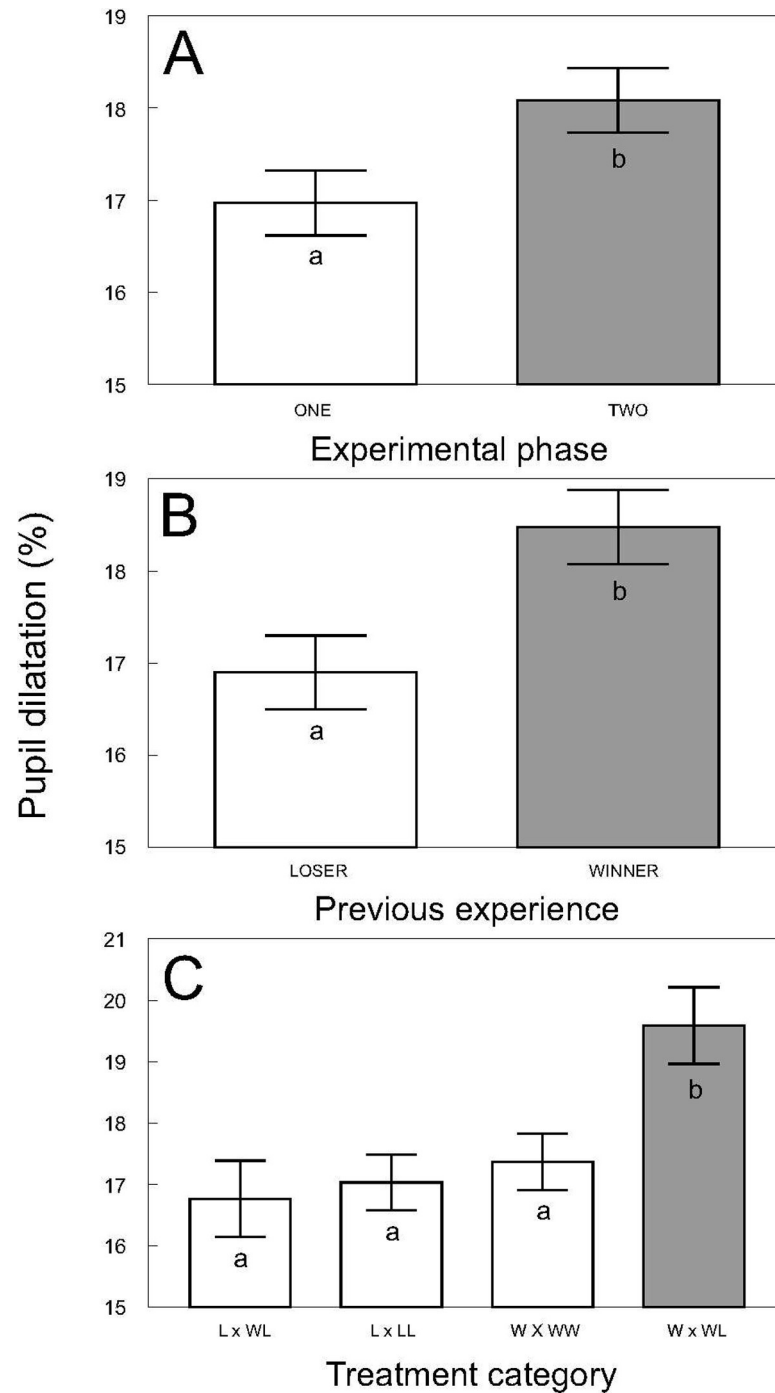
Fig 4. Pupil dilatation according to the experimental phase. A; values according to the experimental phase; 'one' sampled after 15 min, 'two' sampled after 24 h). **B**; values according to catfish previous experience (loser and winner from a penultimate contest) and **C**; values according to treatment combination ('W x WL' winner from the winner/ loser combination, 'W x WW' winner from the winner/winner combination, 'L x LL' loser from the loser/loser combination, and 'L x WL' loser from the winner/loser combination). Values are adjusted means +/- S. E. Different colored and lettered bars within one part of the figure indicate mutually significant differences (Adj. P < 0.05), while differences among the same colored and lettered bars are nonsignificant (Adj. P > 0.05).

https://doi.org/10.1371/journal.pone.0244017.g004

(e.g., of ornamental fish) to evaluate stress level and animal welfare. The present study found that pupil dilatation and cortisol plasma concentrations were increased in fish exposed to stress conditions. These relationships were observed for both albinos and pigmented catfish. Pupil dilatation also increased following social interactions between dominant winners and submissive losers in albinos.

In our study, under stressful conditions, pigmented and albino catfish displayed pupil dilatation accompanied by an increase in cortisol levels in blood plasma. Short-term stressors such as relocation and confinement usually increase cortisol levels in blood plasma [also see 3 for a review, 64, 65]. In fish, increased cortisol concentrations are usually accompanied by other stress indicators, such as intensified gill ventilation [65] and/or subordinate behavior [2]. Both pupil dilatation and higher cortisol levels are associated with fear, stress and/or arousal, as reported in previous studies of human beings [38, 39, 66]. The present results showed a link among PSV, increased levels of cortisol and stress in fish and introduce a possible noninvasive method of stress detection in albino fish.

Our experiment showed that PSV observed during the contact of albino individuals is influenced by the results of their penultimate contest. The highest stress-induced response denoted a winner; the largest dilatation indicated a winner in the WL combination, while the lowest dilatation denoted a loser in the WL combination. The penultimate winning experience tends to modify the behavior of an individual during the next contest in which winning individuals tend to behave more aggressively towards a new contestant and their chance to win again increases (winner effect), while losers tend to behave more submissively and have a higher chance of losing again (loser effect) [67]. In addition, the results of the contest are typically reflected in the physiology of the contestants [68]. An increase in circulating androgens, e.g., testosterone hormone, predesignate winners for a victory in the next contest [69]. Hormones produced by a loser and/or the perception of winning itself can be other reasons for higher emotional arousal of an individual with penultimate winning experience [70]. Our results tended to show perceivable socially induced stress in winner and looser albinos that is reflected by PSV.

A higher stress-induced response of albino catfish with penultimate winning experience can be discussed in terms of hierarchy dominance. Higher stress among dominant individuals during contests results in a facilitated long-term approach to resources, whereas subordinate individuals are subjected to chronic stress as a result of long-term limited availability of resources. These phenomena have been reported in several vertebrates, including fish [4, 5, 71]. However, in fish, the relationship between dominancy and stress may not readily detectable. For example, no difference in acute stress between dominant and subordinate individuals occurred during cooperative breeding in cichlid *Neolamprologus pulcher* (Trewavas and Poll, 1952), while higher chronic stress was associated with dominancy, and its level in subordinate individuals depended on individual behavior, position in a social hierarchy and social stability of a group [72]. Similarly, subordinate *N. pulcher* females that frequently engaged in nonaggressive interactions with dominant females had lower cortisol levels, while subordinate females in groups in which the dominant breeding pair behaved antagonistically towards each had higher cortisol levels [73]. Both examples illustrate the relationship between stress stimuli and dominancy as influenced by species-specific social conditions. The relationship between penultimate winners and losers in albino catfish corresponds to the relationship between dominant and subordinate individuals wherein the higher pupil dilatation of dominant winners reflects both stress and arousal from the perception of the penultimate winning experience. Our results tended to show PSV in albino catfish as a result of socially induced stress perceptible primarily in dominant conspecifics.

Albinism can be considered a behavioral syndrome accompanied by physiological limitations, e.g., reduced adaptation to light resulting in photoreceptor degradation [74], and is often

demonstrated by a reduction in vision and movement perception [75, 76], acrophobia and neophobia [77, 78]. Our results showed that albino and pigmented fish perceive stress similarly, but albinos displayed lower pupil dilatation and higher cortisol level. Lower pupil dilatation can be considered in association with degradation of the photoreceptors resulting in impair visual acuity as reported in physiological studies of rodents [79]. Higher cortisol level can be associated with the inability of albinos to inform conspecifics about their socially induced status by changes in coloring. This limitation can be reflected in behavioral performance and demonstrated by other physiological differences between pigmented and albino individuals. In salmonids, the density of melanin-based and carotenoid-based spots is reflected in behavioral performances [8, 80], e.g., individuals with more spots displayed lower endurance and oxygen consumption [81]. Accordingly, albinos displayed higher sensitivity to stress and diseases [82, 83] and/or lower aggressiveness and different behavior in a group [15]. For example, albino morph cichlid *Metriaclima zebra* (Boulenger, 1899) was more sensitive to brood parasitism of the cuckoo catfish *Synodontis multipunctatus* (Boulenger, 1898), probably due to lower aggressiveness and visual acuity [84]. In general, behavior and physiology in animals, including albinos, correspond to coloring intensity as determined by pleiotropic genetic influence [13, 85, 86]. Additionally, the results showed that socially induced stress in albinos that cannot be signaled by changes in body coloring can be detected from their pupil dilatation.

Conclusions

Pupil dilatation and plasma cortisol increased in fish exposed to stress conditions.

The relationship was observed for both, albinos and pigmented catfish.

Noninvasive method to study stress levels in fish was introduced.

Supporting information

S1 Table. Cortisol and PSV values for the Experiment I. Fish ID, fish color (albino and pigmented fish), photo ID, cortisol concentration and PSV values.
(PDF)

S2 Table. PSV for the Experiment II. Fish ID, Treatment combination, Results of the treatments, photo ID and PSV values.
(PDF)

Acknowledgments

Author Contributions

Conceptualization: Ondřej Slavík, Pavel Horký.

Data curation: Pavel Horký, Josef Velíšek, Tereza Valchářová.

Formal analysis: Pavel Horký, Josef Velíšek.

Investigation: Ondřej Slavík, Pavel Horký, Tereza Valchářová.

Methodology: Pavel Horký, Josef Velíšek.

Writing – original draft: Ondřej Slavík.

References

1. Pottinger TG, Carrick TR. Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. Gen Comp Endocrinol. 1999; 116:122–132. https://doi.org/10.1006/gcen.1999.7355 PMID: 10525368

2. Sloman KA, Metcalfe NB, Taylor AC, Gilmour KM. Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. Physiol Biochem Zool. 2001; 74:383–389. https://doi.org/10.1086/320426 PMID: 11331510

3. Ellis T, Yildiz HY, López-Olmeda J, Spedicato MT, Tort L, Øverli Ø, et al. Cortisol and finfish welfare. Fish Physiol Biochem. 2012; 38:163–188. https://doi.org/10.1007/s10695-011-9568-y PMID: 22113503

4. Øverli Ø, Harris CA, Winberg S. Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. Brain Behav Evol. 1999; 54:263–275. https://doi.org/10.1159/000006627 PMID: 10640786

5. Fernandes-de-Castilho M, Pottinger TG, Volpato GL. Chronic social stress in rainbow trout: Does it promote physiological habituation? Gen Comp Endocrinol. 2008; 155:141–147. https://doi.org/10.1016/j.ygcen.2007.04.008 PMID: 17521651

6. Winberg S, Nilsson GE. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. Comp Biochem Physiol C Toxicol Pharmacol. 1993; 106 (3):597–614. https://doi.org/10.1016/0742-8413(93)90216-8

7. Øverli Ø, Korzan WJ, Höglund E, Winberg S, Bollig H, Watt M, et al. Stress coping style predicts aggression and social dominance in rainbow trout. Horm Behav. 2004; 45(4):235–241. https://doi.org/10.1016/j.yhbeh.2003.12.002 PMID: 15053939

8. Kittilsen S, Schjolden J, Beitnes-Johansen I, Shaw JC, Pottinger TG, Sørensen C, et al. Melanin-based skin spots reflect stress responsiveness in salmonid fish. 2009; Horm Behav. 56(3):292–298. https://doi.org/10.1016/j.yhbeh.2009.06.006 PMID: 19539629

9. Kittilsen S, Johansen IB, Braastad BO, Øverli Ø. Pigments, parasites and personality: Towards a unifying role for steroid hormones? 2012; PLoS One 7(4):e34281. https://doi.org/10.1371/journal.pone.0034281 PMID: 22493685

10. Backström T, Heynen M, Brännäs E, Nilsson J, Winberg S, Magnhagen C. Social stress effects on pigmentation and monoamines in Arctic charr. Behav Brain Res. 2015; 291:103–107. https://doi.org/10.1016/j.bbr.2015.05.011 PMID: 25997582

11. Carden SM, Boissy RE, Schoettker PJ, Good WV. Albinism: modern molecular diagnosis. Br J Ophthalmol. 1998; 82:189–1995. https://doi.org/10.1136/bjo.82.2.189 PMID: 9613388

12. Fuller LJ. Effects of the albino gene upon behaviour of mice. Anim Behav. 1967; 15(4):467–470. https://doi.org/10.1016/0003-3472(67)90045-0 PMID: 6055100

13. DeFries JC. Pleiotropic effects of albinism on open field behaviour in mice. Nature. 1969; 221:65–66. https://doi.org/10.1038/221065a0 PMID: 5782616

14. Slavík O, Horký P, Wackermannová M. How does agonistic behaviour differ in albino and pigmented fish? PeerJ. 2016; 4:e1937. https://doi.org/10.7717/peerj.1937 PMID: 27114883

15. Slavík O, Horký P, Maciak M. Ostracism of an albino individual by a group of pigmented catfish. PLoS One. 2015; 10:e0128279. https://doi.org/10.1371/journal.pone.0128279 PMID: 26018869

16. Uieda W. Behavior of an albino vampire bat, *Desmodus rotundus* (E. Geoffroy) (Chiroptera, Phyllostomidae), in captivity. Rev Bras Zool. 2001; 18(2):641–644. https://doi.org/10.1590/S0101-81752001000200031.

17. Suter HC, Huntingford FA. Eye colour in juvenile Atlantic salmon: effects of social status, aggression and foraging success. J Fish Biol. 2002; 61:606–614. https://doi.org/10.1111/j.1095-8649.2002.tb00899.x

18. Volpato GL, Luchiari AC, Duarte CRA, Barreto RE, Ramanzini GC. Eye color as an indicator of social rank in the fish Nile tilapia. Braz J Med Biol Res. 2003; 36(12):1659–1663. https://doi.org/10.1590/S0100-879X2003001200007 PMID: 14666250

19. Miyai CA, Carretero Sanches FH, Costa TM, Colpo KD, Volpato GL, Barreto RE. The correlation between subordinate fish eye colour and received attacks: a negative social feedback mechanism for the reduction of aggression during the formation of dominance hierarchies. Zoology. 2011; 114(6):335–339. https://doi.org/10.1016/j.zool.2011.07.001 PMID: 21975142

20. Freitas RHA, Negrão CA, Felício AKC, Volpato GL. Eye darkening as a reliable, easy and inexpensive indicator of stress in fish. Zoology. 2014; 117(3):179–184. https://doi.org/10.1016/j.zool.2013.09.005 PMID: 24783997

21. Hubená P, Horký P, Slavík O. Test-dependent expression of behavioral syndromes: A study of aggressiveness, activity, and stress of chub. Aggress Behav. 2020; 46(5):412–424. https://doi.org/10.1002/ab.21909 PMID: 32542801

22. Wang CA, Munoz DP. A circuit for pupil orienting responses: implications for cognitive modulation of pupil size. Curr Opin Neurobiol. 2015; 33:134–140. https://doi.org/10.1016/j.conb.2015.03.018 PMID: 25863645

23. Beatty J. Task-evoked pupillary responses, processing load, and the structure of processing resources. Psychol Bull. 1982; 91(2):276–292. https://doi.org/10.1037/0033-2909.91.2.276 PMID: 7071262

24. Borgdorff P. Respiratory fluctuations in pupil size. Am J Physiol. 1975; 228:1094–1102. https://doi.org/10.1152/ajplegacy.1975.228.4.1094 PMID: 1130509

25. Loewenfeld IE. Mechanisms of reflex dilatation of the pupil; historical review and experimental analysis. Doc Ophthalmol. 1958; 12:185–448. https://doi.org/10.1007/BF00913471 PMID: 13609524

26. Bardeen JR, Daniel TA. An eye-tracking examination of emotion regulation, attentional bias, and pupillary response to threat stimuli. Cognit Ther Res. 2017; 41:853–866. https://doi.org/10.1007/s10608-017-9860-y

27. Macatee RJ, Albanese BJ, Schmidt NB, Cougle JR. The moderating influence of heart rate variability on stressor-elicited change in pupillary and attentional indices of emotional processing: an eye-tracking study. Biol Psychol. 2017; 123:83–93. https://doi.org/10.1016/j.biopsycho.2016.11.013 PMID: 27916689

28. Oka S, Chapman CR, Kim B, Nakajima I, Shimizu O, Oi Y. Pupil dilation response to noxious stimulation: effect of varying nitrous oxide concentration. Clin Neurophysiol. 2007; 118(9):2016–2024. https://doi.org/10.1016/j.clinph.2007.04.023 PMID: 17646133

29. Charier DJ, Zantour D, Pichot V, Chouchou F, Barthelemy J-CM, Roche F, et al. Assessing pain using the variation coefficient of pupillary diameter. J Pain. 2017; 18:1346–1353. https://doi.org/10.1016/j.jpain.2017.06.006 PMID: 28711635

30. Franzen PL, Buysse DJ, Dahl RE, Thompson W, Siegle GJ. Sleep deprivation alters pupillary reactivity to emotional stimuli in healthy young adults. Biol Psychol. 2009; 80(3):300–305. https://doi.org/10.1016/j.biopsycho.2008.10.010 PMID: 19041689

31. Strauss GP, Ossenfort KL, Whearty KM. Reappraisal and distraction emotion regulation strategies are associated with distinct patterns of visual attention and differing levels of cognitive demand. PLoS One. 2016; 11(11):e0162290. https://doi.org/10.1371/journal.pone.0162290 PMID: 27855175

32. Hoehl S, Hellmer K, Johansson M, Gredebäck G. Itsy bitsy spider . . .: Infants react with increased arousal to spiders and snakes. Front Psychol. 2017; 8:1710. https://doi.org/10.3389/fpsyg.2017.01710 PMID: 29093687

33. Partala T, Surakka V. Pupil size variation as an indication of affective processing. Int J Hum Comput Stud. 2003; 59(1–2):185–198. https://doi.org/10.1016/S1071-5819(03)00017-X

34. Bradley MM, Miccoli L, Escrig MA, Lang PJ. The pupil as a measure of emotional arousal and autonomic activation. Psychophysiology 2008; 45(4):602–607. https://doi.org/10.1111/j.1469-8986.2008.00654.x PMID: 18282202

35. Larsen RS, Waters J. Neuromodulatory correlates of pupil dilation. Front Neural Circuits. 2018; 12:21. https://doi.org/10.3389/fncir.2018.00021 PMID: 29593504

36. Schriver BJ, Bagdasarov S, Wang Q. Pupil-linked arousal modulates behavior in rats performing a whisker deflection direction discrimination task. J Neurophysiol. 2018; 120(4):1655–1670. https://doi.org/10.1152/jn.00290.2018 PMID: 29995602

37. Ebitz RB, Pearson MJ, Platt ML. Pupil size and social vigilance in rhesus macaques. Front Neurosci. 2014; 8:100. https://doi.org/10.3389/fnins.2014.00100 PMID: 24834026

38. Levine A, Zagoory-Sharon O, Feldman R, Lewis JG, Weller A. Measuring cortisol in human psychobiological studies. Physiol Behav. 2007; 90(1):43–53. https://doi.org/10.1016/j.physbeh.2006.08.025 PMID: 17055006

39. Finke JB, Behrje A, Schächinger H. Acute stress enhances pupillary responses to erotic nudes: Evidence for differential effects of sympathetic activation and cortisol. Biol Psychol. 2018; 137:73–82. https://doi.org/10.1016/j.biopsycho.2018.07.005 PMID: 30025747

40. Barreto RE, Volpato GL. Evaluating feeding as unconditioned stimulus for conditioning of an endocrine effect in Nile tilapia. Physiol Behav. 2007; 92:867–872. https://doi.org/10.1016/j.physbeh.2007.06.013 PMID: 17689574

41. Barreto RE, Volpato GL. Ventilation rates indicate stress-coping styles in Nile tilapia. J Biosci. 2011; 36:851–855. https://doi.org/10.1007/s12038-011-9111-4 PMID: 22116283

42. Zayan R. The specificity of social stress. Behav Processes. 1991; 25(2–3):81–93. https://doi.org/10.1016/0376-6357(91)90011-N PMID: 24923968

43. Sloman KA, Montpetit CJ, Gilmour KM. Modulation of catecholamine release and cortisol secretion by social interactions in the rainbow trout, Oncorhynchus mykiss. Gen Comp Endocrinol. 2002; 127 (2):136–146. https://doi.org/10.1016/S0016-6480(02)00033-3 PMID: 12383441

44. Backström T, Winberg S. Central corticotropin releasing factor and social stress. Front Neurosci. 2013; 7:117. https://doi.org/10.3389/fnins.2013.00117 PMID: 23847465

45. Dugatkin LA. Winner and loser effects and the structure of dominance hierarchies. Behav Ecol. 1997; 8 (6):583–587. https://doi.org/10.1093/beheco/8.6.583

46. Hsu Y, Wolf LL. The winner and loser effect: integrating multiple experiences. Anim Behav. 1999; 57 (4):903–910. https://doi.org/10.1006/anbe.1998.1049 PMID: 10202098

47. Hsu Y, Wolf LL. The winner and loser effect: what fighting behaviours are influenced? Anim Behav. 2001; 61(4):777–786. https://doi.org/10.1006/anbe.2000.1650

48. Cucherousset J, Horký P, Slavík O, Ovidio M, Arlinghaus R, Boulêtreau S, et al. Ecology, behaviour and management of the European catfish. Rev Fish Biol Fish. 2018; 28:177–190. https://doi.org/10. 1007/s11160-017-9507-9

49. Linhart O, Štěch L, Švarc J, Rodina M, Audebert JP, Grecu J, et al. The culture of the European catfish, Silurus glanis, in the Czech Republic and in France. Aquat Living Resour. 2002; 15(2):139–144. https:// doi.org/10.1016/S0990-7440(02)01153-1

50. Boujard T. Diel rhythms of feeding activity in the European catfish, *Silurus glanis*. Physiol Behav. 1996; 58(4):641–645. https://doi.org/10.1016/0031-9384(95)00109-V

51. Slavík O, Pešta M, Horký P. Effect of grading on energy consumption in European catfish *Silurus glanis*. Aquaculture. 2011; 313: 73–78. https://doi.org/10.1016/j.aquaculture.2011.01.002

52. Slavík O, Maciak M, Horký P. Shelter use of familiar and unfamiliar groups of juvenile European catfish *Silurus glanis*. Appl Anim Behav Sci. 2012; 142(1–2):116–123. https://doi.org/10.1016/j.applanim. 2012.09.005

53. Dingerkus G, Seret B, Guilbert E. The first albino wels, Silurus glanis Linnaeus, 1758, from France, with a review of albinism in catfishes (Teleostei: Siluriformes). Cybium. 1991; 15(3):185–188.

54. Nobile AB, Freitas-Souza D, Pontieri de Lima F, Acosta AA, Da Silva RJ. Partial albinism in Rhinelepis aspera from the Upper Paraná Basin, Brazil, with a review of Albinism in South American freshwater fishes. Rev Mex Biodivers. 2016; 87:531–534. https://doi.org/10.1016/j.rmb.2016.04.005

55. Manoel PS, Ono ER, Alves MIB. First report of albinism in the South American catfish Imparfinis mirini (Siluriformes: Heptapteridae). Rev Mex Biodivers. 2017; 88:471–473. https://doi.org/10.1016/j.rmb. 2017.01.030

56. Buchanan K, Burt de Perera T, Carere C, Carter T, Hailey A, Hubrecht R, et al. Guidelines for the treatment of animals in behavioural research and teaching. Anim Behav. 2012; 83(1):301–309. https://doi. org/10.1016/j.anbehav.2011.10.031

57. Li Z-H, Velíšek J, Žlábek V, Grabic R, Máchová J, Kolářová J, et al. Chronic toxicity of verapamil on juvenile rainbow trout (Oncorhynchus mykiss): Effects on morphological indices, hematological parameters and antioxidant responses. J Hazard Mater. 2011; 185(2–3):870–880. https://doi.org/10.1016/j. jhazmat.2010.09.102 PMID: 20970250

58. Svobodová Z, Pravda D, Palacková J. Unified methods of hematological examination of fish. Vodňany: VÚRH, Edition Methods No. 20, 1991

59. Stará A, Zusková E, Kouba A, Velíšek J. Effects of terbuthylazine-desethyl, a terbuthylazine degradation product, on red swamp crayfish (Procambarus clarkii). Sci Total Environ. 2016; 566–567:733–740. https://doi.org/10.1016/j.scitotenv.2016.05.113 PMID: 27239716

60. Slavík O, Horký P, Douda K, Velíšek J, Kolářová J, Lepič P. Parasite-induced increases in the energy costs of movement of host freshwater fish. Physiol Behav. 2017; 171:127–134. https://doi.org/10.1016/ j.physbeh.2017.01.010 PMID: 28087365

61. Chase ID, Bartolomeo C, Dugatkin LA. Aggressive interactions and inter-contest interval: How long do winners keep winning? Anim Behav. 1994; 48(2): 393–400. https://doi.org/10.1006/anbe.1994.1253

62. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012; 9:671–675. https://doi.org/10.1038/nmeth.2089 PMID: 22930834

63. Kenward MG, Roger JH. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics. 1997; 53(3):983–997. https://doi.org/10.2307/2533558 PMID: 9333350

64. Pottinger TG, Pickering AD. The influence of social interaction on the acclimation of rainbow trout, Oncorhynchus mykiss (Walbaum) to chronic stress. J Fish Biol. 1992; 41(3):435–447. https://doi.org/ 10.1111/j.1095-8649.1992.tb02672.x

65. Barreto RE, Volpato GL. Caution for using ventilatory frequency as an indicator of stress in fish. Behav Processes. 2004; 66(1):43–51. https://doi.org/10.1016/j.beproc.2004.01.001 PMID: 15062970

66. Kramer SE, Teunissen CE, Zekveld AA. Cortisol, chromogranin A, and pupillary responses evoked by speech recognition tasks in normally hearing and hard-of-hearing listeners: a pilot study. Ear Hear. 2016; 37:126–135. https://doi.org/10.1097/AUD.0000000000000311

67. Hsu Y, Earley RL, Wolf LL. Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. Biol Rev. 2006; 81(1):33–74. https://doi.org/10.1017/S146479310500686X PMID: 16460581

68. Earley RL, Lu CK, Lee IH, Wong SC, Hsu Y. Winner and loser effects are modulated by hormonal states. Front Zool. 2013; 10:6. https://doi.org/10.1186/1742-9994-10-6 PMID: 23399457

69. Oliveira RF, Silva A, Canário AVM. Why do winners keep wining? Androgen mediation of winner but not loser effects in cichlid fish. Proc Royal Soc B. 2009; 276(1665):2249–2256. https://doi.org/10.1098/rspb.2009.0132

70. Rutte C, Taborsky M, Brinkhof MWG. What sets the odds of winning and losing? Trends Ecol Evol. 2006; 21(1):16–21. https://doi.org/10.1016/j.tree.2005.10.014 PMID: 16701465

71. Creel S, Dantzer B, Goymann W, Rubenstein DR. The ecology of stress: effects of the social environment. Funct Ecol. 2013; 27(1):66–80. https://doi.org/10.1111/j.1365-2435.2012.02029.x

72. Mileva VR, Fitzpatrick JL, Marsh-Rollo S, Gilmour KM, Wood CM, Balshine S. The stress response of the highly social African cichlid Neolamprologus pulcher. Physiol Biochem Zool. 2009; 82(6):720–729. https://doi.org/10.1086/605937 PMID: 19807269

73. Ligocki YI, Earley RL, Hellmann JK, Hamilton IM. Variation in glucocorticoid levels in relation to direct and third-party interactions in a social cichlid fish. Physiol Behav. 2015; 151:386–394. https://doi.org/10.1016/j.physbeh.2015.08.004 PMID: 26255122

74. Marc RE, Jones BW, Watt CB, Vazquez-Chona F, Vaughan DK, Organisciak DT. Extreme retinal remodeling triggered by light damage: implications for age related macular degeneration. Mol Vis. 2008; 14:782–806. PMID: 18483561

75. Buhusi CV, Perera D, Meck WH. Memory for timing and auditory signals in albino and pigmented rats. J Exp Psychol Anim Behav Process. 2005; 31(1):18–30. https://doi.org/10.1037/0097-7403.31.1.18 PMID: 15656724

76. Hupfeld D, Hoffmann KP. Motion perception in rats (Rattus norvegicus sp.): Deficits in albino Wistar rats compared to pigmented Long-Evans rats. Behav Brain Res. 2006; 170(1):29–33. https://doi.org/10.1016/j.bbr.2006.01.022 PMID: 16563528

77. Abeelen van JHF, Kroes WH. Albinism and mouse behaviour. Genetica. 1967; 38:419–429. https://doi.org/10.1007/BF01507473

78. Owen K, Thiessen DD, Lindzey G. Acrophobic and photophobic responses associated with the albino locus in mice. Behav Genet. 1970; 1:249–255. https://doi.org/10.1007/BF01074656 PMID: 5535859

79. Prusky GT, Harker KT, Douglas MR, Whisaw IQ. Variation in visual acuity within pigmented, and between pigmented and albino rat strains. Behav Brain Res. 2002; 136(2): 339–348. https://doi.org/10.1016/S0166-4328(02)00126-2 PMID: 12429395

80. Backström T, Johansson K, Brännäs E, Nilsson J, Magnhagen C. Short-term stress: effects on cortisol levels and carotenoid spots in Arctic charr (Salvenius alpinus). Can J Zool. 2016; 94:707–712. https://doi.org/10.1139/cjz-2016-0043

81. Magnhagen C, Backström T, Nilsson J, Brännäs E. Oxygen consumption and swimming performance in Arctic charr with different pigmentation patterns. Appl Anim Behav Sci. 2018; 202:119–124. https://doi.org/10.1016/j.applanim.2018.01.006

82. Lund PM, Taylor JS. Lack of adequate sun protection for children with oculocutaneous albinism in South Africa. BMC Public Health. 2008; 8:225. https://doi.org/10.1186/1471-2458-8-225 PMID: 18590551

83. Li Y, Geng X, Bao L, Elaswad A, Huggins KW, Dunham R, et al. A deletion in the Hermansky-Pudlak syndrome 4 (Hps4) gene appears to be responsible for albinism in channel catfish. Mol Genet Genom. 2017; 292:663–670. https://doi.org/10.1007/s00438-017-1302-8 PMID: 28289846

84. Cohen MS, Hawkins MB, Knox-Hayes J, Vinton AC, Cruz A. A laboratory study of host use by the cuckoo catfish Synodontis multipunctatus. Environ Biol Fishes. 2018; 101:1417–1425. https://doi.org/10.1007/s10641-018-0788-1

85. Ducrest A-L, Keller L, Roulin A. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends Ecol Evol. 2008; 23(9):502–510. https://doi.org/10.1016/j.tree.2008.06.001 PMID: 18644658

86. Brännäs E, Backström T, Nilsson J, Carlberg H, Stien LH, Magnhagen C. Distinguishing Arctic charr with different stress coping styles by visual screening of spottiness–reliability and consistency over time. J Zool. 2016; 300:213–220. https://doi.org/10.1111/jzo.12374