Physiology & Behavior 139 (2015) 182–187

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

My stress, our stress: Blunted cortisol response to stress in isolated housed zebrafish

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HIGHLIGHTS

- Long-term isolated zebrafish presented a reduced cortisol response to stress.
- Stress response of isolated fish depends solely on their own stressor perception.
- The introduction of a stressed shoal in a resident non-stressed shoal induces stress in all fish.
- Stress response of grouped fish was augmented by chemical cues from the other members of the shoal.

ARTICLE INFO

Article history:
Received 20 May 2014
Received in revised form 21 October 2014
Accepted 10 November 2014
Available online 14 November 2014

Keywords:
Stress
Cortisol
HPI axis modulation
Shoaling behavior

ABSTRACT

Here, we show that individually housed zebrafish presented a reduced cortisol response to an acute stressor (persecution with a pen net for 120 s) compared to zebrafish housed in groups of 10. We hypothesized that the cortisol response to stress was reduced in individually housed zebrafish because they depend solely on their own perceptions of the stressor, whereas among grouped zebrafish, the stress response might be augmented by chemical and/or behavioral cues from the other members of the shoal. This hypothesis was based on previous described chemical communication of stress in fish as well on individual variation in stressor perception and potential individual differences in fish personality.

1. Introduction

In aquatic ecosystems, intraspecific and interspecific communications are essential for the balance of the communities that make up living things. It is well documented that fish can chemically communicate to the group (conspecifics) the occurrence of risk by chemicals (alarm substance) produced and stored in epidermal cells "club" and released into the water as a result of injury to the skin [1–4] and by the presence of blood in the water [5] or by odor [3,6].

However, studies on the chemical communication against indirect contact where disturbance substances are released without injury are still scarce. Barcellos et al. found that contact with the predator promotes a rise in cortisol in conspecifics by chemical and non-visual signals [7]. In this type of communication, both the recognition of risk and the ability to release the substance used in communication are essential [8,9].

This chemical communication to conspecifics is interpreted as an adaptive mechanism that promotes elevation of cortisol in anticipation of the threat, amplifying the consciousness of the animal in relation to the environment [1,4,7,9–12].

Zebrafish (*Danio rerio*) are studied in a wide range of research fields, such as genetics, embryology, metabolism, and oncology. They are also employed to study neurodegenerative diseases, and behavior and drug responses because of their genetic homology to humans [13–18].

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http://dx.doi.org/10.1016/j.physbeh.2014.11.035
0031-9384/

In the natural environment, zebrafish live in shoals and schools. These aggregations represent important forms of socialization that reduce predation risk [19], and isolation can be a source of stress and anxiety.

Laboratory studies do not reproduce the form of socialization in school, since many experimental protocols are performed with fish individually housed [3,18,20–24]. This situation may compromise the response to stress and interfere with results that explain physiological, neuroendocrine and behavioral mechanisms by lack of chemical signaling among conspecifics. Thus, the stress response, an important homeostatic mechanism may vary according to the form of housing fish.

Short-term isolation is considered a form of stress because cortisol levels have been observed to increase under this condition after transfer to a new tank [23]. Moreover, Parker et al. [22] demonstrated that isolated fish exhibit less anxiety after long-term isolation and that isolation acts as a CNS depressor. Here we evaluate whether the responses to acute stressors differed between grouped and isolated fish over a long-term period (15 to 30 days) and the introduction of stressed fish stress promote a school of fish residents.

2. Materials and methods

2.1. Ethical note

This study was approved by the Ethics Commission for Animal Use (CEUA) of Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #9/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Subjects

A stock population of 876 mixed-sex, adult, wild-type zebrafish (*D. rerio*) of the short-fin (SF) strain was housed in two tanks with constant aeration and biological filtering under a natural photoperiod (approximately 14 h light: 10 h dark). The water was maintained under the following conditions: 26 ± 2 °C; pH 7.0 ± 0.25; dissolved oxygen at 6.5 ± 0.4 mg/L; total ammonia at < 0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22 mg/L $CaCO_3$.

2.3. Experimental protocols

Experiment 1. Acute stress challenge test of isolated and grouped zebrafish

We divided this experiment into two pre-treatment durations: 15 days and 30 days. Individuals were maintained in either isolation or groups of 10 fish prior to applying the acute stress challenge test (persecution with a pen net for 120 s). In each phase, fish from the stock population were distributed into 40 plastic tanks (12 L): 10 containing individually isolated fish without a stress challenge (control), 10 with individually isolated fish subject to an acute stress challenge, 10 with groups of fish without a stress challenge (control) and 10 with groups of fish submitted to an acute stress challenge. Cortisol levels were sampled 15 min after the stress challenge to obtain the peak cortisol concentrations [25]. One fish was sampled at random from each group tank for cortisol level measurement. During the pre-treatment maintenance periods (15 or 30 days), the water quality was monitored and maintained at conditions identical to those in the stock population.

Experiment 2. Transfer of individually isolated or group zebrafish to a novel environment

Fish were distributed into 40 plastic tanks (12 L): 10 tanks with individually isolated fish without transfer to a novel environment (control), 10 tanks with individual fish isolated for 15 days before transfer to a novel environment, 10 tanks with groups of fish without transfer (control) and 10 tanks with individual fish isolated for 15 days before transfer to a novel environment. For the transfer treatments, we killed the fish 15 min after transfer to investigate the effects of the transfer treatment and exploration of the novel environment in individually isolated and grouped fish. One fish was randomly sampled from each group tank for cortisol level measurement.

Experiment 3. Effect of introducing stressed fish into a resident zebrafish shoal

Fish were initially separated into two groups according to fish size: small (0.5 ± 0.05 g) and large (1.0 ± 0.05 g). A total of 72 small fish were categorized as "resident zebrafish (RZf)," and 144 large zebrafish were categorized as "introduced zebrafish (IZf)." The IZf were then distributed into two subgroups of 72 individuals, with each subgroup housed in an 80-L glass aquarium.

The experiment was performed using 24 plastic tanks (12 L). Half of the tanks were stocked with a shoal of six RZf. The remaining 12 tanks received no fish. We sought to investigate the effect of introducing stressed IZf on the cortisol profiles of the RZf. For this purpose, four experimental groups were examined. In the first group, we introduced un-stressed IZf into empty tanks. In the second group, we introduced un-stressed IZf into tanks containing shoals of six RZf. The third group involved introducing stressed IZf into empty tanks, and in the fourth group, we introduced stressed IZf into tanks containing shoals of six RZf. The acute stress was identical to that of the first experiment (persecution with a pen net), and the IZf were transferred immediately after applying the stressor. The IZf and RZf were sampled for cortisol determination 30 min after IZf introduction (i.e., at the peak cortisol moment, [12]; see scheme in Fig. 3).

Experiment 4. Effect of the introduction of individual fish stressed in a shoal of resident zebrafish

As in Experiment 3, fish were initially separated into two groups according to size: small (0.5 ± 0.05 g) and large (1.0 ± 0.05 g). A total of 42 small fish were categorized as "resident zebrafish" (RZf), and 12 large zebrafish were categorized as "introduced zebrafish" (IZf). The experiment was performed using 19 plastic tanks (12 L), 12 tanks with a shoal of six RZf and 7 with IZf, which 6 tanks with one fish (SIso IZf) and one tank with a shoal of six IZf (SG IZf). We sought to investigate the effect of introducing stressed individual IZf on the cortisol profiles of the RZf. For this purpose, two experimental groups were examined. In the first group, we introduced SIso IZf into empty tanks and in the second group, we introduced SG IZf into tanks containing shoals of six RZf. The acute stress was identical to that of the first experiment (persecution with a pen net), and the IZf were transferred immediately after applying the stressor. The IZf and RZf were sampled for cortisol determination 30 min after IZf introduction (i.e., at the peak cortisol moment, [12]; see scheme in Fig. 4).

2.4. General procedures

2.4.1. Cortisol extraction and analysis

Fish were captured and immediately frozen in liquid nitrogen for 10–30 s, followed by storage at −20 °C until cortisol extraction. Whole-body cortisol was extracted following Oliveira et al. [26]. Measurement accuracy was evaluated by calculating the levels recovered from samples spiked with known amounts of cortisol (50, 25 and 12.5 ng/mL). The mean detection of spiked samples was 94.3%. All cortisol values were adjusted for recovery using the following equation: cortisol value = measured value × 1.0604.

Tissue samples were resuspended in 1 mL PBS, and whole-body cortisol levels were measured in duplicate samples of each extract using a commercially available enzyme-linked immunosorbent assay kit (EIAgen™ CORTISOL test, BioChem ImmunoSystems). This kit was