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Eye darkening as a reliable, easy and inexpensive indicator of stress in fish

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ABSTRACT

We expand the use of eye darkening (ED) to indicate non-social stress in the fish Nile tilapia *Oreochromis niloticus* (L.). ED is easily estimated, not requiring any sophisticated equipment, and is non-invasive, facilitating the collection of several measures of stress over time. In the current study, we showed the following: (i) high- and low-ED occur spontaneously, indicating different fish reactions to adjustments to a novel environment; (ii) fish confinement or air exposure clearly increases ED (air exposure is a stronger stressor than confinement), and the time to restore basal values indicates the severity of the impact of the stressor on the fish (this response is not affected by period of the day, e.g., morning or afternoon); and (iii) in adults, females were more responsive (slower recovery) to 2-min air exposure than to 30-min confinement.

1. Introduction

Stress is an important factor that can influence the outcome of laboratory studies (Brydges et al., 2009) and is present in aquaculture practices (Wendelaar Bonga, 1997; Barcellos et al., 2004). Although measurements of fish conditions are needed in such studies and practices, there is an imperative need for non-invasive techniques. The current possible techniques include measuring changes in waterborne (Ellis et al., 2004) and fecal (Lupica and Turner, 2009) steroids, ventilatory frequency (Barreto and Volpato, 2004, 2006), swimming activity (Øverli et al., 2006; Kittilsen et al., 2009), latency to move from an uncomfortable condition (Laursen et al., 2011), skin darkening (Höglund et al., 2000) and eye darkening (ED) (Suter and Huntingford, 2002; Volpato et al., 2003; Vera Cruz and Brown, 2007).

The estimation of ED is a potentially easy, inexpensive, and non-invasive way to measure conditions of fish stress. ED has been associated with social stress in Atlantic salmon (Suter and Huntingford, 2002) and Nile tilapia (Volpato et al., 2003; Vera Cruz and Brown, 2007). Recently, ED was shown to generate negative social feedback, as displayed by subordinate fish, which reduces the dominant's aggression in pearl cichlid (Miyai et al., 2011). Because these studies are all concerned with social stressors, in the present experiment we tested a possible generalization of ED as a response to non-social stressors (confinement and air exposure). These stressors are recognized to be potent stressors in aquaculture management (confinement: Arends et al., 1999; Barcellos et al., 2004; air exposure: Arends et al., 1999; Barcellos et al., 2001, 2004) and research practices (confinement: Cairns et al., 2008; Pottinger, 2010; air exposure: Van der Salm et al., 2006).

Despite the classical increase of plasma cortisol in stressed fish, the release of this hormone is also affected by other factors. Cortisol is released into the bloodstream in a circadian rhythm (Srivastava and Meier, 1972; Garcia and Meier, 1973) and, for some species, sex differences have been reported in plasma cortisol in stressed fish (Garcia and Meier, 1973; Kubokawa et al., 2001; Kling et al., 2008). Because stress measured in terms of cortisol levels is related to sex and the circadian cycle, we also investigated ED in response to stressors in accordance with these factors.

We tested whether ED is a reliable indicator of the imposition of non-social stressors on fish by estimating ED changes in confined or air-exposed fish. To know how broadly useful ED could be as an indicator of stress in fish, we also investigated stressor-induced ED changes at different points in the day (morning and afternoon), during different stages of fish development (juveniles and adult fish), and in the two sexes.

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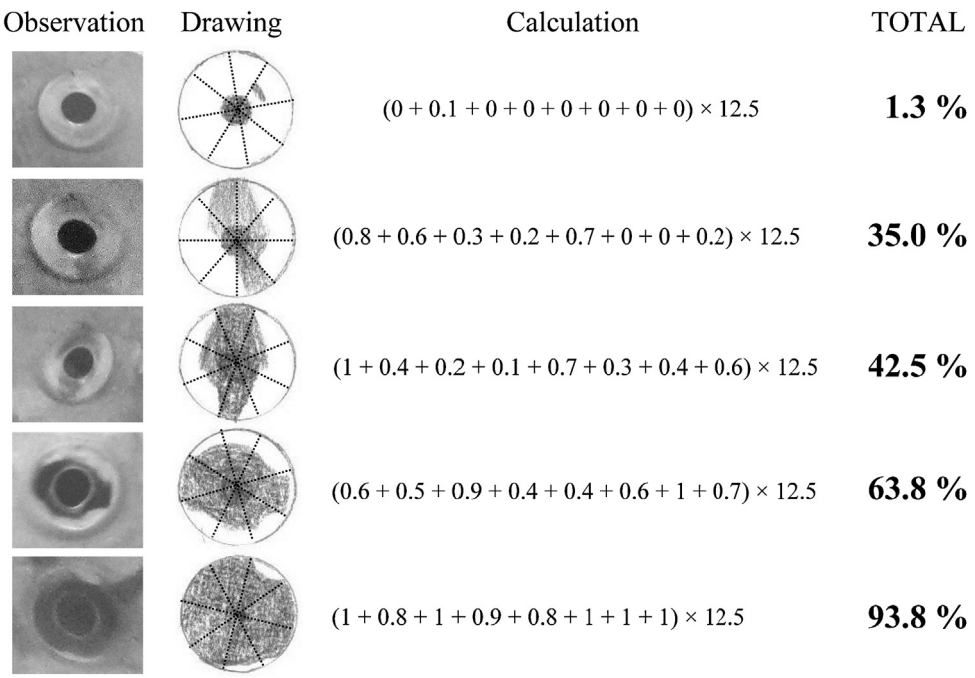

Fig. 1. Examples of the calculation of eye darkening (ED) in Nile tilapia. According to our observations (photos), we drew the dark area in a circle divided into eight equal areas, each one representing 12.5% (based on Volpato et al., 2003). We estimated the percentage that was dark in each of the eight areas, summed them and multiplied by 12.5 to calculate the total dark area per eye.

2. Materials and methods

In our first study, we examined juvenile Nile tilapia in two experiments as follows: (i) we observed spontaneous changes in ED during the day; and (ii) we compared the effects of confinement or air exposure in inducing ED changes, considering also the effect of the time of collection (morning or afternoon period) on the ED response. In a second study, we tested whether the sex of the fish influenced ED recovery after confinement or air exposure.

The protocols for these studies were conducted in accordance with the Ethical Principles in Animal Research guidelines and were approved by the Ethics Committee for Animal Experimentation (CEEA) of the Bioscience Institute, UNESP, Botucatu, SP, Brazil (Protocol #56/08).

2.1. Animal housing

Nile tilapia (*Oreochromis niloticus*) individuals from a monoculture hatchery were transferred and grouped in static indoor tanks with aeration for approximately 1 month prior to experimentation. These fish were kept in the animal care facility at a maximum density of 1 fish/5 l and grouped in size and age classes. Photoperiod was from 6:00 h to 18:00 h; food was offered once a day (~5% of biomass), and the water temperatures ranged from 22.0 to 25.5 °C. We used only tanks and aquaria with no water recirculation among them and no flow-through system but with continuous aeration.

Fish from this stocking population were used in the following two studies.

2.2. Study 1

2.2.1. Experiment 1

Fourteen juvenile fish (mean (±SD) standard length (SL) of 5.9 ± 0.3 cm) were individually isolated in glass aquaria (20 cm × 40 cm base and 25 cm high, ~16 l of static water with aeration) 24 h prior to the observations. Then, each fish was video monitored (without recording) by an observer (who stayed in the laboratory throughout the experiment) using a video camera located 4 m from the wall where all the aquaria were placed. All estimations of ED (see details in Section 2.4.2) were obtained using the zoom function of the camera each 30 min, from 9:00 h to 15:00 h. The water temperature was measured at the end of observations and averaged 24.0 ± 0.5 °C (ranging from 23.0 to 25.0 °C).

2.2.2. Experiment 2

Thirty juvenile fish (different fish from those in Experiment 1) were individually isolated in glass aquaria (15 cm × 60 cm base and 20 cm high, ~16 l of static water with aeration) for 24 h. Because low ED indicates low social stress (Volpato et al., 2003; Vera Cruz and Brown, 2007 for Nile tilapia; Suter and Huntingford, 2002 for Atlantic salmon; Miyai et al., 2011 for pearl cichlid), we used only fish that showed low ED (<~25% of the iris and sclera were dark) at the first observation (24 h after isolation and 2 min before imposing the stressor). We set up two treatment conditions, one in which isolated fish were subjected to confinement ($n = 9$, SL of 7.3 ± 1.6 cm) and another in which isolated fish (similar fish size; unpaired t-test, $t_{(18)} = 0.93$, $P = 0.36$) were subjected to air exposure ($n = 11$, SL of 6.8 ± 0.5 cm). ED was estimated in each fish 2 min before the stressor was imposed (Section 2.4.1. provides details about the stressors), immediately after removing the stressor (time 0; after removing the partition or replacing the fish into the water), and after 15, 30, 60, and 120 min. The tests began at 9:00 h (referred to as Test 1). As in Experiment 1, without imposition of stressors, ED did not spontaneously change in accordance with the time of day (see Fig. 2). To confirm the consistency of the results of Test 1 in this experiment, we repeated the test 6 h later (Test 2; 15:00 h) on the same fish, imposing the same previously described stressors. The temperature was 23.0 ± 1.5 °C (ranging from 21.0 to 26.0 °C) throughout the experimentation and did not differ among treatments (unpaired t-test, $t_{(18)} = 0.27$, $P = 0.79$).

2.3. Study 2

Sixty-four adult fish were individually isolated in glass aquaria (30 cm × 50 cm base and 35 cm high, ~45 l of static water with

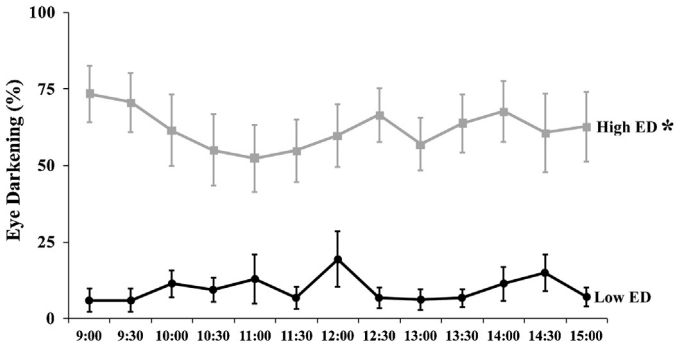

Fig. 2. Spontaneous eye darkening (ED) throughout the light period in undisturbed Nile tilapia (mean ± SEM) in the low ED ($n = 8$) and high ED ($n = 6$) groups. ED group status was maintained independent of the time of day. Likelihood ratio tests revealed no differences in time effects ($\chi^2_{12} = 7.10$, $P = 0.85$), but the ED classes were different (*, $\chi^2_1 = 19.96$, $P < 0.001$).

aeration) for 24 h before experimentation. Size-matched males and females (one-way ANOVA, $F_{(5,41)} = 0.68$, $P = 0.64$) that showed low ED (<25% of the iris and sclera were dark) were subjected to either confinement (males: $n = 11$, SL of 12.0 ± 1.5 cm; females: $n = 10$, SL of 11.9 ± 1.2 cm), air exposure (males: $n = 6$, SL of 12.8 ± 1.9 cm; females: $n = 8$, SL of 11.7 ± 1.2 cm) or were not disturbed (control; males: $n = 6$, SL of 12.7 ± 1.6 cm; females: $n = 6$, SL of 12.3 ± 0.6 cm), and ED was estimated (2 min before stressor, and 0, 15, 30, 60, 120 and 180 min after removing the stressor). In the control treatment, the pre-stressor period was considered to be 2 min before time 0. Each fish was tested once, regardless of the time of day (starting at 9:00 h or 15:00 h), as time did not affect ED in Experiment 1 (see Figs. 2 and 3). The water temperature was 25.1 ± 0.8 °C (ranging from 24.0 to 26.0 °C) and did not differ among treatments (one-way ANOVA, $F_{(5,41)} = 0.09$, $P = 0.99$).

2.4. Specific procedures

2.4.1. Stressors

To impose a confinement stressor, we added an opaque PVC partition to an aquarium for 30 min (based on Barreto and Volpato, 2004, 2006) to restrict the fish to one side of the aquarium (2.5 cm from the lateral border) and to prevent it from turning back to swim. To create an air-exposure stressor, we netted the fish and kept it out of the water for 2 min (based on Barreto and Volpato, 2007).

2.4.2. Estimation of eye darkening

The central black pupil of the Nile tilapia is surrounded by an iris and sclera that can range from pale to dark. We estimated ED as the percent darkened area of the iris and sclera (based on Volpato et al., 2003) by producing drawings of the circular area of the fish eye and dividing this into eight equal areas. The observer approached to a distance of 1 m from each aquarium at every collection period to estimate ED in Experiment 2 of Studies 1 and 2. During the observations of the fish inside the aquarium, the observer filled in each area according to the darkening observed in one eye of the fish (the first and easiest observed), as complementary observations revealed no differences between both eyes in the same fish. For this purpose, we registered ED in the left and right eyes of the same fish in a sample of 15 fish (SL of 10.67 cm ± 0.49 cm) and found that all of the percentages matched exactly for both eyes in the same fish. The mean ED values were 14.5% ± 17% before 2-min air exposure and 97.5% ± 7.0% immediately after abolishing the stressor.

The estimation of ED required 10–40 s each time. The clear results obtained in response to stress confirmed the adequacy of this procedure and that the results cannot be attributed to the interference of the observer. The calculations were made by summing the estimated percentages as illustrated in Fig. 1.

2.4.3. Sex determination

The fish were sexed by staining the oviductal opening of the genital papillae with methylene blue (Castro et al., 2009; Mendonça et al., 2010) before isolation in the experimental aquaria. This method is reliable in older fish but not in small juveniles.

2.5. Statistical analysis

To apply parametric testing, we transformed ED data [$\sqrt{(x + 0.5)}$] to fit a normal distribution (Kolmogorov–Smirnov's test) and to meet the parametric requirement of homoscedasticity (Levene's test). To compare ED, we fit mixed linear models according to two estimation methods (based on the "lme4" package in Bates et al., 2013; R Core Team, 2013): maximum likelihood (ML) was applied to compare models by likelihood ratio tests (LRT); and restricted maximum likelihood (REML) was applied to obtain the parameter estimates of the models. Levels of a significant factor were compared using Tukey's test as a post hoc test in all experiments. In all models, fish ED was considered to be a random factor and its interactions with time series and period were considered only for Experiment 2 of Study 1. Data on fish body size and temperature

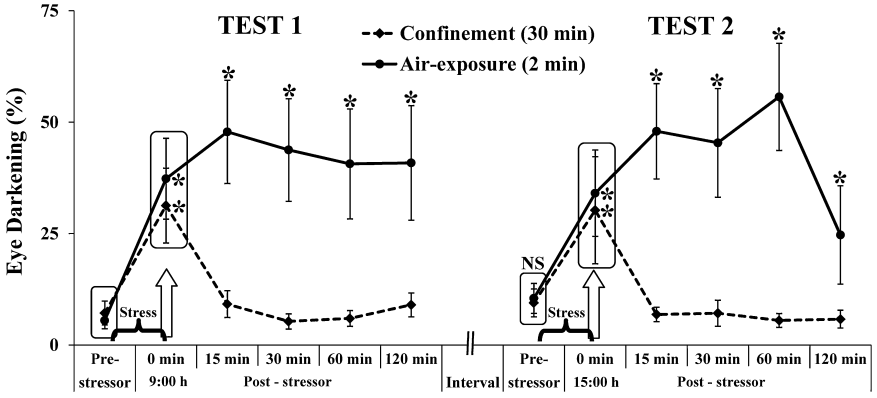

Fig. 3. Stressor-induced changes in eye darkening (ED) in Nile tilapia juveniles (mean ± SEM). Pre-stressor ED was quantified immediately before imposition of the stressor; 0 min post-stressor was measured immediately after removal of the stressor. Brackets indicate the stress periods (30 min for confinement and 2 min for air exposure). The arrow indicates the moment when the stressor was removed (the partition was removed or the fish was replaced into the water). Likelihood ratio tests revealed no differences between Test 1 and Test 2 ($\chi^4_1 = 0.01$, $P = 0.98$), but indicated differences between the stressors ($\chi^4_1 = 6.99$, $P < 0.01$) and in the recovery time after the stressor was removed ($\chi^4_1 = 37.35$, $P < 0.001$). * indicates ED that differs from the respective pre-stressor level. The means inside the rectangles were statistically equal to each other. NS means that pre-stressor levels before Test 2 were not significantly different from pre-stressor levels before Test 1.

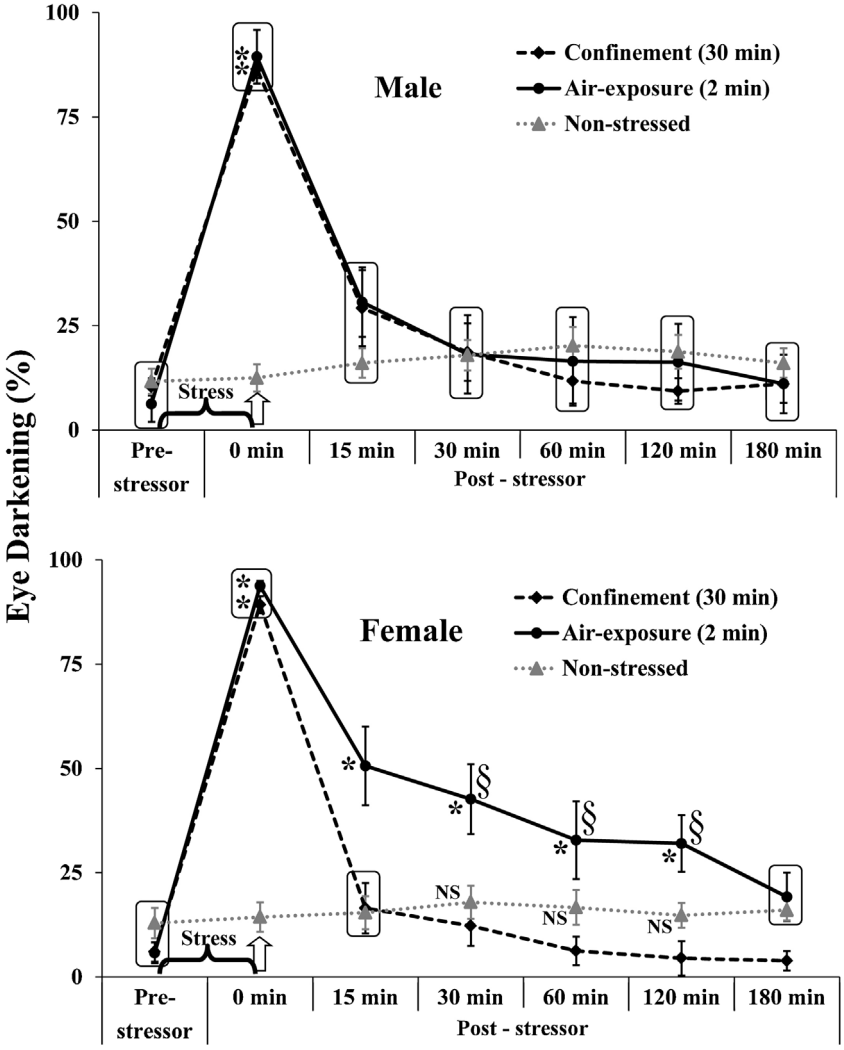

Fig. 4. Stressor-induced changes in eye darkening (ED) in male and female Nile tilapia (mean ± SEM). Pre-stressor ED was quantified immediately before imposition of the stressor; 0 min post-stressor was measured immediately after removal of the stressor. The arrow indicates the moment when the stressor was removed (the partition was removed or the fish was replaced into the water). Brackets indicate the stress period (30 min for confinement and 2 min for air exposure). Likelihood ratio tests revealed differences among treatments ($\chi^2_2 = 6.24$, $P < 0.05$) and time effects ($\chi^2_6 = 210.89$, $P < 0.001$) and no differences between the sexes ($\chi^2_1 = 0.05$, $P = 0.82$), but an interaction between sex and treatment ($\chi^2_2 = 5.46$, $P = 0.065$). * indicates ED that differs from the respective pre-stressor level. § indicates a difference between the sexes in the air-exposure treatment for the respective time interval (30, 60 and 120 min). NS means that ED in the control treatment was not significantly different from ED in both stress treatments; however, there were differences among the stressors. The means inside the rectangles were statistically equal to each other. All pre-stressor levels were equal to each other.

met all parametric assumptions and were tested by an unpaired *t*-test or one-way ANOVA. Statistical significance was set at $\alpha < 0.05$.

3. Results

3.1. Study 1

3.1.1. Experiment 1

After the 24-h isolation period, some fish showed dark eyes and others pale eyes. Thus, fish were separated into two groups: low-ED (ED < 25% dark) and high-ED (ED ≥ 25% dark). We observed that these darkening patterns were maintained in each group throughout the day (Fig. 2). ED was not associated with body length because the mean (±SD) standard length of low-ED fish (5.8 ± 0.4 cm, $n = 8$) did not differ from that of high-ED fish (6.0 ± 0.3 cm, $n = 6$) (unpaired *t*-test, $t_{(12)} = 0.99$, $P = 0.34$).

3.1.2. Experiment 2

Both confinement and air exposure induced eye darkening (Fig. 3). However, the confined fish re-established pre-stressor ED 15 min after the stressor was removed, while the air-exposed fish sustained ED over time (Fig. 3, Test 1). Immediately before starting Test 2, the fish had re-established pre-stressor ED in both treatments. When the respective stressor was imposed again (6 h after the first stressor), the fish showed the same response profiles as in Test 1.

3.2. Study 2

In this study, both stressors led to ED, regardless of sex (Fig. 4). However, recovering the pre-stressor ED profile was sex-dependent for the air-exposure stressor but not for confinement. Males re-established the pre-stressor levels of ED in 15 min for both stressors, while females recovered the pre-stressor ED only after 3 h after being exposed to air (Fig. 4).

4. Discussion

This study expanded the estimation of ED as a response to non-social stress in fish. The non-social stressors increased ED, a

response that was more pronounced in adults than in juveniles but was not affected by the time of the day (morning vs. afternoon). In juveniles, ED indicated 2-min air exposure to be a more severe stressor than 30-min confinement. In adults, the recovery after each stressor was sex-dependent: females recovered ED to non-stress levels after air exposure later than males, suggesting that this stressor is more severe for females. In juveniles, ED varied widely among individuals not experimentally stressed, allowing for the classification of high- and low-ED profiles. As a whole, our findings indicate ED to be an important variable reflecting stress and other adjustment conditions. As this is an easy, non-invasive measure, it is useful for selecting fish before experimentation and for evaluating handled fish in aquaculture practices.

In some fish species, ED has been reported as an effect of social stress (Suter and Huntingford, 2002; Volpato et al., 2003; Vera Cruz and Brown, 2007; Miyai et al., 2011): the darker the eye, the lower the social rank. As social stress is a well-defined stressor, with subordinate fish being more stressed than dominant ones (Suter and Huntingford, 2002; Volpato et al., 2003), darkened eyes in subordinate fish and pale eyes in dominant ones might indicate the stress level.

Although the underlying physiological mechanisms linking stress and ED are not well understood (Sköld et al., 2013), our study corroborates ED as a stress response rather than just a socially mediated darkening response. Vera Cruz and Brown (2007) hypothesized that ED in Nile tilapia may follow a similar mechanism of darkening as the postorbital skin in lizards. In lizards, the postorbital skin is stimulated by the sympathetic activation of β2-andrenergic receptors via adrenal catecholamines (Korzan et al., 2000). Catecholaminergic pathways could also be involved in the control of the skin color of red porgy (*Pagrus pagrus*) subjected to air exposure (Van der Salm et al., 2006). Thus, this pathway may be used here because the mechanism involved can be quickly activated. Our non-social stressors, confinement and air exposure, increased ED in juvenile and adult Nile tilapia, independent of the time of day, life stage and adult sex, and the mechanism bears further study.

Unchanged ED in non-manipulated fish (Fig. 2) is also in line with ED increases (Figs. 3 and 4) as a response to stress. The clear low- and high-ED group differences detected in juveniles might be explained in terms of different fish reactions to adjustment to a novel environment, although all the fish experienced the same handling. All the fish were grouped in a tank and transferred for isolation into a glass aquarium for 24 h. These two ED responses are also in line with Pottinger et al.'s (1992) determination of low and high cortisol responders in trout.

Considering ED as an indicator of stress, the recovery times for ED after confinement and air exposure (Fig. 3) reveal different intensities for these stressors in Nile tilapia juveniles. Nile tilapia juveniles recovered ED far more quickly after confinement than after air exposure. This makes sense because, during confinement, fish respiration was not impaired, while air exposure restricted fish from swimming and impaired respiration. Reinforcing this conclusion, the 30-min confinement of Nile tilapia increased plasma cortisol by a factor of 3–4 (Moreira and Volpato, 2004), while air exposure for 2 min increased this hormone level by a factor of 13 (Barreto and Volpato, 2007) from its respective basal level.

Eye darkening is a response to confinement with a faster recovery than cortisol increase. Nile tilapia confined for 30 min re-established the basal level of cortisol after 35–75 min (Barreto and Volpato, 2004). In our experiment, Nile tilapia confined for 30 min had already re-established pre-stress ED after 15 min. Moreover, we recorded very rapid ED changes, usually in less than a minute or even in a few seconds. Because of the faster response and recovery times, we believe ED to be a more rapid indicator of stress (social and non-social) in fish (i.e., eye darkening appears faster and disappears sooner).

Because we do not know of how many males and females the juvenile samples were composed, it is difficult to attribute juvenile and adult different responses of ED to stressor as a developmental change, and more studies should be addressed to this topic. However, a dependence of recovery to stress on sex was found in adults (Fig. 4). Recovery time after stressor removal has been considered to indicate stressor severity in terms of uncontrolled and unpredictable situations (Koolhaas et al., 2011). De Kloet et al. (2008) suggested that the slow recovery of the stress hormonal response in mammals is caused by a delay in negative feedback mechanisms. A delay would explain the slower ED recovery of females after air exposure compared to confinement (Fig. 4) and might indicate that air exposure was more severe for females. The higher impact of air exposure compared to confinement in females, as reported above, confirms the dependence of ED upon the type (severity) of the stressor. Sex differences in stress responses have also been reported for other fish (e.g., Pottinger and Carrick, 2000; Kubokawa et al., 2001; Figueiredo-Fernandes et al., 2006; Øverli et al., 2006; Kling et al., 2008; Pottinger, 2010). In rats, females are also more responsive to stress than males (Kant et al., 1983; Aloisi et al., 1994). Accordingly, the slower ED recovery reported here in females indicates that these fish are more sensitive to some stressors (at least to air exposure) than males.

On the whole, we conclude that estimates of ED are a reliable indicator of stress in fish. The stronger the ED, the more stressed is the fish. As ED can even be assessed while handling fish, this measure may be useful for quickly evidencing stressful conditions for fish by just evaluating the appearance of their eyes. As we found in the first Study, Experiment 1, fish may be separated according to low or high ED for any reason; they may even differ in their response to holding conditions. This easy technique opens up new possibilities and facilitates stress studies in fish.

Moreover, we demonstrated that exposure to air is a severe stressor for Nile tilapia (at least for juveniles and females), and thus we recommend minimizing the time fish are exposed to air for handling. In fact, Brydges et al. (2009) suggested that handling fish with water-filled scoops, in contrast to traditional dip-nets, appears to be less stressful.

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References

Aloisi, A.M., Steenbergen, H.L., van de Poll, N.E., Farabollini, F., 1994. Sex-dependent effects of restraint on nociception and pituitary–adrenal hormones in the rat. Physiol. Behav. 55, 789–793.

Arends, R.J., Mancera, J.M., Muñoz, J.L., Wendelaar Bonga, S.E., Flik, G., 1999. The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. J. Endocrinol. 163, 149–157.

Barcellos, L.J.G., Woehl, V.M., Wassermann, G.F., Krieger, M.H., Quevedo, R.M., Lulhier, F., 2001. Plasma levels of cortisol and glucose in response to capture and tank transference in *Rhamdia quelen* (Quoy and Gaimard), a South American catfish. Aquacult. Res. 32, 123–125.

Barcellos, L.J.G., Kreutz, L.C., Quevedo, R.M., Fioreze, I., Rodrigues, L.B., Soso, A.B., Ritter, F., Conrad, J., Cericato, L., Fagundes, M., Lacerda, L.A., Terra, S., 2004. Hematological changes in jundiá (*Rhamdia quelen* Quoy and *Gaimard, Pimelodidae*)

provoked by usual aquaculture practices, with emphasis on immunosuppressive effects. Aquaculture 237, 229–236.
Barreto, R.E., Volpato, G.L., 2004. Caution for using ventilatory frequency as an indicator of stress in fish. Behav. Process. 66, 43–51.
Barreto, R.E., Volpato, G.L., 2006. Ventilatory frequency of Nile tilapia subjected to different stressors. J. Exp. Anim. Sci. 43, 189–196.
Barreto, R.E., Volpato, G.L., 2007. Evaluating feeding as unconditioned stimulus for conditioning of an endocrine effect in Nile tilapia. Physiol. Behav. 92, 867–872.
Bates, D., Maechler, M., Bolker, B., Walker, S., 2013. Lme4: linear mixed-effects models using Eigen and S4. R package version 1.0-5. http://CRAN.R-project.org/package=lme4
Brydges, N.M., Boulcott, P., Ellis, T., Braithwaite, V.A., 2009. Quantifying stress responses induced by different handling methods in three species of fish. Appl. Anim. Behav. Sci. 116, 295–301.
Cairns, M.T., Johnson, M.C., Talbot, A.T., Pemmasani, J.K., McNeill, R.E., Houeix, B., Sangrador-Vegas, A., Pottinger, T.G., 2008. A cDNA microarray assessment of gene expression in the liver of rainbow trout (*Oncorhynchus mykiss*) in response to a handling and confinement stressor. Comp. Biochem. Physiol. D 3, 51–66.
Castro, A.L.S., Gonçalves-de-Freitas, E., Volpato, G.L., Oliveira, C., 2009. Visual communication stimulates reproduction in Nile tilapia, *Oreochromis niloticus* (L.). Braz. J. Med. Biol. Res. 42, 368–374.
De Kloet, E.R., Karst, H., Joels, M., 2008. Corticosteroid hormones in the central stress response: quick-and-slow. Front. Neuroendocrinol. 29, 268–272.
Ellis, T., James, J.D., Stewart, C., Scott, A.P., 2004. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. J. Fish Biol. 65, 1233–1252.
Figueiredo-Fernandes, A., Fontaínhas-Fernandes, A., Peixoto, F., Rocha, E., Reis-Henriques, M.A., 2006. Effects of gender and temperature on oxidative stress enzymes in Nile tilapia *Oreochromis niloticus* exposed to paraquat. Pestic. Biochem. Physiol. 85, 97–103.
Garcia, L.E., Meier, A.H., 1973. Daily rhythms in concentration of plasma cortisol in male and female gulf killifish, *Fundulus grandis*. Biol. Bull. 144, 471–479.
Höglund, E., Balm, P.H.M., Winberg, S., 2000. Skin darkening, a potential social signal in subordinate arctic charr (*Salvelinus alpines*): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. J. Exp. Biol. 203, 1711–1721.
Kant, G.J., Lenox, R.H., Bunnell, B.N., Mougey, E.H., Pennington, L.L., Meyerhoff, J.L., 1983. Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. Psychoneuroendocrinology 8, 421–428.
Kittilsen, S., Ellis, T., Schjolden, J., Braastad, B.O., Øverli, Ø., 2009. Determining stress-responsiveness in family groups of Atlantic salmon (*Salmo salar*) using non-invasive measures. Aquaculture 298, 146–152.
Kling, P., Norman, A., Andersson, P.L., Norrgren, L., Förlin, L., 2008. Gender-specific proteomic responses in zebrafish liver following exposure to a selected mixture of brominated flame retardants. Ecotoxicol. Environ. Saf. 71, 319–327.
Koolhaas, J.M., Bartolomucci, A., Buwalda, B., de Boer, S.F., Flügge, G., Korte, S.M., Meerlo, P., Murison, R., Olivier, B., Palanza, P., Richter-Levin, G., Sgoifo, A., Steimer, T., Stiedl, O., van Dijk, G., Wöhr, M., Fuchs, E., 2011. Stress revisited: a critical evaluation of the stress concept. Neurosci. Biobehav. Rev. 35, 1291–1301.
Korzan, W.J., Summers, T.R., Ronan, P.J., Summers, C.H., 2000. Visible sympathetic activity as a social signal in *Anolis carolinensis*: changes in aggression and plasma catecholamines. Horm. Behav. 38, 193–199.
Kubokawa, K., Yoshioka, M., Iwata, M., 2001. Sex-specific cortisol and sex steroids responses in stressed sockeye salmon during spawning period. Zool. Sci. 18, 947–954.
Laursen, D.L., Olsén, H.L., Ruiz-Gomez, M.L., Winberg, S., Höglund, E., 2011. Behavioural responses to hypoxia provide a non-invasive method for distinguishing between stress coping styles in fish. Appl. Anim. Behav. Sci. 132, 211–216.
Lupica, S.J., Turner, J.W., 2009. Validation of enzyme-linked immunosorbent assay for measurement of faecal cortisol in fish. Aquacult. Res. 40, 437–441.
Mendonça, F.Z., Volpato, G.L., Costa-Ferreira, R.S., Gonçalves-de-Freitas, E., 2010. Substratum choice for nesting in male Nile tilapia *Oreochromis niloticus*. J. Fish Biol. 77, 1439–2144.
Miyai, C.A., Sanches, F.H.C., Costa, T.M., Colpo, K.D., Volpato, G.L., Barreto, R.E., 2011. 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 114, 335–339.
Moreira, P.S.A., Volpato, G.L., 2004. Conditioning of stress in Nile tilapia. J. Fish Biol. 64, 961–969.
Øverli, Ø., Sørensen, C., Nilsson, G.E., 2006. Behavioral indicators of stress-coping style in rainbow trout: do males and females react differently to novelty? Physiol. Behav. 87, 506–512.
Pottinger, T.G., 2010. A multivariate comparison of the stress response in three salmonid and three cyprinid species: evidence for inter-family differences. J. Fish Biol. 76, 601–621.
Pottinger, T.G., Carrick, T.R., 2000. Contrasting seasonal modulation of the stress response in male and female rainbow trout. J. Fish Biol. 56, 667–675.
Pottinger, T.G., Pickering, A.D., Hurley, M.A., 1992. Consistency in the stress response of individuals of two strains of rainbow trout, *Oncorhynchus mykiss*. Aquaculture 103, 275–279.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/
Sköld, H.N., Aspengren, S., Wallin, M., 2013. Rapid color change in fish and amphibians – function, regulation, and emerging applications. Pig. Cell Mel. Res. 26, 29–38.
Srivastava, A.K., Meier, A.H., 1972. Daily variation in concentration of cortisol in plasma in intact and hypophysectomized gulf killifish. Science 177, 185–186.
Suter, H.C., Huntingford, F.A., 2002. Eye colour in juvenile Atlantic salmon: effects of social status, aggression and foraging success. J. Fish Biol. 61, 606–614.
Van der Salm, A.L., Pavlidis, M., Flik, G., Wendelaar Bonga, S.E., 2006. The acute stress response of red porgy, *Pagrus pagrus*, kept on a red or white background. Gen. Comp. Endocrinol. 145, 247–253.
Vera Cruz, E.M., Brown, C.L., 2007. The influence of social status on the rate of growth, eye color pattern and insulin-like growth factor-I gene expression in Nile tilapia, *Oreochromis niloticus*. Horm. Behav. 51, 611–619.
Volpato, G.L., Luchiari, A.C., Duarte, C.R.A., Barreto, R.E., Ramanzini, G.C., 2003. Eye color as an indicator of social rank in the fish Nile tilapia. Braz. J. Med. Biol. Res. 36, 1659–1663.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.