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Short-term exposure to repeated chasing stress does not induce habituation in Senegalese sole, *Solea senegalensis*

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ABSTRACT

Animals can habituate to certain repeated stressors and reduce the physiological response that such stressor evoked initially. Studies related to stress habituation in fish are scarce and the available data differ depending on the species and on the type, duration and severity of the stressor. The main objective of this study was to investigate the stress response of juvenile Senegalese sole (*Solea senegalensis*) submitted to repeated chasing stress for 3 days previous to the experiment in order to evaluate the occurrence of habituation to those stress conditions in this fish species. Thus, five different experimental groups were evaluated: not stressed fish (control, C), fish stressed only on the experimental day (ST/naïve), and fish stressed on the experimental day and on the 3 previous days: during the day (ST/Dt), at night (ST/Nt) or both (ST/Dt + Nt). Parameters related to primary and secondary responses to chasing were evaluated in plasma, liver and brain. Chasing in ST/naïve group induced incremented values of plasma cortisol, glucose and lactate but no changes in catecholamine levels compared to controls. In trained fish, higher cortisol but decreased glucose, lactate and catecholamine levels were observed after stress compared to controls and to ST/naïve groups. In the liver, stress did not induce any changes with respect to controls whereas ST/Dt and ST/Dt + Nt showed lower values of glucose and glycogen than stressed naïve fish. In the brain, ST/naïve group presented no significant changes in serotonergic activity. However, incremented serotonergic activity was detected in fish previously trained. Furthermore, dopaminergic activity decreased in diurnal trained and nocturnal trained groups with respect to ST/naïve fish. *Crh* expression in hypothalamus was higher in ST/naïve fish but not in fish submitted to repeated stress compared to controls. In summary, it seems that there was no habituation to the repeated acute stress protocol in *Solea senegalensis* in terms of serotonergic activity and cortisol release during the physiological stress response. However, the decreased levels of plasma catecholamines and energy metabolites, and of the hypothalamic *crh* mRNA abundance and dopaminergic activity, indicate a modulation of the stress response in trained fish. Altogether, the results suggest that either the chasing stressor was too strong or the training period too short for the animals to habituate, indicating that repeated chasing within short periods should be avoided when manipulating fish in order to keep proper welfare conditions in this species.

1. Introduction

International concern about fish welfare is increasing and presently, the control of the stressors experienced by fish in aquaculture or research facilities is an important issue to be considered from both the legal and ethical points of view. Besides, uncontrolled stressors may also result in productivity losses in aquaculture. Nevertheless, different kind of stressors are inherent to the life in captivity and a research effort should be made to minimize the impact of different routine procedures on the health and welfare of the fish, especially when the stress suffered by the animals could become chronic.

It is known that animals, to certain extent, can habituate to relatively mild stressors in such a way that they present modified, most usually reduced physiological and behavioral responses to stressors that are repeated in time (Grissom and Bhatnagar, 2009; Nilsson et al., 2012; Rose and Rankin, 2001; Viblanc et al., 2012). Habituation has

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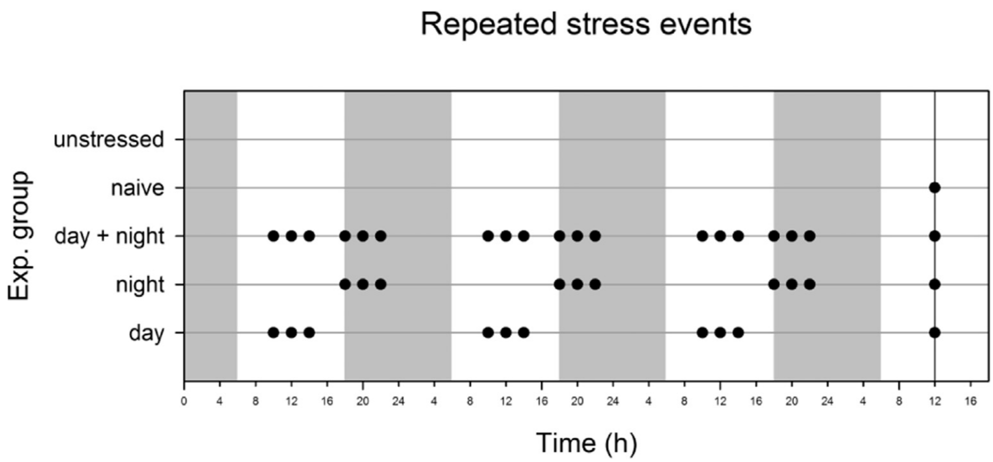

Fig. 1. Scheme of the experimental design showing the chasing protocols applied to each of the experimental groups.

been traditionally considered a nonassociative form of learning, and it is believed to have strong evolutionary utility for the conservation of energy and resources by dampening responses to stressors that are not life-threatening (Grissom and Bhatnagar, 2009; McCarty, 2016; Nesse et al., 2010). Information about the ability of fish to habituate to repeated stressors is very scarce since most studies dealing with stress exposure in fish usually comprise laboratory-based trials in which the stressors are applied only once and in which the fish have no or little previous experience with the stressor. Inherent stressors to fish rearing facilities include handling, changes in water quality, tank cleaning, sizing, vaccination/treatment administration, among others. The repeated exposure to the same or different stressor could result in a situation of chronic stress that can become maladaptive for the fish (Wendelaar Bonga, 1997). However, little is known about the ability of the fish to habituate to those stressors. Any kind of habituation or sensitization would be of relevance to the assessment of the actual welfare status of the fish in a facility, since habituation ability is part of fish natural robustness against stress exposure (Noakes and Jones, 2016). Some data suggest that fish are able to habituate to certain stressors (Johansson et al., 2016; Madaro et al., 2016b) but not to others (Barcellos et al., 2006; Koakoski et al., 2013). The physiological capacity for habituation in fish seems to depend on the type of stressor, the severity and the species involved. It has been shown for example that the hypothalamus-pituitary-interrenal (HPI) axis response in Atlantic salmon was reduced upon exposure to a repeated stressor (5-min chasing, twice a day) for 5 days (Madaro et al., 2016b).

The Senegalese sole, *Solea senegalensis* is a relatively new species in aquaculture and the knowledge regarding potential stressors and the species stress resilience is still limited when compared to other fish species such as salmonids. Recent research demonstrated that the HPI axis of *S. senegalensis* is sensitive to acute stressors such as handling, high stocking density, salinity changes, poor water quality or air exposure (Costas et al., 2008, 2011; Gesto et al., 2016; López-Olmeda et al., 2013; López-Patiño et al., 2013; Weber et al., 2012; Wunderink et al., 2011) but almost no information is available regarding the adaptability to repeated and chronic stressors in this species.

In the present study we evaluated the ability of the Senegalese sole to habituate to a chasing protocol after repeated exposure. Brain (serotonergic and dopaminergic activity in telencephalon and hypothalamus, hypothalamic *crh* and *crhbp* transcript levels), plasma stress markers (adrenaline, noradrenaline, cortisol, glucose and lactate levels) and liver metabolites (glucose, glycogen and lactate levels) were evaluated after a chasing protocol in fish that had been repeatedly exposed to the same stressor as well as in naïve fish. Since the stress sensitivity of the fish could vary depending on the moment of the day (López-Olmeda et al., 2013; Oliveira et al., 2013), repeated stress was applied during the day, during the night, or during day and night.

2. Materials and methods

2.1. Animals

Senegalese sole obtained from a commercial fish farm (Aquacria Piscícolas, S.A., Aveiro, Portugal) were kept under quarantine conditions for a 3-week period. The fish were then individually weighed (88.3 ± 1.5 g), measured and distributed (5 fish per tank) among 10 fiberglass rectangular tanks (0.5 m × 0.4 m × 0.3; 60 L) in a closed recirculation system. The system was supplied with filtered and heated (20.0 ± 1.0 °C) seawater (24 ppm) at a flow rate of 1.5 L·min^{-1}. The daily water renovation of the system was set up at 20% to help keeping good standards in water quality. An artificial photoperiod of 12 h light:12 h dark was established. Fish were fed by hand once daily (16.00 h) to satiety with a diet manufactured by Sparos, Portugal (proximate feed analysis was 57.9% crude protein, 0.8% fibre, 15.5% starch, 8.6% crude fat, and 8.5% ash; 20.3 MJ/kg of feed). The experiment was directed by trained scientists (following category C FELASA recommendations) and conducted according to the European guidelines on protection of animals used for scientific purposes (directive 2010/63/EU).

2.2. Experimental design

Following a 2-week acclimation period and during the 3 previous days of the experiment, fish of different tanks were stressed by chasing (forcing the fish to move with a net during 5 min) at different times and per duplicated tanks, according to the following experimental groups (Fig. 1): Diurnal trained fish (ST/Dt) composed by fish stressed during the day (at 10.00, 12.00 and 14.00 h); Nocturnal trained fish (ST/Nt) including fish stressed during the night (18.00, 20.00 and 22.00 h); and diurnal and nocturnal trained fish (ST/Dt + Nt) with fish stressed by chasing during whole day (10.00, 12.00, 14.00, 18.00, 20.00 and 22.00 h). On the day of the experiment, 24 h fasted fish from the different experimental tanks were stressed by chasing them with a dip net during 5 min. Moreover, two additional experimental groups were added: a control group (Control) consisting of undisturbed fish and a stressed group (ST/naïve) including naïve fish just exposed to the stressor the day of sampling. On the sampling day, all groups except the controls were submitted to a 5 min-chasing protocol. Thirty minutes after stress, fish were removed from their holding tanks, anesthetized (175 mg/L MS222) and sampled (5 fish per tank, in duplicate); netting was performed by experienced staff in a gentle way. Samples of blood, hypothalamus and telencephalon were removed firstly and then samples of liver were taken, lasting around 15 min for sampling each experimental tank. Tissue samples were immediately frozen on dry ice and stored at −80 °C until further analyses. Plasma samples were