The impact of chronic unpredictable early-life stress (CUELS) on boldness and stress-reactivity: differential effects of stress duration and context of testing

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Abstract

Early-life stress (ELS) has been shown to result in a diverse array of long-lasting impacts; for example, increasing vulnerability to disease or building 'resilience' in adulthood. Previously, zebrafish (*Danio rerio*) have been used to understand the mechanisms by which ELS induces different behavioral phenotypes in adults, with alterations in both learning and anxiety observed in exposed individuals. Here, we subjected zebrafish larvae to chronic unpredictable early-life stress (CUELS) for 7 or 14 days, to investigate the impact on boldness towards a new environment and novel object, and stress-reactivity. We observed that 7 days of CUELS resulted in increased time spent in the top of a novel tank (indicating boldness) but did not alter approach to a novel object. Although CUELS did not affect stress-reactivity in terms of cortisol levels, decreased anxiety-like response to conspecific alarm substance (CAS) was observed in both ELS groups (7 and 14 days of CUELS). Therefore, for the first time, we observe a potential negative effect of CUELS by dampening the behavioral stress response following exposure to CAS. Overall, these data support the use of zebrafish as a translational model to study the broad range of ELS-induced permanent changes in behavior. It could also be used to investigate the mechanisms underlying both the positive and the negative effects of early-life adversity.

Keywords: alarm pheromone; conspecific alarm substance; cortisol levels; novel tank; zebrafish.

1. Introduction

Adverse early-life experiences or early-life stress (ELS) permanently alter metabolism (*e.g.* glucocorticoid production) [1], brain function (*e.g.* abnormal dopaminergic and serotoninergic activity)[2, 3] and several behavioral domains (*e.g.* anxiety, aggression and social behavior)[3, 4]. Stress can modulate behavior differently depending on its duration and intensity. Severe acute experience/traumatic experiences and chronic unpredictable stress can lead to abnormal behavior such as disrupted affective behavior and cognitive deficits [5, 6]. Meanwhile, moderate or mild stress can lead to positive adaptations, known as 'resilience' [7, 8]. This resilience can be defined as a process or phenomenon that reflects positive adjustments despite previous negative experiences [9]. The outcomes of ELS in humans and animal models are not only linked to stress intensity and duration, but also to the environment in which they are subsequently reared [10, 11]. For example, if the environment is neutral or positive the individual can enhance their ability to cope with stressful situations building resilience [10, 12-14]. Meanwhile, if the environment is insufficient or maladaptive, an individual will be more likely to develop vulnerability to diseases [15-19].

Zebrafish (*Danio rerio*) is recognized as a species that presents high genetic and physiological similarities to mammals [20, 21]. The long-term outcomes of ELS have been previously described in zebrafish: stress differently affects adult behavior depending on the animal's developmental stage and the stressors type/intensity/duration [22-24]. For example, social isolation at early-life stages results in isolated zebrafish showing decreased shoal cohesion but also decreased anxiety-like phenotypes in the open-field task [23].

The evidence, therefore, for development of resilience following early-life stress in zebrafish appears clear: stress "positively" affects two different behavioral domains by increasing working memory [24] and decreasing anxiety [22, 23]. However, the extent to which the measured behavioral endpoints represent 'positive' behavioral change is a matter of some debate. For example, although the increased time spent in the top and lit zones are often linked to decreased anxiety in zebrafish [25-29], these behavioral phenotypes can be associated to increase risk-taking due to animals' higher predation exposure [30]. Therefore, it may be that the observed 'positive' effects are indicative of impairment of impulse control, with the exposed animals being more 'risk prone'. This may have implications for the development of impulse control disorders and the role of early-life stress.

Here, we investigated whether the effects of ELS on decreased anxiety is linked to increased risk-taking or 'boldness', or on stress reactivity. In order to test this, we exposed larval zebrafish to either 7 or 14 days of chronic unpredictable early life stress (CUELS), using a previously described protocol [22] and reared them to adulthood. We then analyzed boldness and stress-reactivity responses as adults. Adult behavior was analyzed in the novel object boldness task and in the response to CAS in the novel tank task. Stress reactivity was induced by exposing the animals to conspecific alarm substance (CAS) [31, 32]. As well as behavioral endpoints, cortisol was examined to investigate the physiological responses to CAS following CUELS.

2. Material and Methods

2.1. Animal husbandry and experimental design

Larval AB wild-type zebrafish were bred in-house from multiple tanks (3 tanks per batch) by adding a small box with marbles to the breeding tank, then eggs were collected and kept in a petri dish (100 x 15 mm) until 7 days post fertilization (dpf). Afterwards, animals were transferred to a small plastic container (15×8×2 cm length x height x water depth) until they reach 14 dpf when animals were introduced to the re-circulating (Aquaneering, USA) system in groups of 40 animals per 1.4L. Juvenile animals (>30dpf) were kept in the re-circulating system in groups of 10 animals per 2.8 L until 7 months when fish were test behaviorally. During all life-stages animals were kept on a 14/10-hour light/dark cycle (lights on at 9:00 a.m.), pH 8.4, at 28 °C (±1 °C). Animals were fed twice a day during 5 to 10 dpf with rotifers during mornings and fry food (ZM-000, ZM Ltd.) at noon, meanwhile juvenile animals were fed with small brine shrimp in the mornings and three times with dry food depending on their age (10 – 20 dpf; ZM-100 and 20 – 28 dpf; ZM-200). Adult animals were fed with flake food (ZM-flake and ZM-300, ZM Ltd.) three times a day and adult brine shrimp during the mornings.

All behavioral tests were performed between 10:00 and 16:00 h. Animals took part in two different experimental designs: 1) novel object boldness task to analyze the boldness of fish exposed to the CUELS protocol; 2) exposure to CAS for 5 min and then novel-tank diving task to analyze the anxiety-like phenotype after acute stress exposure. For cortisol analysis, fish were exposed to CAS for 5 min, then fish were transferred to a new tank containing only system water for 15 min, the peak time for cortisol response after stressful situations in adult zebrafish [33]. Fish were killed using rapid cooling (submersion in 2°C water), their bodies were collected and immediately frozen in liquid nitrogen. Samples were kept at -80 °C for further analysis. Animals used in the novel object boldness task and novel-tank diving test were not used for sample collection and were euthanized using 2-phenoxyethanol from Aqua-Sed (Aqua-Sed™, Vetark, Winchester, UK). To ensure data reliability, two independent batches/replicates were tested ($n = 6 – 7$ per task and per group in each batch). Animals from each batch were ELS at different times, within a 2 weeks interval between batches. Researchers and technical staff were blind to the experimental conditions to avoid biased handling during the behavioral sessions and animals were randomly selected from one of four tanks for each experimental design.

The number of animals per group were calculated a prior (d = 0.35, power = 0.8, alpha = 0.05) following extensive published work from our laboratory using zebrafish as a translational model and considering our primary outcomes. Thus, the initial number of animals per group and primary outcome for each task were: novel object boldness task (n = 12/group; time spent close to the object) and novel-tank diving test (n=14/per group; time spent in the bottom zone). For the behavioral response, no animal was excluded from the statistical analysis. For cortisol analysis, 1 animal were excluded in total (1 detected as outlier due extreme values [>3*IQR]). All experiments were carried out following approval from the University of Portsmouth Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F].

2.2. Chronic unpredictable early-life stress (CUELS)

The CUELS protocol was performed from 7 dpf to 14 or 21 dpf, except for the group control animals that were not handled during these days. Animals were exposed to a variety of stressors as follows: individually transfer animal to well in a white 96-well plate for 45 minutes (social isolation); transfer 40 animals to a well in a 12-well plate for 45 minutes (overcrowding); light/dark cycle changes for 60 minutes (light/dark cycle); change animal to a new tank with new water 3 times (water transfer); transfer animal tanks to an incubator until the water temperature reaches 23 ºC for 30 minutes (cooling); transfer to an incubator until the water reaches 33 ºC for 30 minutes (heating); stir the water for 5 minutes using a Pasteur pipette (mechanical stirring); removal of water until fish body is exposed, and leave in this condition for 2 minutes (shallow water). The protocol for CUELS in zebrafish was performed as described in previous work [22] and the experiment schedule is described in **Table 1**.

2.3. Experiment 1 – Novel Object Boldness Task

Boldness is commonly defined as the willingness among individuals to take risks, especially in novel environments [34]. In zebrafish, this risk-taking behavior can be assessed by analyzing the amount of time that the fish explore a novel object [35, 36]. Here, to assess whether animals (n = 36) exposed to the CUELS protocol are more risk-taking or not, we tested boldness in a novel environment by measuring the time that individuals spend close to the object (2 cm distance around the object) and number of entries to the object zone. The object was made with dark modeling clay in the shape of a 15-cm falcon tube and the tank dimensions were 27 cm x 36 cm x 10 cm (W x L x D) (**Fig. 1a**). Animals were recorded for 6 minutes and animals were immediately culled after the protocol. Data for the novel object boldness task was analyzed using the behavioral tracking software ANY-maze™.

2.4. Experiment 2 – Stress-reactivity

In the second experiment, we investigated how animals exposed to CUELS respond to an acute stressor later in life by analyzing their adult behavioral response in the novel-tank diving test and measuring their cortisol levels.

2.4.1. Conspecific alarm substance (CAS)

The CAS is a fear-cue that has been successfully used to trigger stress-related responses at physiological and behavioral levels in different fish species [25, 31, 32, 37-40]. Briefly, CAS exposure was performed by individually exposing fish to 3.5mL/L of CAS preparation for 5 minutes. The CAS preparation is obtained by damaging epidermal cells with 10 shallow slices on both sides of the phenotypically similar donor fish body using a razor blade. 10 mL of distilled water was them added into a Petri dish and mixed to fully cover the fish's body. Fish were previously culled using rapid cooling (submersion in 2°C water) for the preparation of CAS. For the behavioral analysis, a total of 21 fish were exposed to CAS per experimental day (2 batches in total), thus, CAS substance was prepared 3 times and in each CAS extraction $n = 2 – 3$ fish per group were exposed to the same preparation (1.05 mL was added in a 300 mL beaker). Similarly, for cortisol analysis, a total of 18 fish were exposed to CAS and 2 donor fish were used (total of 20 mL of CAS preparation). All procedures were performed on ice and controlled to avoid drawing blood, to avoid contamination and keep CAS stability in ice. CAS substance was used at no longer than 15 min after preparation [28, 31, 37, 41]. After CAS exposure, fish were tested in the novel tank diving test or transferred to a new tank for 15 min for further cortisol analysis.

2.4.2. Novel Tank Test

The novel tank test is a common behavioral task used to measure locomotion, boldness and anxiety-related phenotypes stimulated when fish are introduced to a new environment. This test was previously used to characterize several drug responses showing a cross-species behavioral response similarities [25-28]. Immediately after CAS exposure, animals (*n* = 42) were individually placed in a novel tank (20 cm x 17.5 cm x 5 cm; L x H x W) containing 1 L of aquarium water (**Fig. 2a**). Control animals (*n* = 42) were removed from their original housing groups prior the novel tank test. Behavioral activity was analyzed using the Zantiks automatic system (Zantiks Ltd., Cambridge, UK) for 6 minutes [28, 42, 43]. For further analysis, the tank was virtually divided in three areas (bottom, middle and top), time spend on bottom was used to measure anxiety-related phenotypes and distance traveled was used to evaluate locomotion during the test.

2.4.3. Whole-body cortisol

To assess hormonal stress-reactivity response, 7-month-old fish (*n* = 36) were killed by immersion in ice-water (2ºC) after 15 min of the exposure to CAS (stressed groups) or immediately after removal of their housing tanks (controls). The whole bodies were snap-frozen using liquid nitrogen and kept in −80ºC until assay. Cortisol levels were evaluated using a human salivary cortisol ELISA kit (Salimetrics Salivary Cortisol ELISA, Stratech, Cambridge, UK) [42, 44]. Samples were homogenized in 5 ml ice-cold PBS. 5 ml of diethyl ether was added, then centrifuged (3500 x g) for 30 minutes, and the organic layer (top layer) was removed. The process was repeated, and the diethyl ether was evaporated overnight. The resulting cortisol was reconstituted in 1 ml ice cold PBS and then ELISA was performed in 96-well plates as manufacturer's instructions. Cortisol concentrations (ng/g^{-1}) were determined from optical density (OD) readings and compared against manufacturer’s standards. All samples were run in duplicate and the inter- and intra-assay coefficients of variation were determined.

2.5. Statistics

Data were analyzed in GraphPad Prism® and the results were expressed as means ± standard error of the mean (S.E.M). Normality of data and homogeneity of variance were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. One-way ANOVA was used to analyze the effects of the CUELS in the novel object boldness test. Furthermore, the interaction between

CUELS and CAS in the novel tank-diving test and cortisol levels was assessed using two-way ANOVA with CUELS (three levels – 0, 7 and 14 days of CUELS) and CAS (two levels – control *vs.* stress) as the fixed factors. Tukey's test was used as post-hoc analysis, and results were considered significant when $p \leq 0.05$.

3. Results

3.1. Behavioral effects of CUELS in a novel object boldness test and stress-reactivity response

Fig. 1b shows the effects of CUELS in the novel object boldness task. No significant effect was observed between groups for the time spent close to the object ($F_{(2,33)} = 0.4734$; $p = 0.6270$) or for the number of entries in the object zone ($F_{(2,33)} = 0.8623$; $p = 0.4315$). Regarding the response of early-life stressed animals to CAS in the novel tank diving task (**Fig. 2b**), no significant interaction (CUELS *vs.* CAS) ($F_{(2,78)} = 0.998$; $p = 0.9060$), CUELS ($F_{(2,78)} = 0.4589$; $p = 0.6337$) or effect of CAS ($F_{(1,78)} = 0.2129$; $p = 0.6458$) was observed. However, two-way ANOVA yielded a significant interaction (CUELS *vs.* CAS) ($F_{(2,78)} = 7.447$; $p^{**} = 0.0011$), CUELS ($F_{(2,78)} = 10.78$; $p^{****} < 0.001$) and effect of CAS ($F_{(1,78)} = 14.38$; $p^{***} = 0.003$) for the time spent in the bottom zone. A significant decrease in time spent in bottom was observed for animals exposed to CUELS for 7 days compared to control non-stressed ($p^{**} = 0.0056$) and 14 days CUELS ($p^{***} = 0.0009$). CAS significantly increased the time spent in bottom compared to two non-stressed groups, 0 ($p^{*} = 0.0149$) and 7 days of CUELS ($p^{****} < 0.0001$), except when compared to 14 days CUELS ($p = 0.0669$). Importantly, only animals exposed to 14 days of CUELS + CAS had a significant decrease in the time spent in the bottom zone when compared to 0 days of CUELS + CAS ($p^{*} < 0.0046$).

3.2. Stress-reactivity and cortisol response in animals previously exposed to CUELS

The cortisol response of animals exposed to early-life stress, and their stress-reactivity response is displayed in **Fig. 3**. Although no significant effect was observed for interaction (CUELS *vs.* CAS; $F_{(2,25)} = 0.7612$; $p = 0.4776$) or CUELS ($F_{(2,25)} = 0.8889$; $p = 0.4237$) through two-way ANOVA analysis, a significant effect of CAS ($F_{(1,25)} = 52.69$; $p^{****} < 0.0001$) was observed. A post-hoc Tukey's test showed that a significant effect was observed comparing non-stressed groups (0, 7 and 14 days of CUELS; $p^{*}= 0.0189$, $p^{***}= 0.0002$ and $p^{**}= 0.0028$, respectively) to 0 days of CUELS + CAS. No significant difference was observed between non-stress animals or between CAS exposed animals.

4. Discussion

In this study we aimed to investigate the impact of early-life stress on stress reactivity and boldness. We found that there were no differences between controls and 7 or 14-day CUELS exposure in approach to a novel object, suggesting that boldness is differently expressed by 7 and 14-days CUELS animals depending on context. In addition, we found that fish exposed to 7 days CUELS showed increased exploration in the novel tank test, when exposed to CAS although there is a significant response compared to their own group, fear/anxiety-related behavior were not similar to control response. Regarding zebrafish exposed to 14 days of CUELS, when animals were exposed to CAS no behavioral change was observed, indicating that both groups may have a disrupted stress-reactivity response in terms of behavioral analysis. However, in terms of cortisol-responses after exposure to an acute stressor (CAS) no significant differences were found in ELS animals. Overall, it appeared that 7 days of CUELS building resilience and 14 days of CUELS (Fontana et al., 2021) also affect stress-reactivity responses showing decreased response to CAS compared to controls. Thus, for the first time, we showed that 7 and 14 days of CUELS may have a negative impact on the animal's response to fear cues.

Boldness and risk-taking can be assessed by several tasks (*e.g.* the shelter task, novel tank diving test, new object boldness test and light/dark test) in zebrafish and the choice of test has a large impact on boldness-related behavior. The interpretation of this behavior can be highly contextual varying between environment, conditions, and individuals [45-48]. Here, we used the novel tank and novel object boldness test to investigate the boldness of fish previously exposed to a CUELS protocol for different durations (7 and 14 days of CUELS starting from 7 dpf). As previously shown (Fontana et al., 2018), animals subjected to CUELS for 7 days showed decreased time spent in bottom which can reflect in animals decreased anxiety-like behavior. The decreased time spent in bottom can be also directly correlated to boldness [49]; however, in the novel object boldness test, ELS fish did not show any difference in the time spent and number of times approaching the object. Bold individuals are usually explorative and 'risk-taking', while shy individuals are characterized by low exploration and a passive response [34]. The differences between boldness patterns were previously explored in zebrafish, and there is considerable variation in behavioral phenotypes on different tasks [50]. The animals exposed to CUELS for 7 days were previously measured in two behavioral tasks to assess anxiety/boldness responses, and we saw consistency in their behavioral patterns across tasks were animals showed increased boldness and decreased anxiety-like behavior [22]. It remains unclear, however, whether these responses are linked predominantly to the animals' boldness or risk-taking behavior, as no increased boldness was observed in the novel object task, suggesting that animals exposed to CUELS show different patterns of behavior depending on context.

To further evaluate if those fish would have increased risk-taking, we exposed fish to CAS for 5 minutes, a treatment that is known to induce increased fear and anxiety-related responses including increasing the time that fish spend in the bottom and decreasing their activity. When a fear-cue (CAS) is presented, fish exposed to CUELS for 7 days have a normal anxiogenic response compared to control fish. CAS is an effective acute stressor which is produced and stored in the epidermal "club" cells, and released into the water after skin injuries provoked by predator attacks [51, 52]. The different concentrations and effects of this molecule in zebrafish fear and anxiety-related behavior was first described by Speedie and Gerlai [31]. For example, in the light-dark test zebrafish exposed to CAS for 5 minutes increased scototaxis (preference for dark areas)[37, 40] which is a behavioral change often observed after the exposure to anxiogenic drugs [53]. CAS also affects fish social behavior, increasing zebrafish cohesion in a shoal test [38], which is known to confer multiple advantages in reducing predation and facilitating foraging in other fish species [54]. These data support CAS as being efficient for inducing fear-related responses.

As previously discussed, early-life stress protocols have produced ‘resilient’ effects in adults, showing no clear negative effects later in life [22-24]. This has included data from animals exposed to 7 and 14 days of CUELS and tested later at adult stages. However, here for the first time we saw some evidence that adult animals exposed to the CUELS protocol for 7 and 14 days may have a disrupted behavioral response to stress, by showing no differences in the time spent in bottom when exposed to the CAS stressor. Blunted responses to stress stimuli have previously been observed in children that experienced ELS and the underlying mechanism is related to inhibition of hypothalamic–pituitary–adrenal axis (HPA) axis reactivity [55, 56]. Although the mechanisms in which ELS alter HPA activity still unknown, hypothesis involving altered amygdala activity to different responses to threats later in life have been proposed as a potential mechanisms underlying lack of stress-reactivity in mice and humans [57]. Thus, the mechanisms underlying decreased stress responses after CUELS exposure may have association with alterations in the zebrafish pallial amygdala, however further investigation looking at other HPI associated pathways and brain activity are still necessary.

Importantly, the analysis of behavioral responses during CAS exposure is often used to evaluate differences in stress response being complementary to the animals’ response to CAS in the novel tank [37]. Because here we did not evaluate this behavioral response, being a limitation of our study, future analysis during CAS exposure can help to elucidate if CUELS may have negative or positive effects on adult phenotypes. Other limitations of this study include that only two behavioral tasks were used to evaluate boldness-like behavior (novel tank and novel object boldness task) using different animals in each test, limiting the correlational analysis between tasks in animals exposed to CUELS protocol. Moreover, further studies looking at other behavioral tasks in an unfamiliar environment with no shelter (open field) and in the presence of a shelter [58], combined to analysis of behavior in ‘dangerous’ situations in the presence of a predator [59] may help to better understand the fully complexity of boldness, risk-taking and anxious behavior in zebrafish exposed to CUELS. Besides the limitations presented in this study, our study strength the hypothesis that boldness and anxiety-like behavior may be differently modulated depending on environment, which is consistent in animals exposed to CUELS. In addition, the novel tank diving task is a reliable behavioral task to assess decreased anxiety-like behavior in animals exposed to 7 days of CUELS and to evaluate zebrafish response to acute stress increasing anxiety.

Here, however, we saw no difference from controls in the cortisol response for 7- and 14-day CUELS animals exposed to CAS. It therefore appears that although the physiological response to the stressor was normal (no different to controls) CUELS animals show a blunted behavioral response to CAS. This is potentially very interesting, as it indicates a negative impact of CUELS that could be psychological in nature, and potentially more complex than a simple alteration in HPI activation. Additionally, it may be that the CAS represents a strong stressor that may cause a physiological ceiling effect.

5. Conclusion

Here we reported, for the first time, that ELS-induced increase boldness/decrease anxiety-like behavior (7 days CUELS) may be differently expressed depending on the task. These conclusions are based on, although increased time spent in the top of a tank can be associated with increased boldness, no significant difference was found in the novel object boldness test. We also showed a potential negative impact of CUELS for 7 and 14 days, where animals showed a blunted behavioral response after being exposed to an acute stressor. However, animals ELS for 7 or 14 days showed normal increased cortisol response to CAS exposure indicates a negative impact of CUELS that could be psychological in nature, and potentially more complex than a simple alteration in HPI activation. Further studies looking at other behavioral tasks when presented with an array of stress challenges, coupled with a more in-depth analysis of molecular markers, would help researchers to understand the mechanisms underlying the positive and negative effects of CUELS. Overall, these data suggest that CUELS can positively or negatively modulate behavior depending on context, where all ELS groups showed blunted increased anxiety-like behavior in the novel tank diving test.

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Conflict of Interest

The authors declare no conflict of interest exists.

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Table 1

Day	1st Stressor (between 7:00 – 12:00)	2nd Stressor (between 12:00 – 16:00)
1	10:00 – Heating 33ºC *(30 min)*	16:00 – Mechanical stirring *(5 min)*
2	07:00 – Lights on *(60 min)*	14:00 – Social isolation *(45 min)*
3	11:00 – Overcrowding *(45 min)*	16:00 – Cooling 23ºC *(30 min)*
4	09:00 – Shallow water *(2 min)*	13:00 – Water change *(3 times)*
5	12:00 – Mechanical stirring *(5 min)*	14:00 – Heating 33ºC *(30 min)*
6	11:00 – Lights off *(60 min)*	12:00 – Water change *(3 times)*
7	09:00 – Cooling 23ºC *(30 min)*	15:00 – Social isolation *(45 min)*
8	11:00 – Shallow water *(2 min)*	16:00 – Overcrowding *(45 min)*
9	09:00 – Mechanical stirring *(5 min*)	13:00 – Water change *(3 times)*
10	10:00 – Heating 33ºC *(30 min)*	15:00 – Overcrowding *(45 min)*
11	08:00 – Lights on *(60 min)*	16:00 – Shallow water *(2 min)*
12	10:00 – Cooling 23ºC *(60 min)*	12:00 – Social isolation *(45 min)*
13	12:00 – Mechanical stirring *(5 min)*	13:00 – Lights off *(60 min)*
14	11:00 – Water change *(3 times)*	15:00 – Heating 33ºC *(30 min)*

Table 1. Procedure of the unpredictable chronic early life stress protocol in zebrafish larvae from 7 days post fertilization (dpf) to 21 dpf (adapted from Fontana, Gibbon [22])

Figures

Figure 1. Novel object boldness task response of animals previously exposed to 7 or 14 days of CUELS. Data were represented as mean ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test multiple comparison test (*n = 12 per group*).

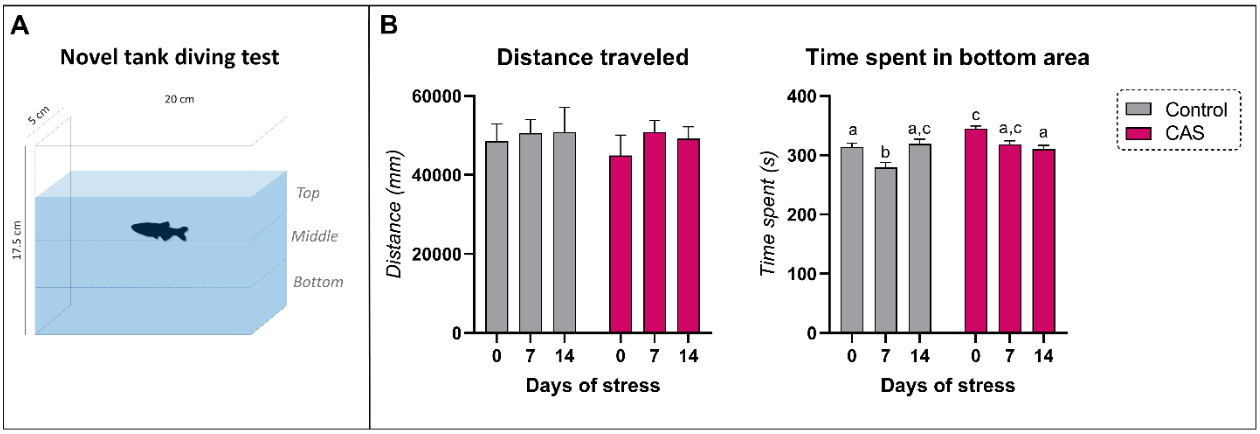

Figure 2. Behavioral response of adult animals acutely stressed to CAS and previously exposed to CUELS protocol for 7 or 14 days in the novel tank diving task. Data were represented as mean ± S.E.M and analyzed by two-way ANOVA (CAS and CUELS as factors), followed by Tukey's test multiple comparison test (*n = 14 per group*).

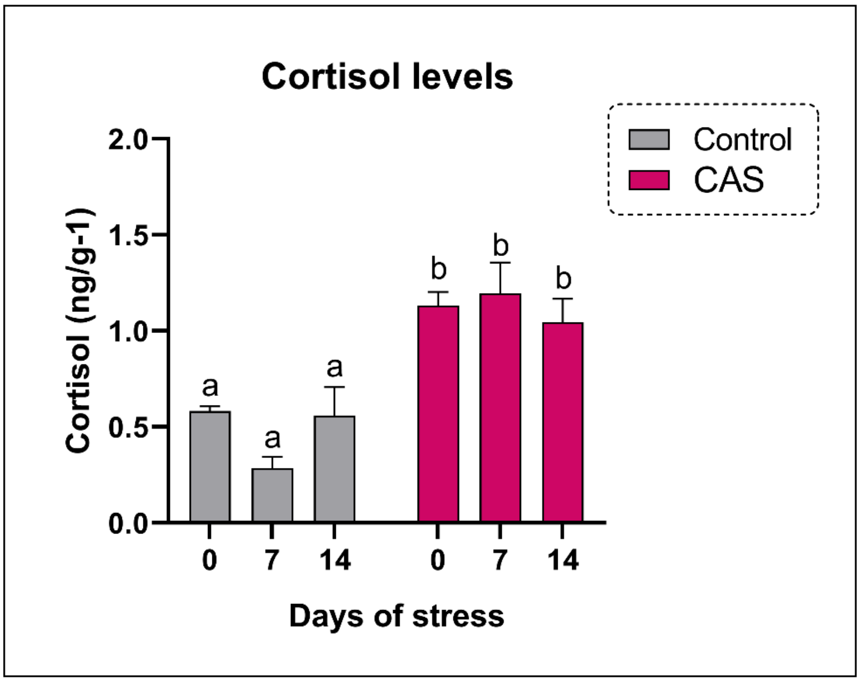

Figure 3. Control *vs.* CAS cortisol levels for adult animals previously exposed to CUELS protocol. Data were represented as mean ± S.E.M and analyzed by two-way ANOVA (CAS and CUELS as factors), followed by Tukey's test multiple comparison test (*n = 5 – 6 per group*).