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Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress

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HIGHLIGHTS

- UCS increased aggression behavior in males, but not in females.
- Females displayed more aggressive behavior at baseline than control males.
- Stressed females showed increased locomotion compared to stressed and control males.
- Stressed males had higher cortisol levels compared to all other groups.
- No differences in cortisol levels were found between stressed and control females.

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ABSTRACT

Chronic stress may cause physical, behavioral and neuropsychiatric changes, affecting the health condition of an individual. Aggression is a universal behavior with great relevance on human and animal social systems. Despite studies showing the influence of chronic stress on aggression, the effects of unpredictable chronic stress (UCS) on aggressive behavior in male and female zebrafish remain unknown. Thus, the aim of this study was to evaluate the effects of UCS on the aggressive behavior and cortisol levels in adult zebrafish of both sexes. Our results showed that UCS increased aggression in males, but not in females, which displayed more aggressive behavior at baseline than control males. Increased whole-body cortisol levels were observed in stressed males; however, no differences were found between female groups. In conclusion, we reported for the first time gender differences on behavioral parameters and cortisol levels in response to UCS in zebrafish. These results highlight the relevance of studying behavioral and physiological parameters in both sexes separately.

1. Introduction

Stress is characterized as a process whereby the organism reacts to the threat or stressor by physiological and behavioral changes that, largely, affect the health condition of an individual [5,18]. Stress can induce behavioral and metabolic changes, which may result in somatic and mental disorders [5]. Chronic stress may cause anxiety, depression, executive and/or cognitive dysfunction, atherosclerotic cardiovascular disease, metabolic disorders, neurovascular degenerative disease, osteopenia, osteoporosis and sleep disorders [7,20]. Thus, excessive stressors and/or chronic exposure can harm a number of essential physiological functions, such as metabolism, growth, reproduction and immune competence, as well as the development of personality and aggressive behavior [17,18,23,30,39].

Aggression is a universal behavior which influences both human and animal social systems [10,11,16,36,37]. This behavior typically occurs in the competition for resources, such as food, mate selection and nesting sites, and is important for protecting offspring and establishing territories and dominance hierarchies [1,10,11,19,24,36–38]. However, intensified aggression can cause harm to an individual and others [16,25], meaning that this behavior must be carefully controlled [16]. Thus, aggression is a complex set of adaptive behaviors [25,27] that

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may have important effects on both the success and evolutionary fitness of individuals [24,28,36].

The zebrafish is a social fish that forms dominance hierarchies in both sexes [32,38]. In this species, aggression is commonly used to monopolize resources like food and mates, occupy nesting sites and protect their status [27,32]. Both males and females may exhibit aggressive behavior, including erection of dorsal, pectoral, caudal and anal fins, dancing or undulating movements, attacks towards the opponent and bites [12,19,38]. They can also display visible color changes after the establishment of dominance, during the fight or during spawning by getting darker and/or more colored [11,12]. This model organism has been used to evaluate the effects of unpredictable chronic stress (UCS) on behavioral, molecular and physiological parameters [33]; however, the effects of UCS on aggressive behavior in male and female zebrafish remain unknown.

Thus, here we evaluate the effects of UCS on aggressive behavior and whole-body cortisol of male and female zebrafish.

2. Materials and Methods

2.1. Animals

A total of 128 adult male ($n = 64$) and female ($n = 64$) 6–8 months old wild-type zebrafish (*Danio rerio*) were obtained from a local commercial supplier (Red Fish, Porto Alegre, Brazil) and acclimated for at least 2 weeks in the experimental room before the experiments. The animals were housed in a 30-L thermostated aquarium filled with unchlorinated water constantly aerated at a targeted temperature of 26 ± 2 °C. Fish were kept under a 14-h light/10-h dark cycle photoperiod and fed twice a day with commercial flake fish food (Alcon BASIC®, Alcon, Brazil). All protocols were approved by the Institutional Animal Care Committee (14/00419, CEUA-PUCRS).

2.2. Unpredictable chronic stress (UCS)

Males and females were divided in two groups: control and stressed. The control and stressed male and female groups were divided in two tanks for each group to minimize a possible tank effect. UCS was performed according to the protocol previously established by Piato et al. [33]. Following a two-week habituation period, the stressed group was subjected twice a day to one of the following stressors during 7 days (Table 1): crowding of 10 animals for 50 min in a 250 mL beaker; social isolation, maintaining animals alone for 45 min in a 250 mL beaker; tank change, three consecutive times; cooling tank water up to 23 °C for 30 min; low water level on housing tanks until animals' dorsal body wall was exposed for 2 min; heating tank water up to 33 °C for 30 min; and chasing animals for 8 min with a net. To prevent habituation and maintain unpredictability, time and sequence of stressors presentation were changed daily. Except during heating and cooling stress, the temperature was controlled during each stressor. Despite the stressful conditions intermittently presented to the fish, no extreme suffering or deaths were observed. A control group (non-stressed) remained in the same room during the equivalent 7-day period.

2.3. Aggression

Twenty-four hours after the UCS, control and stressed fish were subjected to mirror-induced aggression (MIA) protocol previously described by Gerlai [11]. Each fish was individually netted into a small experimental tank (30 × 15 × 10 cm length × height × width). A mirror was placed inclined at 22.5 degrees to the back wall of the tank so that the left vertical edge of the mirror was touching the side of the tank and the right edge was further away. After 30 s for habituation, experimental fish was recorded for 11 min and later analyzed by the ANY-Maze tracking software (Stoelting Co., USA). For the data analysis, the tank was divided in four equal sections and the amount of time spent in the segment closer to the mirror was quantified (Segment 1). In addition, locomotor parameters such as total distance traveled (m), mean speed (m/s) and number of crossings in the tank were evaluated.

2.4. Cortisol extraction and analysis

After aggressive interaction with a mirror, fish were captured and immediately frozen in liquid nitrogen for 10–30 s, followed by storage at −20 °C until cortisol extraction. A pool of 2 fish was used per sample. Whole-body cortisol was extracted using the method previously described by Oliveira et al. [29]. Fish were weighed, minced and placed in a disposable stomacher bag with 2-mL phosphate buffered saline (PBS, pH 7.4) for 6 min. The contents were then transferred to a 10-mL screw top disposable test tube, to which 5 mL of laboratory grade ethyl ether was added. The tube was vortexed for 1 min and centrifuged for 10 min at 3000 rpm, after which the sample was immediately frozen in liquid nitrogen. The unfrozen portion (ethyl ether containing cortisol) was decanted and transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol, which was stored at −20 °C. 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 extracts were re-suspended 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). The accuracy was tested by repeating the assay 12 times using seven randomly chosen samples on the same plate and calculating the intra-assay coefficient of variation (CV). The reproducibility was tested by assaying the same samples on different plates and calculating the inter-assay CV. To test for linearity and parallelism, the tissue samples were subjected to serial dilutions in the buffer provided with the kit. A strong positive correlation between the curves was observed ($R^2 = 0.8918$), and the samples yielded low inter- and intra-assay CV values (7–10% and 5–9%, respectively).

2.5. Statistical analysis

The data are expressed as mean ± standard error of mean (S.E.M.). Results were analyzed by two-way ANOVA followed by Tukey's post

Table 1
Procedure of the unpredictable chronic stress protocol in males and females zebrafish.

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
11:50 am	08:00 am	10:30 am	09:15 am	08:45 am	9:30 am	11:30 am
Crowding	Tank change	Low water level	Heating	Social isolation	Cooling	Chasing
4:00 pm	3:30 pm	1:30 pm	5:30 pm	2:30 pm	3:00 pm	4:30 pm
Social isolation	Cooling	Crowding	Chasing	Tank change	Low water level	Heating