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# Involvement of anxiety-like behaviors and brain oxidative stress in the chronic effects of alarm reaction in zebrafish populations

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ABSTRACT

Aversive conditions elicit anxiety responses that prepare the organism to an eventual threat. Nonetheless, prolonged anxiety is a pathological condition associated with various neuropsychiatric disorders. Here, we evaluated whether the conspecific alarm substance (CAS), a chemical cue that elicits aversion, influences anxiety-like behaviors and modulates brain oxidative stress-related parameters in *wild-type* (*WT*) and *leopard* (*leo*) zebrafish following a repeated exposure protocol. CAS exposure was performed for 5 min, once daily for 7 consecutive days. In the 8$^{th}$ day, animals were tested in the light/dark and novel tank tests and their brains were further dissected for biochemical analyses. CAS chronically induced anxiogenic-like states in *WT* and *leo* populations when their behaviors were analyzed in the light/dark and novel tank tests. CAS also increased catalase (CAT) and glutathione S-transferase (GST) activities, as well as non-protein thiol (NPSH) content in *WT* and *leo*, but only *leo* had increased thiobarbituric reactive substance (TBARS) levels in the brain. At baseline conditions, *leo* was more 'anxious' when compared to *WT*, displaying lower CAT activity and carbonylated protein (CP) levels. Overall, CAS chronically triggers anxiety-like behavior in zebrafish populations, which may be associated with changes in oxidative stress-related parameters. Furthermore, the use of different zebrafish populations may serve as an interesting tool in future research aiming to investigate the neurobehavioral bases of neuropsychiatric disorders in vertebrates.

## 1. Introduction

Anxiety- trauma- and stressor-related disorders constitute serious public health problems worldwide (Kessler et al., 2005; Riaza Bermudo-Soriano et al., 2012). Patients with such disorders may present exacerbated fear and/or anxiety, which differ according to the proximity of the threat (Baldwin et al., 2010, 2005; Smaga et al., 2015). Thus, measuring fear/anxiety-like behaviors in animal models represents an interesting strategy to understand how aversive conditions affect defensive behaviors and help elucidate the mechanisms underlying various neuropsychiatric disorders (Atmaca et al., 2008; Kalueff et al., 2007; Kuloglu et al., 2002b, 2002a; Maximino et al., 2010b; Ozdemir et al., 2009).

Although reactive oxygen species (ROS) are produced during aerobic metabolism, their excessive formation associated with reduced antioxidant defenses culminates in oxidative stress (Moniczewski et al., 2015; Rocha et al., 2003). Oxidative damage affects cellular structures, neurotransmission, as well as transduction signaling pathways (Halliwell, 2006; Hovatta et al., 2010), and correlates with various neuropsychiatric conditions (Rammal et al., 2008; Smaga et al., 2015). Cellular antioxidant defenses, such as glutathione, thioredoxin, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase activities modulate ROS levels, thereby maintaining redox status (Hagedorn et al., 2012). Mounting evidence shows that SOD and CAT activities in the blood are stimulated in patients with social phobia (Atmaca et al., 2008), panic disorder (Kuloglu et al., 2002b), and

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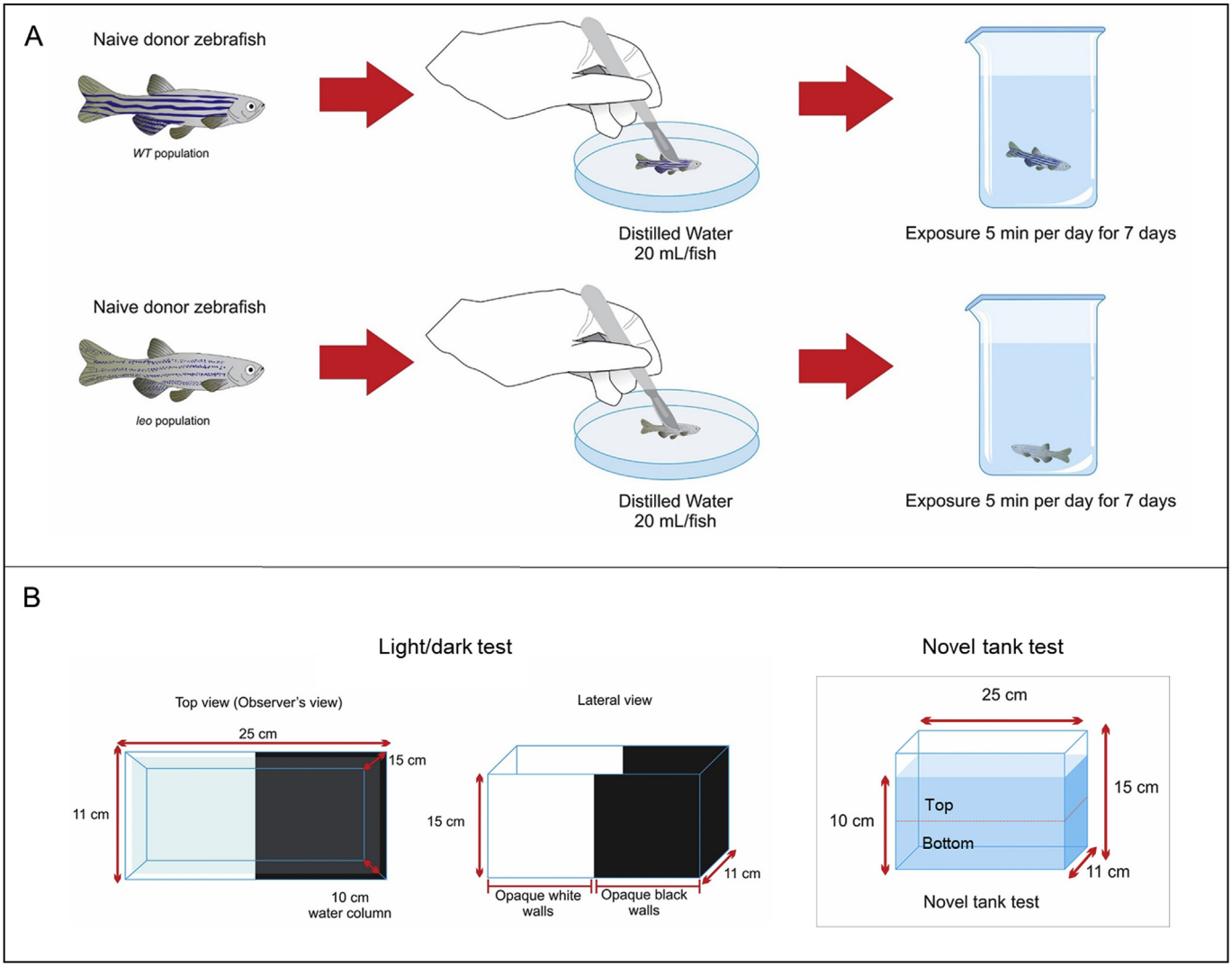


**Fig. 1.** Schematic representation of experimental procedures and apparatus. (A) CAS extraction from naïve *WT* and *leo* donors and exposure protocol. The skin extract was prepared on ice and 3.5 mL/L were used for the experiments. (B) Experimental apparatuses. The light/dark tank consisted of a glass apparatus divided into two equally sized dark and lit areas. The compartments were delimited by opaque plastic self-adhesive films in black or white colors externally covering walls and floor. The novel tank was divided into two areas (top and bottom) to assess vertical exploration.

transient-compulsive disorder (Kuloglu et al., 2002b, 2002a), suggesting a close relationship between neuropsychiatric diseases and oxidative stress. Therefore, measuring behavioral phenotypes and brain oxidant status in animal models help investigate evolutionarily conserved mechanisms involved in the pathophysiology of anxiety- trauma- and stressor-related disorders.

The zebrafish (*Danio rerio*) is a well-established model organism in biochemical and neurobehavioral research (Fontana et al., 2018; Kalueff et al., 2014; Rosemberg et al., 2012; Stewart et al., 2014). The high degree of genetic conservation when compared to the human genes (~70%) (Howe et al., 2013) and the well-characterized behaviors (Kalueff et al., 2013) provide relevant insights into the genetic bases involved in anxiety-related disorders (Egan et al., 2009; Shin and Fishman, 2002). Moreover, behavioral endpoints that reflect anxiety-like responses have been described in both *wild-type* (*WT*) and *leopard* (*leo*) populations (Egan et al., 2009; Maximino et al., 2013; Quadros et al., 2018, 2016). As compared to *WT*, *leo* spends more time in the bottom area of the tank, displays increased shoaling, and prominent scototaxis, suggesting a more 'anxious' behavior (Canzian et al., 2017; Egan et al., 2009; Maximino et al., 2013). Both *WT* and *leo* present different responses when exposed to the conspecific alarm substance (CAS), a chemical cue that elicits defensive behaviors (Quadros et al., 2018, 2016). CAS increases habenular *c-fos* expression and promotes sympathetic activation, which stimulates adrenaline release and increases blood glucose levels (Maximino et al., 2014; Ogawa et al., 2014). In zebrafish, CAS exposure evokes freezing, erratic movements, escaping behavior (Maximino et al., 2018a) and stimulates aggressive behavior following a single exposure, suggesting activation of 'fight-or-flight' responses (Quadros et al., 2018). Conversely, repeated CAS promotes long-term changes in the CNS by inducing depressant-like behaviors without changing aggression (Quadros et al., 2018). When animals face aversive conditions, biochemical and physiological alterations occur to maintain homeostasis (Chrousos, 2009; Miller and Raison, 2016; Mocelin et al., 2018). However, maladaptive responses following prolonged aversive situations may play a role in anxiety-related disorders and correlated neurochemical mechanisms (Hassan et al., 2016; Hovatta et al., 2010; Mocelin et al., 2015). Therefore, we sought to investigate whether repeated CAS exposure (7 days) modulates defensive behaviors in *WT* and *leo*, as well as to explore the putative involvement of oxidative stress in CAS-mediated responses.

## 2. Materials and methods

### 2.1. Animals

Adult zebrafish (*Danio rerio*) of the short fin *wild type* (*WT*) and *leopard* (*leo*) populations were obtained from a local supplier (Hobby Aquários, RS, Brazil). Animals were 4–6 months-old and a 50:50 (male:female) proportion was used for the experiments. Because the genetic bases were not fully elucidated, we used of the term "population" to define both zebrafish phenotypes tested here. Animals were separated by their respective phenotypes at a maximum density of four fish per liter. Fish were allocated in 40 L tanks filled with non-chlorinated water and maintained under mechanical, biological, and chemical filtration (temperature and pH set at 27 ± 1 °C and 7.2 ± 0.1, respectively) for 2 weeks before the experiments to acclimate to laboratory conditions. Illumination was provided by fluorescent lamps under a photoperiod cycle (14h/10h, light/dark respectively). All animals used were fed thrice daily with commercial flake fish food (Alcon BASIC™, Alcon, Brazil). The experiments fully adhered to the National Institute of Health Guide for Care and Use of Laboratory. All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (106/2014).

### 2.2. Conspecific alarm substance extraction and exposure protocol

CAS was extracted from donor fish previously euthanized, as described elsewhere (Egan et al., 2009; Quadros et al., 2016; Maximino et al., 2018b), by damaging the epithelial cells with a razor blade (10–15 shallow slices on each side of the body). Fish were then washed with 20 mL distilled water in a Petri dish kept on ice, avoiding blood contamination into solution. For chronic CAS exposure, zebrafish were exposed individually to 1.75 mL skin extract preparation into 500 mL tanks for 5 min once daily during 7 consecutive days and tested on the 8$^{th}$ day (Quadros et al., 2018) (Fig. 1A). *WT* and *leo* populations were exposed to CAS obtained from their phenotypically similar donor fish. Control groups were handled in a similar manner, except that only distilled water was added to the tank.

### 2.3. Behavioral measurements

All behavioral tests were performed between 09:00 a.m. and 4:00 p.m. Behavioral activities were registered in a single 6 min session and the apparatus was positioned on a stable surface. Throughout the experiments, animals were handled carefully to minimize stress and the water was changed after each trial. Behaviors were recorded using a webcam connected to a laptop at 30 frames/s, and subsequently analyzed using appropriate video-tracking system (ANY-maze™, Stoelting CO, USA). All tests were performed using two independent batches.

### 2.4. Behavioral tests

#### 2.4.1. Light/dark test

Anxiety-like behaviors were determined using the light/dark test as described previously (Maximino et al., 2010a, 2010c; Quadros et al., 2016). The apparatus consisted of a rectangular tank (15 × 10 × 25 cm, height, depth and length respectively), divided in two equally-sized dark and lit areas (Fig. 1B). The compartments were covered externally by opaque black or white self-adhesive films in the walls and floor of the apparatus. Experimental tanks were filled with 2 L non-chlorinated water and animals ($n$ = 12–14 per group) were placed individually in the lit area of the apparatus before starting the test. Behaviors were analyzed for 6 min and the following endpoints were measured: time spent in lit area, number of transitions to the lit area, latency to enter the dark area, and number of risk assessments. Risk assessment episodes were computed manually by two trained observers (inter-rater reliability > 0.85) blinded to the experimental condition. This behavior was counted when fish approximate the lit area without crossing the pectoral fin, associated with a fast return to the dark compartment (Kalueff et al., 2013; Maximino et al., 2014).

#### 2.4.2. Novel tank test

Locomotor and vertical exploratory activity of fish, which reflects habituation of novelty stress, were analyzed in rectangular tanks (25 cm × 15 cm × 11 cm length, height and width respectively), filled with 1.5 L of non-chlorinated water and divided into two equal horizontal areas (bottom and top) (Egan et al., 2009; Rosemberg et al., 2011, 2012) (Fig. 1B). Animals were individually placed in the test tanks and their behaviors further analyzed for 6 min. We measured the distance traveled and absolute turn angle (locomotor-related endpoints), time spent in top area, transitions to top area, and the latency to enter the top (vertical activity parameters). Both erratic movements and freezing (aversive behavioral responses) were computed manually by trained observers (inter-rater reliability > 0.85) blinded to the experimental condition. Freezing was defined as complete immobility associated to increased opercular beat rate for at least 2 s, while erratic movements were considered sharp changes in direction or velocity and repeated rapid darting behavior (Kalueff et al., 2013).

### 2.5. Biochemical parameters

#### 2.5.1. Tissue preparation

After CAS exposure, fish were previously anesthetized in water at 4 °C and subsequently euthanized by decapitation. Brains were dissected on ice and transferred to microtubes for storage at −80 °C. For each sample, two brains were pooled, homogenized in 500 μL of 50 mM Tris-HCl buffer, pH 7.4, and posteriorly centrifuged at 3000 × $g$ for 10 min at 4 °C. Supernatants were removed for biochemical assays. All tests were run in duplicate.

#### 2.5.2. Antioxidant enzymatic defenses

Superoxide dismutase (SOD) activity was assessed based on the inhibition of superoxide radical reaction with adrenaline (Misra and Fridovich, 1972). Samples (20–30 μg protein) were incubated with 50 mM glycine-NaOH buffer, pH 10.0, in the presence of 1 mM adrenaline. The rate of adenochrome formation was measured on a microplate reader and data were expressed as U SOD/milligram protein.

Catalase (CAT) activity was evaluated based on the rate of $H_2O_2$ decrease at 240 nm ultraviolet spectrophotometry (Aebi, 1984). Samples (0.01 mL, 20–30 μg protein) were mixed with 1 mL of 50 mM potassium phosphate buffer, pH 7.0 and 0.05 mL $H_2O_2$ (0.3 M) was used as substrate. Results were expressed as micromole/min/milligram protein.

Glutathione S-transferase (GST) activity was measured using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, 10 mM reduced glutathione, 20 mM potassium phosphate buffer, pH 6.5, and 20 μL of samples (40–60 μg protein) (Müller et al., 2017; Nunes et al., 2017). Enzyme activity was determined by variations in absorbance at 340 nm using the molar extinction coefficient of 9.6 mM/cm (Habig et al., 1974). GST activity was measured according to the amount of enzyme required to catalyze 1 mol CDNB conjugate with GSH/min at 25 °C. Results were expressed in nanomole GS-DNB/min/milligram protein.

#### 2.5.3. Non-protein thiol content quantification

Non-protein thiol (NPSH) levels were determined as described previously (Ellman, 1959). Briefly, 100 μL of sample (60–80 μg of protein) was mixed in 100 μL of TCA 10% and then centrifuged at 3000 × $g$ for 10 min. Later, 30 μL of the supernatant was mixed with 10 μL of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) (0.01 M) dissolved in ethanol. The intense of yellow color developed was measured at 412 nm after 30 min in a microplate reader. Results were expressed as nanomole NPSH/milligram protein.

#### 2.5.4. Measurement of lipid peroxidation and protein carbonylation

Lipid peroxides were estimated by thiobarbituric reactive substance (TBARS) production (Draper and Hadley, 1990). Briefly, 160 μL of 10% Trichloroacetic acid (TCA) was added in 80 μL of samples (80–100 μg protein) and subsequently centrifuged at 10,000 × *g* for 10 min. Later, 100 μL of the supernatant was added to an equal volume of 0.67% thiobarbituric acid (TBA, 4,6-dihydroxypyrimidine-2-thiol) and heated for 30 min at 100 °C. TBARS levels were determined using malondialdehyde (MDA) as standard in a microplate reader at 532 nm. Data were expressed as nanomole MDA/milligram protein.

Carbonylated protein (CP) levels were measured based on the protocol described elsewhere (Yan et al., 1995). Initially, 200 μL of protein was added to 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid and incubated for 1 h in dark. Later, 0.15 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8 containing SDS 3.0%), 0.5 mL of heptane (99.5%) and 0.5 mL of ethanol (99.8%) were added, mixed for 40 s, and centrifuged for 15 min at 3000 × *g*. Proteins were isolated, suspended twice in ethanol/ethyl acetate (1:1) and mixed with 0.25 mL of denaturing buffer. Data were measured colorimetrically at 370 nm and calculated using the molar extinction coefficient of 22,000 M/cm. Protein carbonylation was expressed as nanomole carbonylated proteins/milligram protein.

### 2.6. Protein quantification

Protein was determined using the Coomassie blue method and bovine serum albumin was used as standard (Bradford, 1976). Samples were run in duplicate and the absorbance was measured at 595 nm.

### 2.7. Statistical analyses

Normality of data and homogeneity of variances were analyzed using Kolmogorov–Smirnov and Bartlett's tests, respectively. Results were expressed as means ± standard error of mean (S.E.M) and analyzed by two-way analysis of variance (ANOVA) (considering population and treatment as factors), followed by Student–Newman–Keuls multiple comparison test whenever necessary. Differences among groups were set at $p \leq 0.05$ level.

## 3. Results

### 3.1. Repeated CAS exposure increases anxiety-like behaviors in WT and leo populations

In the light/dark test (Fig. 2), two-way ANOVA yielded significant effects of population and treatment for the time spent in lit area ($F_{1,48} = 10.35$, $p = 0.0023$ and $F_{1.48} = 57.51$, $p < 0.0001$ respectively), transitions to the lit area ($F_{1,48} = 5.773$, $p = 0.0202$ and $F_{1.48} = 61.55$, $p < 0.0001$ respectively), and latency to enter the dark area ($F_{1,48} = 6.485$, $p = 0.0141$ and $F_{1.48} = 28.01$, $p < 0.0001$ respectively). Repeated CAS exposure reduced the time spent in the lit area and the number of transitions to the respective compartment in both *WT* and *leo*. At the baseline, *WT* population spent more time in the lit area, showing fewer transitions to the lit compartment and higher latency to enter the dark area than *leo*. Furthermore, *leo* displayed more risk assessment episodes than *WT*, but CAS only increased such behavior in *WT* population ($F_{1,48} = 15.53$, $p = 0.0003$ for the interaction term).

In the novel tank test (Fig. 3), although *leo* spent more time in bottom than *WT* at the baseline, CAS reduced the time spent in top for both populations ($F_{1,25} = 21.47$, $p < 0.0001$ for the interaction term). A significant population and treatment interaction for the transitions to top area was observed ($F_{1,25} = 7.321$, $p = 0.0121$), in which only *WT* showed decreased vertical activity following repeated CAS exposure. Moreover, CAS increased the latency to enter the top area in both populations ($F_{1,25} = 55.92$, $p < 0.0001$ for the treatment term). Two-way ANOVA revealed significant effects of population, in which *leo* showed more erratic movements than *WT* at the baseline ($F_{1,25} = 5.737$, $p = 0.0244$). No differences were observed in distance traveled, absolute turn angle, and freezing episodes among groups (data not shown).

### 3.2. CAS chronically alters antioxidant parameters and lipid peroxidation

Fig. 4 shows the effects of repeated CAS exposure on antioxidant responses and oxidative stress biomarkers. Although SOD activity did not differ among groups (Fig. 4A), significant effects of population ($F_{1,18} = 8.514$, $p = 0.0092$) and treatment ($F_{1,18} = 6.488$, $p = 0.0202$) were observed for CAT activity. CAS increased CAT activity only in *leo*, which showed lower enzyme activity than *WT* at the baseline (Fig. 4A). For GST activity (Fig. 4A) and NPSH levels (Fig. 4B), we verified significant effects of treatment ($F_{1,12} = 33.11$, $p < 0.0001$ and $F_{1,20} = 20.47$, $p = 0.0002$ respectively), where both populations showed a markedly increase in these antioxidant responses following CAS exposure. Fig. 4C shows the effects of CAS on lipid peroxidation and protein carbonylation. We observed higher TBARS levels in CAS-exposed *leo* ($F_{1,12} = 10.5$, $p = 0.0071$ for the interaction term). Although CAS did not induce protein carbonylation, CP levels were lower in *leo* than *WT* at the baseline ($F_{1,15} = 4.703$, $p = 0.0466$ for the population term).

## 4. Discussion

In this study, our novel data showed that repeated CAS exposure induces anxiogenic-like behaviors and modulates oxidative stress parameters in *WT* and *leo* populations. Basically, in the light/dark test, CAS reduced the frequency of entries in the lit area, but decreased the latency to enter the dark area and increased risk assessments only in *WT*. In the novel tank test, CAS decreased the time spent in top area and increased the latency to enter the top, while only *WT* population showed reduced transitions to top area after CAS exposure. Although CAS increased CAT and GST activities, as well as NPSH levels, in both populations, only *leo* showed pronounced lipid peroxidation following the exposure period. At baseline conditions, *leo* displayed a more 'anxious' phenotype, with reduced CAT activity and CP levels in the brain. Overall, we suggest that repeated CAS exposure induces anxiogenic-like behaviors in zebrafish, accompanied by changes in oxidant status in the brain.

In ostariophysans, CAS plays a key role as a chemical cue of predation risk (Maximino et al., 2018a; Speedie and Gerlai, 2008). This substance is released when epithelial cells are ruptured, triggering robust alert responses when fish face a potential threat (Egan et al., 2009; Quadros et al., 2016; Sanches et al., 2015; Speedie and Gerlai, 2008). CAS elicits anxiogenic-like behaviors since it markedly increases social cohesion, geotaxis, and *c-fos* expression in habenula (Canzian et al., 2017; Ogawa et al., 2014; Oliveira et al., 2017; Quadros et al., 2016). Moreover, CAS increases blood glucose, ephinephrine, and serotonin levels in the brain, suggesting activation of sympathetic nervous system (Maximino et al., 2014). We have previously showed that CAS acutely increases aggression in *WT* and *leo*, but induces depressant-like phenotypes following a repeated exposure protocol (Quadros et al., 2018). Here, we hypothesized that CAS would chronically induce anxiogenic-like effects causing an imbalance of oxidant status in the brain, in such a way that differences in zebrafish populations could be predicted using two behavioral tasks with a good correlation and similar sensitivity to zebrafish anxiety-like states *in vivo* (Kysil et al., 2017). Our data confirmed that *leo* has a more 'anxious' behavior than *WT* and, after repeated exposure, both populations showed increased scototaxis and reduced vertical activity in the light/dark and novel tank tests, respectively. However, only *WT* had decreased latency to enter the dark area and increased risk assessment episodes in the light/dark test, as well as showed fewer transitions to top area in the novel tank test, reinforcing a stronger sensitivity of the respective population following

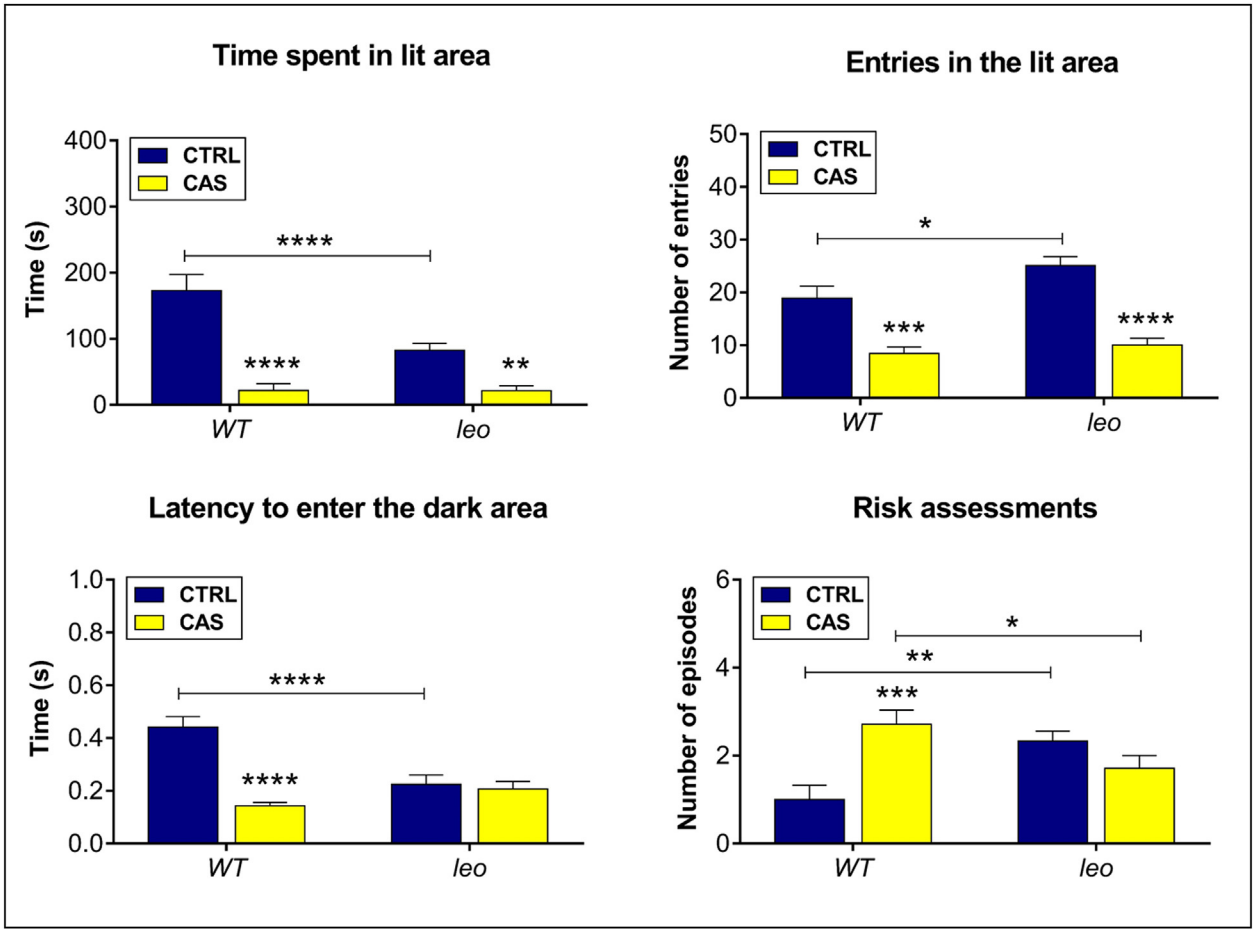


**Fig. 2.** Repeated CAS exposure induces anxiety-like states in *WT* and *leo* populations in the light/dark test. Animals were exposed to CAS (5 min daily for 7 consecutive days) and their behavioral activities were further recorded for 6 min in the 8$^{th}$ day. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA followed by Student–Neuman–Keuls multiple test whenever appropriate (*$p < 0.01$; **$p < 0.01$; ***$p < 0.005$, ****$p < 0.001$; $n = 12–14$ per group).

chronic CAS exposure. Although one could attribute the absence of some effects in *leo* to a floor effect, anxiogenic-like states in *WT* and *leo* were observed in both light/dark and novel tank tests using the protocol described here. Importantly, a single CAS exposure increases shoaling in *WT*, but not in *leo* (Canzian et al., 2017), while caffeine, another anxiogenic substance, elicits anxiety-like behaviors in *leo* by increasing bottom-dwelling and whole-body cortisol levels depending on the concentration tested (Rosa et al., 2018). These set of data reinforce the utility of zebrafish populations when different behavioral domains are assessed. Although we did not perform a relationship between genes and behavior, our results suggest that genetic variations between populations may contribute, at least partially, to the behavioral differences observed when aversive stimuli are presented.

Aversive situations trigger an innate response that activates transduction signaling pathways aiming to restore homeostasis (Chrousos and Gold, 1992; McEwen, 2007). Thus, the maintenance of redox status is extremely important for brain physiology since the imbalance between oxidants and antioxidant defenses culminates in oxidative stress (Langley and Ratan, 2004; Zhang and Yao, 2013). Prolonged stressful conditions are maladaptive, culminating in neurochemical impairments that affect behavioral responses. For example, increased ROS may damage various biomolecules (*e.g.*, lipids, proteins, and DNA), thereby affecting cellular viability, which are often associated with neurodegenerative diseases (Behl and Moosmann, 2002; Gutowicz, 2011; Niedzielska et al., 2016), and anxiety-related disorders (Guney et al., 2014; Hovatta et al., 2010; Niedzielska et al., 2016).

To investigate whether changes in redox status could influence CAS-induced behaviors, we measured oxidative stress-related parameters in the brain following a 7-day exposure protocol. Physiologically, SOD and CAT play a synergic role to neutralize superoxide anion and hydrogen peroxide, respectively (Gutteridge, 1984; Halliwell, 2006), while GST catalyzes the conjugation of the reduced glutathione (GSH) to xenobiotic substrates for the purpose of detoxification (Dringen et al., 2000; Pisoschi and Pop, 2015). Although the increased NPSH levels, CAT, and GST activities in CAS-exposed *leo* suggest compensatory non-enzymatic and enzymatic mechanisms aiming to regulate homeostasis, these effects do not protect against the lipid peroxidation in this population. Although further experiments are needed to understand how anxiety-like behaviors correlate with oxidant processes following chronic CAS exposure, the differences in enzymatic antioxidant defenses at baseline level observed here could reflect a higher susceptibility of oxidative damage in *leo* and/or genetic variability between populations.

## 5. Conclusion

In conclusion, our novel data show that repeated CAS exposure induces anxiety-like behaviors and modulates oxidative stress-related biomarkers in zebrafish. Some behavioral and biochemical parameters were substantially different in *WT* and *leo*, both at baseline and after CAS exposure, suggesting population-dependent responses. Furthermore, the anxious behavior following stressful situations could be related to changes in oxidant status in the brain, since we demonstrated a modulatory effect of CAS on oxidative stress-related endpoints. Although additional empirical data are necessary to elucidate the mechanisms underlying alarm responses in zebrafish, we reinforce the growing utility of this aquatic species in modeling anxiety-related

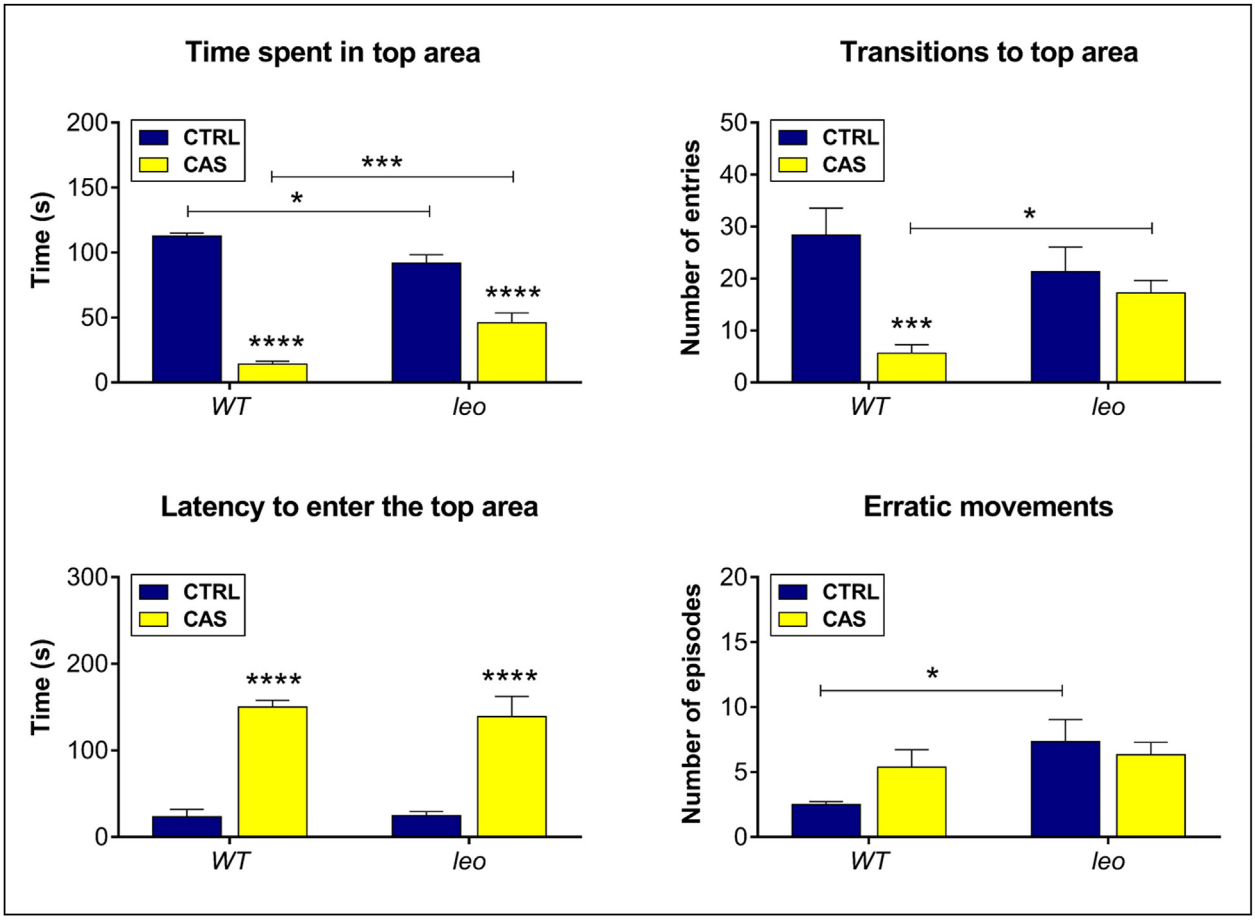


**Fig. 3.** Repeated CAS exposure modulates exploratory activities of *WT* and *leo* measured in the novel tank test. Animals were exposed to CAS (5 min daily for 7 consecutive days) and their behavioral activities were further recorded for 6 min in the 8$^{th}$ day. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA followed by Student–Neuman–Keuls multiple test whenever appropriate (*$p < 0.05$; ***$p < 0.005$, ****$p < 0.001$; $n$ = 7–9 per group).

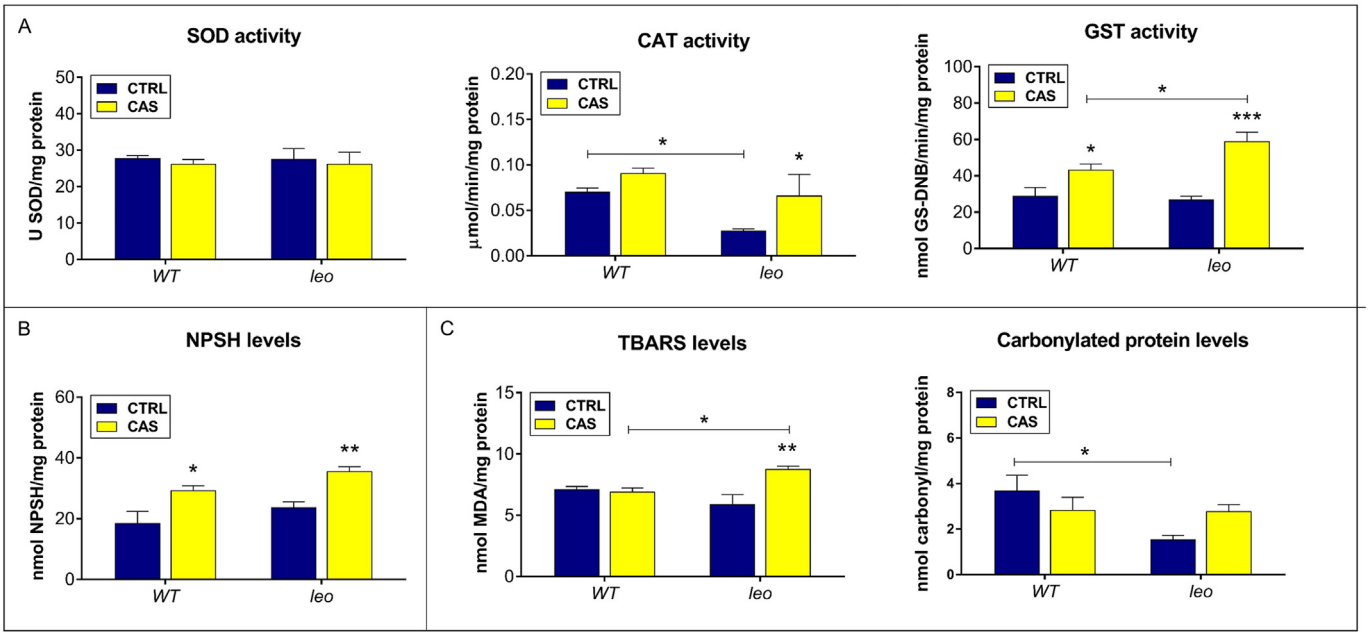


**Fig. 4.** Antioxidant parameters and biomarkers of lipid and protein damage in the brain of *WT* and *leo* populations following repeated CAS exposure. (A) Enzymatic antioxidant defenses (SOD, CAT, and GST activities); (B) Non-enzymatic antioxidant responses (NPSH amounts); (C) TBARS and CP levels. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA followed by Student–Neuman–Keuls multiple test whenever appropriate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; $n$ = 4–6 per group).

disorders. Thus, future studies aiming to correlate oxidative stress with changes in neurotransmitter signaling pathways will bring novel opportunities to understand how conspecific alarm cues affects brain functions.

## Conflicts of interest

The authors declare that no competing interests exist.

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