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Effects of carbamazepine on cortisol levels and behavioral responses to stress in the fish *Jenynsia multidentata*

Emilia Calcagno [a], Patricia Durando [b,*], M. Eugenia Valdés [c], Liliana Franchioni [d], María de los Ángeles Bistoni [e,*]

[a] Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, X2500HUA Córdoba, Argentina
[b] Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Ing. Agr. Félix Aldo Marrone 746, X2500HUA Córdoba, Argentina
[c] Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), CONICET-UNC, Medina Allende y Haya de la Torre, Ciudad Universitaria, X2500HUA Córdoba, Argentina
[d] Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Medina Allende y Haya de la Torre, Ciudad Universitaria, X2500HUA Córdoba, Argentina
[e] IDEA-Instituto de Diversidad y Ecología Animal, CONICET, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, X2500HUA Córdoba, Argentina

HIGHLIGHTS

- Restraint stress disrupts scototaxis and increases cortisol in *Jenynsia multidentata*
- In the light/dark test, CBZ decreases cortisol in fish under restraint stress
- CBZ at its lowest concentration reduces behavioral endpoints in stressed fish
- In the shoaling test, neither CBZ nor stress modify shoaling behavior or cortisol
- CBZ induces diverse responses depending on test, concentration or endpoint evaluated

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ABSTRACT

Carbamazepine (CBZ) is an anticonvulsant drug, prescribed worldwide for the treatment of epilepsy, bipolar disorder and trigeminal neuralgia, which has been frequently detected in aquatic environments. The objective of this study was to analyze if CBZ modifies scototaxis and shoaling behaviors and/or whole-body cortisol levels of the one-sided livebearing fish *Jenynsia multidentata* under stress condition. Female adults of *J. multidentata* were exposed to 0, 10, 50 and 200 μg CBZ/L during 14 days. After CBZ exposure, fish were subjected to restraint stress during 15 min. Control animals were not exposed to CBZ or stress. In the light/dark preference test (scototaxis), the individuals under acute restraint stress (without CBZ) exhibited a significant increase in the mean speed and in the time spent both in the light compartment and in the bottom of the tank with respect to controls. They also showed a tendency to stay longer frozen in the light compartment. Fish exposed to 10 and 50 μg CBZ/L showed a significant reduction in mean speed compared to stressed fish without CBZ. A reduction in the time spent in the bottom of the tank was also observed in fish exposed to 10 μg CBZ/L. Fish exposed to 200 μg CBZ/L showed a decreasing tendency in all behavioral endpoints (time spent in the light compartment, mean speed, time spent at the bottom and freezing) in comparison to stressed fish not exposed to CBZ. Considering whole-body cortisol results, fish under acute restraint stress (without CBZ) significantly increased their hormone levels with respect to the control group, while fish exposed to CBZ and acute restraint stress, significantly decreased their whole-body cortisol levels. There were no significant changes in shoaling behavior due to either stress or CBZ exposure and no significant differences in whole-body cortisol levels between experimental groups. Considering that the light/dark and shoaling tests measure different stress response behaviors regulated by different neuroendocrine systems, these results could indicate that CBZ has a differential effect on fish behavioral stress response and cortisol levels, depending on the behavioral test used and stressor applied.

1. Introduction

Research in the last decade has demonstrated that water pollution with psychiatric drugs, such as anxiolytics, sedatives, hypnotics and antidepressants, disturbs fish behavior as these directly affect the central nervous system and disrupt neuroendocrine signaling

* Corresponding authors.
E-mail addresses: pdurando@agro.unc.edu.ar (P. Durando), mbistoni@com.uncor.edu (M.Á. Bistoni).

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[1]. Carbamazepine (5H-dibenzo[*b*,*f*]azepine-5-carboxamide, CBZ) is one of these neuroactive compounds, frequently detected in surface water courses polluted by wastewater treatment plant effluents [2–5]. CBZ is an anticonvulsant drug prescribed worldwide for the treatment of epilepsy, bipolar disorder and trigeminal neuralgia [6,7]. It affects central nervous system function by various probable mechanisms, such as decreasing neuronal excitability by rapid inactivation of voltage-gated sodium channels [8], modulation of neurotransmitter action [9] and decreasing arachidonic acid turnover [10]. In the human body, most of this drug is metabolized and excreted as a mixture of the unchanged parent compound, metabolites and conjugates [7,11].

In terms of ecological impact, CBZ has low elimination rates by wastewater treatment plant processes (<50%) and slow degradation in aquatic environments (half-life: 82 ± 11 days) [5,12]. In Argentina, CBZ has been detected in sewage and wastewater treatment plant effluents from six locations in Córdoba and Buenos Aires provinces, within the range of 0.2–2.3 μg/L [13]. In the river Suquía (Córdoba, Argentina), CBZ was measured within the range of 15–113 ng/L in sites located downstream of the Córdoba City wastewater treatment plant outflow [4].

The presence of CBZ in aquatic environments is of serious concern since it is a lipophilic and persistent drug that remains bioactive in the environment, with the necessary properties to bioaccumulate and provoke effects in aquatic or terrestrial ecosystems [14]. CBZ bioaccumulation has been detected in muscle, liver, brain, plasma and other tissues from different fish species exposed under both laboratory and field conditions [4,15]. In channel catfish (*Ictalurus punctatus*) exposed 14 days to 83 μg CBZ/L, Garcia et al. [15] reported CBZ accumulation in brain, plasma and muscle. In mosquitofish (*Gambusia affinis*), CBZ was detected in the entire body of fish exposed to 10 and 100 μg CBZ/L during 96 h [4]. Rainbow trout (*Oncorhynchus mykiss*) exposed to higher levels of CBZ showed inhibition of oxidative stress enzymes in liver and muscle [16,17].

Human neuroactive compounds affect fish behavior, since their target receptors/molecules are highly conserved during evolution [18]. Thus, CBZ exposure decreased swimming speed and inhibited feeding behavior of Japanese medaka (*Oryzias latipes*) [19]. Brandão et al. [18] found a significant positive correlation between CBZ concentration and time spent in motion and a negative correlation with time spent in the black compartment in sunfish (*Lepomis gibbosus*) exposed to 62.5–1000 μg CBZ/L, in the light/dark preference test.

Special attention should be paid to behaviors originated in response to stressful conditions because they contribute to appropriate adaptation to environmental changes [20,21]. In teleost fish, several behavioral parameters have been identified through different tasks as indicators of stress, including scototaxis, defined as "the natural preference for dark (or avoidance of bright) lighting/environment" [22]. This behavior reflects the struggle between the natural exploratory motivation (i.e., activity in the white compartment) and the animal's preference for protected areas (e.g., black substrata) in a process of crypsis to avoid predators [23]. Scototaxis can be quantified in the dark–light box tests by assessing behavioral endpoints, such as time spent in the light or dark compartments and distance traveled [22]. Also in this task, it is possible to measure the time spent in the bottom of the tank and freezing as stress responsive behaviors [22]. Several lines of evidence indicate that fish under stress show a significant decrease in exploration, longer latency to reach the top, longer and more frequent freezing and elevated erratic movements [24–28].

Shoaling behavior in social fish can be altered by stress [22,29]. Shoaling is defined as the formation of a relatively non-polarized group of fish, held together by social pressures (i.e., not by individual attraction to an external stimulus) [22]. Stressful conditions cause the shoal to 'tighten' (the fish swim closer together) [22]. In zebrafish (*Danio rerio*), acute restraint stress under laboratory conditions induced an enhancement in mean and maximum swimming speed and in whole-body cortisol levels [30]. A stress-induced increase in cortisol concentration was likewise detected in wild-caught bluegill sunfish (*Lepomis macrochirus*) subjected to air exposure during 3 min [31].

The aim of this study was to determine if CBZ modifies whole body cortisol levels, scototaxis and/or the shoaling behavior of the one-sided livebearing fish (*Jenynsia multidentata*) under stress conditions. This species is widely distributed in the neotropics [32] and it has been used as an excellent model in both laboratory and field studies [43–45], mainly because of its ability to adapt to a wide variety of environments, including poor water quality conditions [32]. Female adults of *J. multidentata* were selected given their usefulness as a bioindicator of water pollution [33–35] and also their inexpensive maintenance and housing.

2. Materials and methods

2.1. Animals and housing

Female adults of *J. multidentata* (total weight: 0.5 ± 0.2 g; standard length: 29 ± 2 mm) were collected with a net at a reference site (Yuspe River, 64°32′W; 31°17′S, Córdoba, Argentina) where no pharmaceuticals were detected in a previous study [4]. They were kept in aerated 20 L water tanks (n = 40 per tank) at least 7 days as an acclimation period (pre-treatment period) at constant temperature (21 ± 1 °C) and photoperiod (12:12 hour light/dark). Fish were fed ad libitum twice a day with commercial fish pellets (TetraMin, USA). The specimens used in all experiments were of similar length to avoid variations in basal cortisol levels and behavior due to size [36] and thus minimize variability of response to the test material [37].

All experimental procedures were approved by the local Committee on Animal Bioethics and Welfare of the Facultad de Ciencias Exactas, Físicas y Naturales, of the Universidad Nacional de Córdoba.

2.2. Chemicals and solutions

Carbamazepine was purchased from Sigma-Aldrich (Buenos Aires, Argentina, purity ≥ 98%). Individual stock solution at 1 mg/mL was prepared in methanol (HPLC grade) and stored in the dark at −20 °C until use. Carbamazepine solutions for exposure experiments were prepared by proper dilution of stock solution in dechlorinated water at the moment of exposure. Calibration plots were obtained from fresh working solutions, prepared daily by proper dilution of stock solution in the initial composition of HPLC mobile phase.

2.3. Experimental design

2.3.1. Exposure conditions

After acclimation, a total of 100 fish (n = 50 for light/dark box test and n = 50 for shoaling test) were tested, evaluating their responses to 5 treatments: control (0 μg CBZ/L and without stress); 0CBZ + S (0 μg CBZ/L and stress); 10CBZ + S (10 μg CBZ/L and stress); 50CBZ + S (50 μg CBZ/L and stress); 200CBZ + S (200 μg CBZ/L and stress). These concentrations were chosen according to previous CBZ bioaccumulation and effects studies [4,15,18]. The control group (0 μg CBZ/L and without stress) enabled the behavioral and endocrine effects of stress to be contrasted with the group not exposed to CBZ but stressed (0CBZ + S). The 0CBZ + S group enabled the contrasting of those effects in stressed fish exposed to different CBZ concentrations (10CBZ + S, 50CBZ + S and 200CBZ + S).

For each behavioral test, two replicate aquaria were used for each treatment (n = 5 fish for each replicate) (Fig. 1). Fish were exposed during 14 days in tanks where the test organism loading did not exceed 1 g/L following the conventional protocols [37]. The test organism loading should not be exceeded, to avoid the concentration of dissolved oxygen decreases below acceptable limits and crowding stress of the test animals. CBZ stability in tanks with dechlorinated water has been

previously evaluated, with recovery percentages of above 85% after 48 h of application [4]. Therefore, during the exposure experiment, the water in tanks (with or without CBZ) was renewed every 48 h. Water samples were taken from each aquarium solution at time 0 and 48 h for CBZ concentration analysis by high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC-HRMS), according to Valdés et al. [4]. Briefly, water samples were filtered (0.45 μm cellulose filters) and concentrated by off-line solid phase extraction (SPE). Strata-X® cartridges (500 mg/6 mL) were conditioned with 10 mL methanol, followed by 10 mL ultrapure water at 1 mL/min. Samples were percolated at 5 mL/min. Finally, cartridges were rinsed with 6 mL ultrapure water and air-dried for 20 min under vacuum. Elution was carried out with 10 mL HPLC grade methanol. Eluates were completely dried under a gentle stream of nitrogen, reconstituted in methanol: ultrapure water 15:85 and transferred to HPLC vials after syringe filtration by 0.22 μm polyvinylidene fluoride (PVDF) filters. The analysis of CBZ in the extracts was accomplished using a high performance liquid chromatograph system coupled to a high resolution mass spectrometer with quadrupole time-of-flight analyzer, using an electrospray ionization source operated in positive mode (HPLC–ESI–qTOF, Agilent Series 1200 LC System – MicrOTOF Q II Bruker Daltonics, USA). CBZ was separated on a reversed phase analytical column (3.0 × 50 mm, 1.8 μm, Zorbax Eclipse C18 XDB, Agilent, USA), heated to 40 °C with a gradient elution program using a binary mobile phase (solvent A: 0.1% formic acid, solvent B: methanol). The injection volume was 40 μL and the flow-rate was kept at 0.2 mL/min. The qTOF was operated in full-scan mode. An internal calibrant (10 mM sodium formate solution) was delivered by an external syringe pump during 1 min in each run for post-run mass internal calibration. Recorded data were processed with the Compass 1.3 software (Bruker Daltonics, USA). Identification of CBZ was based on accurate mass measurement of the base ion ($[M + H]^+$: 237.1028 *m/z*) and a product ion ($[C_{14}H_{12}N]^+$: 194.097 *m/z*) with a mass error < 5 ppm, and comparison of the retention time with a standard (±2%). External standard calibration was used to determine precise concentrations in test samples. The limit of detection (LOD) and limit of quantification (LOQ) for the method were 0.2 and 0.6 μg/L, respectively.

2.3.2. Acute restraint stress procedure

Immediately after CBZ exposure, fish were subjected to a restraint stress procedure, according to Champagne et al. [28], consisting of keeping each fish for 15 min in a small net immersed in clean water to limit locomotion without restricting the opercular movements. This time-period was chosen based on previous works in *D. rerio* [28] and *O. mykiss* [38], determining that motion restraint stress induced a cortisol peak at 15 min from its application.

The following experimental groups were established (Fig. 1).

2.4. Behavioral assessment

Immediately after the stress procedure, behavioral tests were performed. Each fish was recorded individually for 10 min in a glass tank (32 × 25 × 8.5 cm, length × height × width, water column was kept at 15 cm) with clean water, located in an isolated room to avoid disturbance. Each tank was illuminated by environmental light (located on the ceiling of the filming room) to achieve uniform illumination, since the intensity of light can alter the fish's preference for light or dark areas [23]. Filming was carried out in the same time slot (from 9:00 to 12:00 h) to avoid fluctuations due to natural cortisol circadian rhythm [36]. Each behavioral session was filmed by a digital camera (Sony W730) placed in front of the fish tank. The videos were analyzed with ANY-maze (ANY-maze®, Stoelting CO, USA). After each test, water in the tanks was renewed to eliminate chemosensory and stress factors that may have been secreted during testing [25,28]. In the light/dark preference test, several behaviors (scototaxis, mean speed, freezing and time spent on the bottom of the tank) were evaluated and shoaling behavior was analyzed in the shoaling test. Light/dark tests and shoaling tests were conducted separately on different days.

2.4.1. Light/dark preference test

The apparatus consisted of a tank divided equally into one black and one white compartment [39] (Fig. 2). For the dark compartment, the inner walls, bottom and the surface of the tank were covered with opaque black plastic. The other compartment was covered with light, opaque plastic to prevent reflection of the fish in the glass and prevent animals from displaying social behavior in relation to their own reflections [23]. There was no physical barrier between the two compartments and fish moved freely between them. Each individual was initially placed in the clear compartment in order to enable the detection and monitoring of fish by the software. The fish were not previously acclimatized to the test tank.

The position of the digital camera in front of the light/dark apparatus meant the fish could not be recorded in the dark area, so all behavioral variables were analyzed in the light area. At the beginning of each test, the individual was placed in the middle of the light compartment.

Measured variables included: 1. Time spent in the light area (in s); 2. mean speed (in cm/s): total distance traveled by the fish in the light compartment divided by the time that fish spent in this area; 3. freezing duration (in s): amount of time that fish spent frozen with respect to total time spent in the light area. Freezing behavior was considered as complete or partial immobility, with the animal performing only opercular beats, oculomotor responses and those fin movements

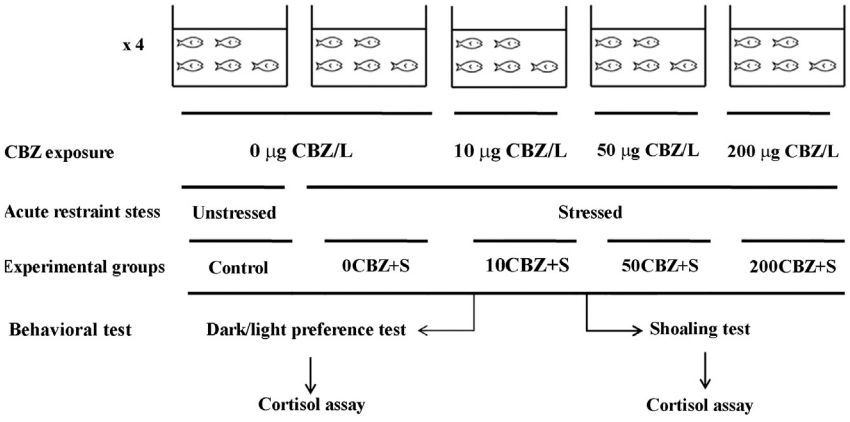

Fig. 1. Schematic representation of the experimental design. For each behavioral test, two replicate aquaria were used (n = 5 fish per replicate tank). Control: fish exposed to 0 μg CBZ/L and unstressed; 0CBZ + S: fish exposed to 0 μg CBZ/L and acute restraint stress; 10CBZ + S: fish exposed to 10 μg CBZ/L and acute restraint stress; 50CBZ + S: fish exposed to 50 μg CBZ/L and acute restraint stress; 200CBZ + S: fish exposed to 200 μg CBZ/L and acute restraint stress.

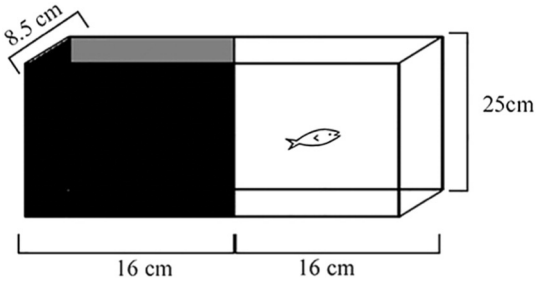

Fig. 2. Schematic representation of the light/dark box test apparatus.

needed to maintain a dorsal posture [23]; 4. time spent on the bottom of the tank (in s): time that the fish remained in the bottom half of the tank. These variables were proposed as predictive markers of stress-response [23,40].

2.4.2. Shoaling test

The apparatus consisted of two tanks: a larger rectangular tank (with same dimensions listed above) placed next to a smaller tank (length: 15 cm, height: 20 cm and width: 8.5 cm) (Fig. 3). The test fish was introduced into the larger tank while a group of 5 fish of the same strain (shoal) was put into the smaller one. Both tanks were in contact on one side to let the test fish see the shoal [41]. The test fish was acclimatized together with the shoal fish in the pre-treatment period.

The larger tank was covered with opaque plastic, leaving the contact zone between tanks without covering as well as the front wall to allow filming. The rectangle defined by the water column (length: 32 cm, height: 15 cm) was divided into two areas: close proximity zone (the area closest to the shoal stimulus) and a neutral zone. The latter was subdivided into two sections: neutral zone close to the shoal (in the middle of the tank) and neutral zone far from the shoal. At the beginning of the assay, the test fish was placed in the neutral zone close to the shoal. In this test, the time (in s) that the fish remained in the different zones, near or far from the shoal, was recorded.

2.5. Whole-body cortisol measurements

After behavioral tests, fish were captured and immediately frozen in liquid nitrogen for 10–30 s, and stored at −80 °C until cortisol extraction [24]. Cortisol extraction was performed following the procedure proposed by Cachat et al. [25]. Briefly, each fish was weighed and its body was cut on ice into smaller pieces to facilitate homogenization. Individual body samples from experimental groups were homogenized in 3 mL ice-cold phosphate-buffered saline (PBS) solution using a tissue homogenizer (ULTRATURRAX T18, IKA, Germany) set at 20,000 rpm during 1 min. Samples were transferred to glass tubes and cortisol was extracted three times with 3 mL of diethyl ether (Cicarelli, Argentina). After solvent evaporation, extracts were reconstituted in 1 mL PBS and quantified by electrochemiluminescence immunoassay (ECLIA) (Cobas analyzer, Roche) [42]. Cortisol analyses were carried out according to the manufacturer's instructions and standard curves were run in each assay. Pilot assays using three different dilutions of ten samples (two samples per treatment) were run to establish the appropriate working dilution. The slope of this dilution series was parallel to the standard curve of the kit. Intra- and inter-assay tests were also performed and inter-assay coefficients of variation did not exceed 14.5%. Whole body cortisol levels were considered as ng cortisol/g body weight (wet weight).

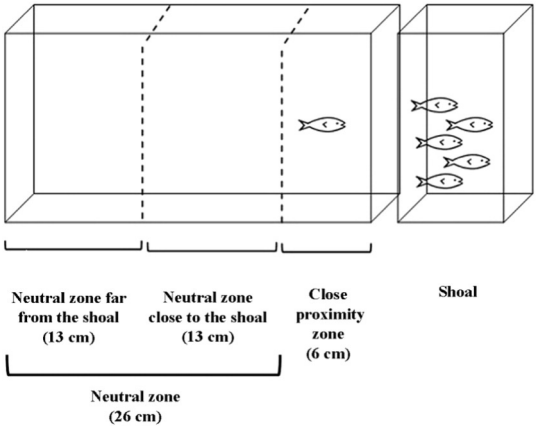

Fig. 3. Schematic representation of the shoaling test apparatus.

2.6. Statistical analysis

Statistical analyses were performed using the Infostat Software Package [43]. Behavioral variables recorded in the light/dark and shoaling tests were analyzed by Kruskal–Wallis and Dunn's posthoc tests. Cortisol concentrations of fish subjected to the light/dark test were analyzed by ANOVA followed by Fisher's LSD test, while cortisol concentrations of fish subjected to the shoaling test were analyzed by Kruskal–Wallis and Dunn's posthoc tests. The differences between cortisol levels detected in both behavioral studies (light/dark and shoaling test) were assessed by ANOVA followed by Fisher's LSD test. Statistical significance was established at $P < 0.05$ level.

3. Results

3.1. Measured CBZ test concentrations

The experimental CBZ concentrations measured in exposure solutions at time 0 h were: 11.5 ± 0.7, 41 ± 3 and 217 ± 12 μg CBZ/L for nominal concentrations 10, 50 and 200 μg CBZ/L, respectively. After 48 h of exposure, these concentrations were: 6 ± 1, 48 ± 1 and 225 ± 37 μg CBZ/L, respectively. CBZ was not detected in control treatment solutions (<LOD: 0.2 ng CBZ/L). Initially, CBZ concentrations were within 15–20% of the nominal values. Before water renewal, the levels remained quite stable, except for the lowest concentration (52% of the initial value). The stability of CBZ in water during 48 h is in agreement with previous reports [4,18].

3.2. Behavioral assessment

3.2.1. Light/dark preference test

In order to assess the effect of CBZ on stress-induced behavior, scototaxis and several parameters of *J. multidentata* swimming activity were registered in the dark–light box test. In this test, a significant increase in time spent in the light compartment was observed in 0CBZ + S with respect to control ($H(5, 49) = 15.67$, $P = 0.0031$). However, no significant differences were found between fish exposed to CBZ in relation to 0CBZ + S (Fig. 4A). In fish exposed to 200CBZ + S, there was a tendency to decrease the time spent in the light compartment, and this endpoint was not significantly different from the control group.

In mean speed results, a significant increase was measured in 0CBZ + S with respect to the control ($H(5, 47) = 22.04$, $P = 0.0002$) (Fig. 4B). In CBZ-exposed groups, mean speed in the 10CBZ + S and 50CBZ + S treatments was significantly lower than in the 0CBZ + S. At the lowest concentration (10CBZ + S), mean speed was not significantly different from the control. Individuals exposed to the highest concentration (200CBZ + S) showed a tendency to reduce mean speed with respect to 0CBZ + S.

There were no significant changes in freezing behavior due to stress or CBZ exposure with respect to the control group ($H(5, 48) = 2.91$, $P = 0.50$) (Fig. 4C). However, this variable tended to increase in stressed

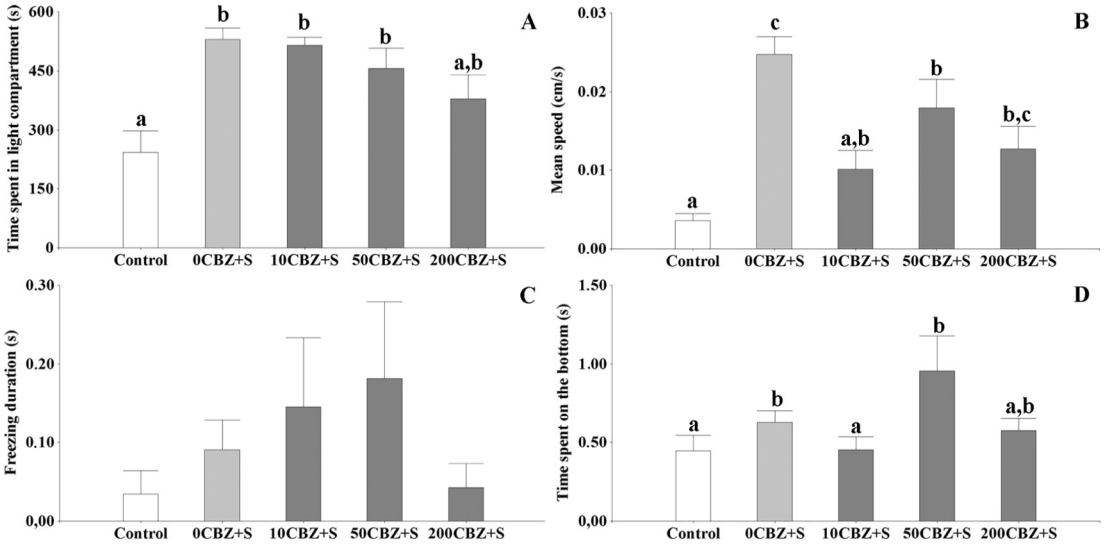

Fig. 4. Effects of carbamazepine on scototaxis of female adult *Jenynsia multidentata* under acute restraint stress. (A) Time spent in the light compartment (in s). (B) Mean speed (in cm/s). (C) Freezing duration (in s). (D) Time spent in the bottom of the tank (in s). Each column represents mean ± SEM (standard error of the mean). Different superscript letters indicate statistical differences ($P < 0.05$).

fish, except for those treated with the highest concentration of CBZ (200CBZ + S), where the trend was downward.

The time spent in the bottom of the tank was significantly longer in 0CBZ + S with respect to control ($H(5, 47) = 10.18$, $P = 0.037$) (Fig. 4D). In fish exposed to 10CBZ + S, there was a significant reduction in this endpoint with respect to 0CBZ + S, being similar to the control group. At the highest concentration of CBZ (200CBZ + S), a tendency to less time in the bottom was observed in relation to 0CBZ + S and 50CBZ + S.

3.2.2. Shoaling test

To analyze the effect of CBZ on stress-induced shoaling behavior, a female *J. multidentata* individual was placed in visual contact with a shoal of five fish of the same strain and swimming activity was recorded. No significant changes due to CBZ exposure or stress were observed in time spent in the close proximity zone (near the shoal) ($H(5, 48) = 4.73$, $P = 0.316$) (Fig. 5). The same results were observed in the neutral zone of the tank far from the shoal ($H(5, 47) = -3.93$, $P = 0.999$).

3.3. Cortisol content

The mean whole-body cortisol levels obtained from fish after the dark–light box test are indicated in Fig. 6. In this experiment, whole-body cortisol levels increased significantly in 0CBZ + S compared to the control treatment ($F_{4,45} = 4.07$; $P = 0.007$). In fish exposed to different doses of CBZ, this hormone was significantly lower with respect to 0CBZ + S, with values similar to the control group.

In the shoaling test, there were no significant differences in whole-body cortisol concentrations between experimental groups ($H(5, 43) = 1.64$, $P = 0.802$) (Fig. 7).

Comparing both behavioral tests, individuals subjected to the shoaling test showed significantly higher whole-body cortisol levels than those from light/dark test ($F_{9,95} = 3.96$; $P = 0.002$). Only 0CBZ + S group individuals from the light/dark test showed no significant differences with respect to those in the shoaling test (Table 1).

4. Discussion

In natural environments, fish respond to stressful conditions through appropriate behavioral and physiological responses. Those stress responses could be altered by the presence of psychiatric drugs as water contaminants. This study was to determine if the anticonvulsant drug CBZ modifies the whole body cortisol levels, scototaxis and/or shoaling behavior of fish *J. multidentata* under stress conditions.

With regard to scototaxis, fish without stress (control group) spent less time in the light than in the dark compartment in the light/dark box test. This behavior matched previous findings in other species [22,23,40,44]. Maximino et al. [44] reported that different fish species perceive the light compartment as an aversive zone, and so prefer the dark environment. In this regard, *J. multidentata* is associated with

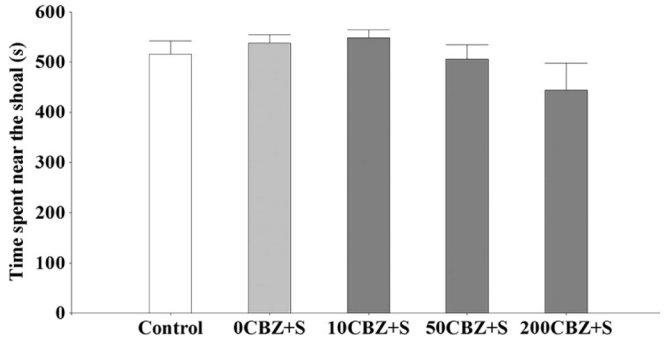

Fig. 5. Effects of carbamazepine on shoaling of female adult *Jenynsia multidentata* under acute restraint stress. Time (in s) spent near the shoal. Each column represents mean ± SEM.

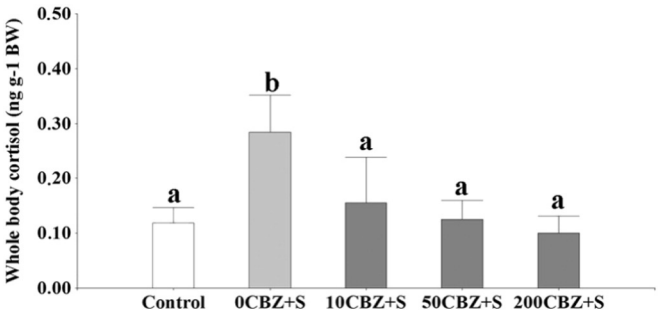

Fig. 6. Whole body cortisol levels (ng/g body weight, BW) obtained from female adult *Jenynsia multidentata* after the dark–light box test experiment. Each column represents mean ± SEM. Different superscript letters indicate statistical differences ($P < 0.05$).

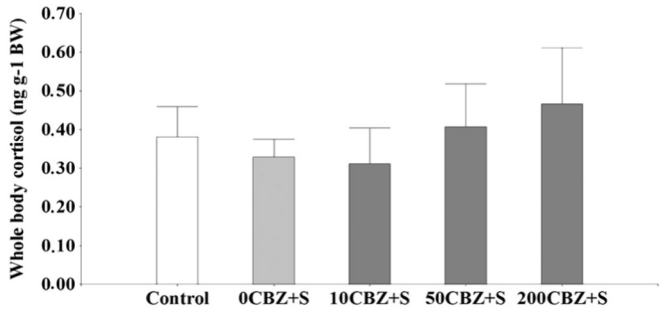

Fig. 7. Whole body cortisol levels (ng/g body weight, BW) obtained from female adult *Jenynsia multidentata* after the shoaling test. Each column represents mean ± SEM.

riparian vegetation in natural environments, indicating a preference for protected habitats [45,46].

The individuals under acute restraint stress (dark–light preference test) exhibited a significant increase in the time spent both in the light compartment and in the bottom of this, with respect to controls. They also showed a significant increase in mean speed and a tendency to freeze longer in the light compartment. Acute stress thus seems to disrupt their normal scototaxis. Using the same stressor (acute restraint stress), Champagne et al. [28] demonstrated that *D. rerio* display an opposite preference behavior to controls, and proposed that they are behaving against their natural tendency. Moreover, the fact that stressed individuals spent more time in the bottom and that their mean velocity was faster than that of controls supports the suggestion of various authors that these endpoints are biomarkers of stress response [22,23,40,44,47]. With respect to the time spent in the bottom of the tank, *D. rerio* tend to seek protection in an unfamiliar environment by diving and remaining in that position until it feels safe enough to explore its new environment [22,26,48,49]. This endpoint increased in stressed fish [22,26,48,49].

The increase in mean speed observed in stressed fish in our study matched other authors' findings using acute restraint stress [28,30]. Champagne et al. [28] pointed out that a significant increase in swimming speed in stressed *D. rerio* could be considered as an indirect index of erratic movements, since this behavior occurs with sharp increases in speed. The erratic movement is considered an accurate indicator of acute stress response [22].

Freezing behavior is one of the most common endpoints used to determine stress response [22,23]. Contrary to our expectations, we did not find significant differences ($P = 0.15$) in the time that stressed fish spent frozen with respect to the control ones. We think that this may be because of variability in the dataset due to the differential responses of each individual. To explain those differential stress responses, we could hypothesize that individuals show different reaction patterns in response to stress [50]. It has been demonstrated that individuals have a different "temperament" or "personality" that could influence an animal's reactivity to a variety of situations, being commonly classified as either "reactive" or "proactive" based on their distribution along a shy-bold continuum. Thus, shy individuals tend to cope with stress situations more reactively (e.g. freezing) while bold individuals do so in a proactive manner (e.g. fighting) [50,51].

Table 1
Comparative analysis of whole-body cortisol concentrations (mean ± SEM) obtained from female adult *Jenynsia multidentata* after each behavioral test.

Treatment	Cortisol concentrations (ng/g body weight)	
	Light/dark box test	Shoaling test
Control	0.12 ± 0.03 (a)	0.38 ± 0.08 (b)
0CBZ + S	0.28 ± 0.07 (b)	0.33 ± 0.05 (b)
10CBZ + S	0.16 ± 0.08 (a)	0.31 ± 0.09 (b)
50CBZ + S	0.12 ± 0.03 (a)	0.41 ± 0.11 (b)
200CBZ + S	0.10 ± 0.03 (a)	0.47 ± 0.15 (b)

Different letters indicate differences between treatments ($P < 0.05$).

In order to evaluate if CBZ could disrupt the behavioral stress response in *J. multidentata*, we exposed fish to increasing concentration of this drug (10, 50 and 200 μg CBZ/L). Although fish exposed to CBZ displayed different behavioral stress-responses, these behaviors did not show a similar pattern of drug concentration-response. The mean speed in fish exposed to 10 and 50 μg CBZ/L was significantly reduced compared to the 0CBZ + S group. However, the reduction in time spent in the bottom of the tank was observed only in fish exposed to 10 μg CBZ/L. It is important to mention that fish exposed to the highest CBZ level (200 μg CBZ/L) showed a decreasing tendency in all behavioral endpoints (time spent in the light compartment, mean speed, time spent in the bottom and freezing) in comparison to the 0CBZ + S group. A non-linear relation between behavior and drug concentration was described by other authors with different anxiolytic compounds [52]. Consistent with this, the lower mean speed and time spent in the bottom of the light compartment suggest that the lowest CBZ concentrations significantly decreased the stress-response. The same tendency (even though not significantly different) was observed in all the behaviors analyzed of fish exposed to the highest CBZ concentration and stress.

Other authors have proposed that CBZ treatment reduces anxious behaviors. For example, in *O. latipes*, CBZ exposure (6.15 mg/L) produced a significant reduction in swimming speed with respect to control fish [19]. In *L. gibbosus*, Brandão et al. [18] found a significant positive correlation between CBZ concentration and time spent in motion and a negative correlation with time spent in the black compartment in the light/dark preference test. Kulkarni et al. [53] demonstrated that oral CBZ administration (5 mg/kg body weight) to *D. rerio* reduced the number of erratic movements and total number of freezing bouts (tendency) and increased the time spent in the upper half with respect to control fish.

In this study, we also measured whole-body cortisol levels in *J. multidentata* as a biomarker of stress response in order to evaluate if CBZ could disrupt this response. As expected, we found a significant increase in whole-body cortisol levels in fish exposed only to acute restraint stress (0CBZ + S) with respect to the control group. This agrees with findings in other fish species exposed to different acute stressors [30,31,38,54]. In fish exposed to CBZ and acute restraint stress, we observed a significant reduction in whole-body cortisol levels compared to only stressed fish. This hormone reached values similar to those of controls in all CBZ-exposed treatments.

To our knowledge, this is the first study on behavioral responses and cortisol secretion under acute restraint stress in the species *J. multidentata*. Speculatively, we may hypothesize that CBZ stimulates fish cortisol biotransformation, as has been already proposed in humans. CBZ administration activates hepatic cytochrome P450 (CYPs) enzymes, particularly the isoenzyme CYP3A4, in a dose-dependent manner [55]. CYP3A4 stimulates catabolism of CBZ by oxidation and also cortisol biotransformation to 6β-hydroxy-cortisol [56,57]. Thus, in patients under chronic CBZ treatment, this drug increases 1.5–3 fold the urinary 6β-hydroxy-cortisol/free cortisol ratio, as a function of dose and duration of treatment [58]. It should be noted that CYP3A also participates in fish phase 1 metabolism (i.e. catabolism) of lipophilic organic chemicals and its function may be modified by pollutants [59].

Even though behavioral data has been correlated with physiological function in the literature, it is well known that it has some limitations. Our study could not establish a clear relationship between behavioral responses and changes in cortisol levels. While cortisol levels decreased in all fish exposed to CBZ (in the light/dark box test), not all behavioral variables showed a decrease in the stress response. These findings suggest that CBZ could affect fish behavioral stress response by two possible mechanisms: acting at central nervous system level and/or decreasing cortisol. In the first case, it has been demonstrated that CBZ crosses the *D. rerio* blood–brain barrier and is bioconcentrated in the brain

[15,53]. As mentioned, several mechanisms of action have been proposed for CBZ effects on the human nervous system [8–10]. The second hypothesis is supported by the cortisol results obtained in our work and the background of evidence reported in humans [56,57]. These hypotheses should be probed in future studies.

In natural environments, *J. multidentata* displays gregarious behavior and movement in groups [45,46]. Shoaling behavior is essential for survival because shoaling is a potent anti-predator mechanism for both individual and group and is a good survival strategy in the presence of danger and stress [29,60]. Stress exposure causes the shoal to 'tighten' (the fish swim closer together) [22]. Visual stimuli alone are able to induce strong shoaling responses in *D. rerio* [61,62]. Shoal formation and the stress response are adaptive behaviors in wild fish and exposure to pharmaceuticals could disrupt fish fitness [29]. Therefore, in this work we analyzed if CBZ disrupts the shoaling behavior stress response in *J. multidentata*. We could not detect significant changes in the time spent in close proximity to the shoal zone or in the neutral zone far from the shoal due to either stress or CBZ exposure. All experimental fish spent most of the analyzed time period near the shoal, regardless of stress exposure. Contrary to our expectations, whole-body cortisol levels did not show significant differences between experimental groups. Moreover, if we compare whole-body cortisol levels in fish from this experiment with hormone levels in fish from the light/dark preference test, we observe that hormone levels in the shoaling test had similar values to those of the stressed group without CBZ of the first test (Table 1). These findings indicate that all fish in the shoaling behavior study were stressed.

It has been demonstrated that *D. rerio* express a visually mediated shoaling preference [61]. As *J. multidentata* rely on visual cues to capture macroinvertebrates for feeding [63], we expected that conspecific visual contact would be enough to make them feel a member of a shoal and to decrease stress-induced behavior. However, in our experimental protocol there was a solid barrier between each fish and the shoal, so they could not interact. The inability of the individual to join and interact with other individuals could have been a stress factor for the fish, which goes along with increased cortisol levels. We can thus hypothesize that *J. multidentata* need other stimuli besides sight, e.g. olfactory, auditory and lateral line cues, from conspecifics to decrease the stress caused by being alone. This should be tested in future experiments. As has been reported, the absence of social interaction in *D. rerio* appears to be stressful; when tested individually, fish showed increased cortisol levels and behavioral variability compared to group-tested animals [64].

Organisms respond to different stressors by mounting a stress response that involves a complex set of behavioral and physiological changes at multiple levels of biological organization [21]. Considering our results in the light/dark and shoaling tests, we cannot demonstrate a similar effect of CBZ on fish behavioral response to stress and on cortisol secretion. A possible explanation for the discrepancy of our behavioral results is that the methods employed (the light/dark and shoaling tests) measure different behavioral endpoints. The light/dark test evaluates anxious behaviors [23] while the shoaling test measures social behavior [62]. Several authors propose that these behaviors are regulated by different areas of the fish central nervous system [65,66]. CBZ might therefore be having differential effects on different brain areas. In accordance with this hypothesis, Maximino et al. [23] stated: "inconsistencies on the effects of pharmaceuticals on different behavioral endpoints on this test arise, and the considerable amount of "borderline" significant effects raises the possibility that these different endpoints are mediated by different motivational systems".

Another possible explanation for these discrepancies may be the different kind of stressors present in each assay and the stress duration. In the shoaling test, the physical barrier between the fish analyzed and the rest of the shoal may be considered as an added stressor, which could explain the increased cortisol levels in all treatment groups including controls. The individual remaining isolated from the shoal represented a psychosocial stressor. In mammals, it has been demonstrated that physical stressors directly activate the sympathetic nervous system and generate a stress response that is clearly distinct from the response induced by a psychological stressor [67]. Due to the high homology between the neuroendocrine system of fish and mammals [68], CBZ may differentially affect the stress responses induced by physical and social stressors. In addition, CBZ may decrease the cortisol levels and behavioral stress response once the stressor is removed (as observed in light/dark test), probably by activation of cortisol metabolism and/or affecting brain function. However, this drug cannot inhibit the response when the stress remains active, as observed in the shoaling test.

This study suggests that CBZ has a differential effect on fish behavioral stress response and cortisol levels depending on the behavioral test used, the stressor quality and the duration of the stress. Further studies are needed to investigate if CBZ modifies the stress response by acting directly on the central nervous system and/or the hypothalamus–pituitary–adrenal axis.

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