Chronic unpredictable early-life stress (CUELS) protocol: early-life stress changes anxiety levels of adult zebrafish

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Abstract

Early-life stress can lead to two different behavioral responses: (1) increased susceptibility to psychiatric disorders or (2) resilience. Here, we created a chronic unpredictable early-life stress (CUELS) protocol to assess the effects of early experiences in adult zebrafish. Animals were exposed to mild stressors twice a day and the duration was varied between groups (0, 1, 3, 7 and 14 days of stress). The stressor consisted of light/dark cycle changes; social isolation; overcrowding; water changes; water cooling; mechanical stirring; water heating; and immersion in shallow water. Behavior was assessed at young stages (21 days post-fertilization – open field analysis) and adulthood (4-months-old - novel tank diving test, light/dark task, shoaling, free movement pattern Y-maze and Pavlovian fear conditioning). Cortisol levels were assessed to evaluate the impact of CUELS in the HPA axis. Zebrafish exposed to 7 days of CUELS showed a decreased anxiety-like phenotype in two behavioral tasks, presenting increased time spent in top and decreased time spent in the dark area. Animals exposed to 14 days of CUELS showed an opposite anxious phenotype compared to 3 and 7 days of CUELS. No significant changes were observed in memory and cognition, social behavior and cortisol levels. In general, 7 days of CUELS protocol decreased anxiety in young and adult zebrafish, and could be used to understand the mechanisms underlying early-life experiences-derived alterations in neural circuits of anxiety.

Keywords: anxiety; cognition; early-life stress; memory; resilience; social behavior

1. Introduction

The negative consequences of Early-Life Stress (ELS) are well recognized in contemporary perspectives on mental health. Childhood seems to be particularly sensitive to environmental disturbances that are linked to changes in adult behavior, such as development of mood, anger, anxiety, and substance abuse disorders (Anda et al., 2006; Heim et al., 2008; Repetti et al., 2002; Turner and Lloyd, 2003). Numerous epidemiologic and clinical studies have provided compelling evidence for a strong association between various forms of ELS and neuropsychiatric disorders (Carr et al., 2013; Schiavone et al., 2015; Syed and Nemeroff, 2017). For example, there is a strong dose–response relationship between the frequency of childhood adversities (sexual abuse, physical abuse, witnessing paternal violence) and general mental health problems in adulthood (Edwards et al., 2003). Although ELS experiences are generally associated with negative impacts in adulthood, ELS can also have positive impacts in later life by building resilience (*i.e.* adapting well in the face of adversity, trauma, tragedy, threats or even significant sources of stress) (Russo et al., 2012; Sapolsky, 2015). Changes in physiology and behavioral responses (e.g. increased or decreased anxiety-like phenotypes) are also observed in animal models of ELS (Goldman-Mellor et al., 2012; Maniam et al., 2014; O'Mahony et al., 2009).

The effects of ELS on adult metabolism and neural circuits are various, including neuroendocrine (increased reactivity of hypothalamic-pituitary-adrenal axis) (Macri et al., 2008), neurochemical (higher level of catecholamines)(De Bellis et al., 1999), electrophysiological (decreased power across the EEG spectrum)(McFarlane et al., 2005) and brain morphology (smaller volumes of the lateral orbitofrontal cortex and caudate, and decreased cortical thickness in multiple areas such as the insula bilaterally)(Saleh et al., 2017) changes. For example, adults that experienced early-life adversity present a consistently high, flat cortisol trajectory instead the typical physiological response (increased cortisol levels in response to stress and then a return to the baseline) (Taylor, 2010). Moreover, ELS can differently affect physiology depending on the adult behavior phenotypes and associated psychiatric disorders. For example, sexually abused girls with history of depression and suicidal behavior have increased urinary concentration of catecholamines

88   but similar cortisol response to corticotropin-releasing hormone injections compared to controls (De

89   Bellis et al., 1994). Meanwhile, children and adolescents exposed to a trauma, and who show

90   persistent Post-Traumatic Stress Disorder (PTSD) symptoms, show elevated circulating

91   norepinephrine, but low peripheral cortisol levels, suggesting a dysfunction of homeostatic

92   responses to stress (Pervanidou, 2008; Pervanidou and Chrousos, 2012). Thus, ELS seems to result

93   in lasting autonomic dysfunction, permanently affecting hypothalamic pituitary-adrenal axis (HPA)

94   function both during the non-activated state, and in its response to stressful situations. Collectively,

95   this dysfunction could be a possible mechanism underlying different behavioral patterns observed,

96   and psychiatric disorders that often occur, in adulthood. Despite considerable knowledge about the

97   impact of ELS in adulthood, the data about the underlying mechanisms of the elicited effects are

98   still conflicting. In humans, studying the pathogenesis of ELS-induced problems would be both

99   impractical and unethical; thus, the characterization of new ELS animal models play an important

100   role in advancing our understanding of ELS effects on the developmental trajectory. Zebrafish are

101   an important model organism, sharing genetic (70% of genes have one human orthologue) and

102   endocrine (cortisol is the primary hormone response) homology with humans. The behavioral

103   repertoire of zebrafish is well-characterized, and their quantification has been (for the most part)

104   fully automated. For example, there are well-defined operational definitions of social behavior

105   (Saverino and Gerlai, 2008; Suriyampola et al., 2016), anxiety-like behavior (Stewart et al., 2011)

106   and memory and cognition (Bailey et al., 2015). In adult zebrafish, unpredictable chronic stress

107   (UCS) impairs both cognitive responses and anxiety levels, and increases the level of cortisol (Piato

108   et al., 2011). Moreover, we have previously shown that young zebrafish have an immediate

109   elevation of cortisol levels after stressful manipulations, and exposure to short-term stress in 1-

110   month old animals can affect behavior later in life (Fontana et al., 2020). In summary, zebrafish

111   are a suitable model organism to validate an ELS model using chronic unpredictable stress.

112   Overall, prolonged stress in early life is strongly associated to the increased risk of

113   developing psychiatric disorders (Schiavone et al., 2015). Although many UCS protocols for adults

114   have been described in literature, an unpredictable stress protocol in larvae has never performed and

115   could be used as valid model for ELS. Zebrafish have high-throughput screening potential and share

116   the autonomic stress-response (i.e. cortisol-release), and the use of zebrafish to understand the

117   impacts of ELS from a translational perspective can benefit the discovery of new alternative

118   treatments and ultimately help to understand the mechanisms underlying how ELS impact later-life.

119   Thus, here we designed a chronic unpredictable early-life stress (CUELS) for zebrafish larvae (7

120   dpf) and investigated the effects of this protocol on 4-month-old adult animals. We also investigated

121   changes to basal cortisol levels as a marker of physiological stress.

122

123   **2. Material and Methods**

124   *2.1. Animal husbandry and experimental design*

125   Larval AB wild-type zebrafish were bred in-house from multiple tanks (5 tanks per batch)

126   by adding a small container with marbles, the eggs were then collected and kept in a petri dish until

127   5 dpf. Animals were kept in an incubator (28.5°C), dead animals were removed and 50% of the

128   water was replaced daily to keep ideal oxygen levels. At larval stage animals were transferred for a

129   small plastic container (15×8×2 cm length x height x water depth) until they reach 14 dpf. Animals

130   were introduced to the re-circulating (Aquaneering, USA) system at 15 dpf in groups of 40 animals

131   per 1.4 L. Zebrafish larvae (5 to 10 dpf) were fed twice a day with rotifers during the mornings and

132   fry food (ZM-000, ZM Ltd.) at noon. Young zebrafish were fed three times a day with ZM-100 (10

133   – 20 dpf; ZM Ltd.) and ZM-200 (20 – 28 dpf; ZM Ltd.) and small brine shrimp at mornings. Adult

134   animals (>28dpf) were fed with flake food (ZM-flake and ZM-300, ZM Ltd.) three times and adult

135   brine shrimp during the mornings. The sex of adult zebrafish was determined visually by the

136   experimenter and independently verified by a trained zebrafish technician. The sex of the animals

137   was determined blind to the experimental groups.

138   Adult animals were kept in the re-circulating system on a 14/10-hour light/dark cycle (lights

139   on at 9:00 a.m.), pH 8.4, at 28 °C (±1 °C) in groups of 10 animals per 2.8 L until 4 months

140   fertilization (adult age). All behavioral tests were performed between 10:00 and 16:00 h. Adult

141   zebrafish were submitted to a shoal task and immediately transferred to the novel tank task or light-

dark task (anxiety-related tasks). 24 h later fish were randomly selected **(see below)** from one of

four housing tanks and tested individually in the free movement pattern (FMP) Y-maze or Pavlovian

fear conditioning task (memory and cognition related-tasks). After behavioral recording fish were

killed using rapid cooling (submersion in 2°C water) and their bodies were collected and

immediately frozen in liquid nitrogen (Fig.1 contains the experimental design timeline). Samples

were kept at -80 °C for further analysis. Animals used in the Pavlovian fear conditioning 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 were tested (*n*

= 8 per task and per group in each batch).

We predicted medium to large effect sizes (d = 0.35, power = 0.8, alpha = 0.05) following

extensive published work from our laboratory and considering our primary outcomes. Thus, the

initial number of animals per group and primary outcome for each task were: open field test

(n=24/per group; thigmotaxis), novel tank test (n = 18/group; time spent in the top), light/dark test

(n = 16/group; time spent in the dark), Pavlovian conditioning (n = 16/group; preference for

conditioned stimulus) and FMP Y-maze (n = 16/group; alternations). The sample size for the

juvenile open field was 120 and no animal was excluded. Initial sample size used for the novel tank

test was n = 92 (final sample size n = 84 following removal of non-responders/outliers [see below]).

For the light/dark test, an initial sample of n = 80 was recruited (final sample size n = 75). For the

Pavlovian conditioning n = 80 was recruited and 4 animals were excluded (final sample size n=76).

For the FMP Y Maze, we initially recruited n = 80 and 13 were excluded leaving n = 67. Finally,

we predicted a medium effect size for the shoaling test, and calculated that we required n = 8

shoals/group (primary outcome: shoal area) based on extensive previous published work from our

group and others using this test. No shoals were excluded meaning that a total of n = 160 fish was

used for the shoaling assay.

To avoid biased handling, the researchers and technical staff were blind to the experimental

groups while performing the behavioral tasks. All behavioral testing was carried out in a pseudo-

randomized order, with fish randomly tested from one of four group housing tanks. Randomization

169 was achieved by selecting one fish from each tank in a different order each time (ie A, B, C, D; B,

170 C, D, A; etc). Once all data were collected and screened for aberrant data value (ie, values of '0' or

171 extreme values [>3*IQR] for behavioral parameters indicate non-engagement with the task or

172 software/tracking problems, and are therefore excluded), the data was analyzed in full (< 1% of the

173 dataset). All experiments were carried out following approval from the University of Portsmouth

174 Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals

175 (Scientific Procedures) Act, 1986) [PPL: P9D87106F].

176

177 *2.2. Chronic unpredictable early-life stress (CUELS)*

178 Animals were subjected to a variety of chronic stressors, including changes in light/dark

179 cycle; social isolation; overcrowding; water changes; water cooling; mechanical stirring; water

180 heating; and immersion in shallow water. The CUELS protocol and stressors were chosen based

181 upon previous experiments using adult zebrafish (Piato et al., 2011). The protocol was performed

182 for 1, 3, 7 or 14 days of stress using young animals (7 days post-fertilization (dpf)) and the

183 experiment schedule is described in **Table 1**. The stressors were administered as follows:

184 individually transfer animal to well in a white 96-well plate for 45 minutes (social isolation – **Fig.**

185 **2A**); transfer 40 animals to a well in a 12-well plate for 45 minutes (overcrowding – **Fig. 2B**);

186 light/dark cycle changes for 60 minutes (light/dark cycle – **Fig. 2C**); change animal to a new tank

187 with new water 3 times (water change – **Fig. 2D**); transfer animal tanks to an incubator until the

188 water temperature reaches 23 ºC for 30 minutes (water cooling – **Fig. 2E**); transfer to an incubator

189 until the water reaches 33 ºC for 30 minutes (water heating – **Fig. 2F**); stir the water for 5 minutes

190 using a Pasteur pipette (mechanical stirring – **Fig. 2G**); transfer animal to tanks with water removed

191 such that the body is exposed, and leave in this condition for 2 minutes (immersion in shallow water

192 – **Fig. 2H**).

193

194 *2.3. Open field and survival rates of young animals*

195   To investigate whether the ELS affected young animals, locomotion patterns were analyzed

196   when animals were 22 days-old (one-day after the 14-day stress protocol for all groups) by using

197   the Zantiks MWP automated tracking system for larval zebrafish (Zantiks Ltd., Cambridge, UK).

198   Animals were individually transferred to a 6-well plate (well diameter 34.8 mm) and their

199   locomotion and anxiety-related behavior was recorded for 5 minutes as described in (Ingebretson

200   and Masino, 2013). The distance traveled was used to investigate changes in locomotion;

201   meanwhile, time in outer zone (5.5 mm) was used to assess any anxiety-related changes

202   (thigmotaxis). After the behavioral protocol, animals were transferred back to their home tanks and

203   grown to 4 months old to analyze impact of the stress protocol in adulthood. The survival rates were

204   also analyzed to investigate if the CUELS protocol was affecting the animals' survival during the

205   21 dpf.

206

207   *2.4. Shoaling test*

208   Fish (4-fish per shoal) were simultaneously placed in the test tank (25×15×10 cm length x

209   height x width) and group behavior was analyzed for 5 minutes (Canzian et al., 2017; Green et al.,

210   2012; Muller et al., 2017; Schmidel et al., 2014). The Zantiks AD unit (Zantiks Ltd., Cambridge,

211   UK) was used to record the fish behavior and videos were exported to Image J 1.49 software to

212   assess shoaling using screenshots taken every 15 s during the 5-min trials (20 screenshots *per* trial)

213   (Green et al., 2012; Schmidel et al., 2014). Screenshots were calibrated proportionally to the size of

214   the tank to allow the quantification of total inter-fish distance and shoal area.

215

216   *2.5. Novel tank diving test*

217   The novel tank test is used to measure exploratory, locomotor and anxiety-like phenotypes

218   in zebrafish. It is sensitive to both, anxiolytic and anxiogenic drugs (Egan et al., 2009; Kalueff et

219   al., 2013; Levin et al., 2007; Wong et al., 2010). Animals *(n = 16 – 18)* were placed individually in

220   a novel tank (20 cm length x 17.5 cm height x 5 cm width) containing 1 L of aquarium water.

221   Behavioral activity was analyzed using the Zantiks automatic system (Zantiks Ltd., Cambridge,

222   UK) for 5 minutes (Egan et al., 2009; Parker et al., 2012; Rosemberg et al., 2012). The tank was

223   separated in three virtual areas (bottom, middle and top) to provide a detailed evaluation of vertical

224   activity. The following endpoints were measured: total distance traveled, and time spent in top zone.

225

226   *2.6. Light-dark preference task*

227   The light-dark preference task has been extensively used to screen anxiolytic drugs and

228   investigate mechanisms of anxiety in different animal models such as zebrafish (Gerlai et al., 2000;

229   Maximino et al., 2010). The light-dark preference task was performed in a black tank (20 cm length

230   x 15 cm height x 15 cm width) divided into two equally sized partitions where half of the tank area

231   contained a bright white light and the other area was covered with a black partition to avoid light

232   exposure. Animals *(n = 14 – 16)* were place individually into the behavioral apparatus and its

233   activity was analyzed using Zantiks AD automated tracking systems (Zantiks Ltd., Cambridge, UK)

234   for 5-min to determine the time spent in dark area (Blaser and Rosemberg, 2012; Maximino et al.,

235   2010; Mezzomo et al., 2016).

236

237   *2.7. FMP Y-maze test*

238   The FMP Y-maze is used to investigate a series of discrete choices as would be the case in

239   a series of trials in a T-maze, or in a two-choice guessing task (Cleal and Parker, 2018; Fontana et

240   al., 2019a; Fontana et al., 2019b; Cleal et al., 2020). Previously, we have seen showed that

241   alternations are reduced following administration of memory-impairing drugs (eg MK801 and

242   scopolamine), suggesting that alternations are indicative of working memory (Cleal et al., 2020).

243   Fish *(n =12)* were individually placed in a Y-shaped maze (6.5 cm length x 1 cm width; three

244   identical arms at a 120º angle from each other; standard equipment with the Zantiks AD unit) with

245   white opaque walls and recorded for 1 hour. Ambient light (1 lux) allowed some visibility in the

246   maze, but there were no intra-maze cues. During the task, left- or right-turn choices were organized

247   in behavioral tetragrams (blocks of four trials) and to assess zebrafish search strategies we used the

248   number of alternations (rlrl + lrlr) and repetitions (rrrr + llll) as a proportion of total number of turns

249 which are highly expressed during a 1-hour recording (% of total turns) (Gross et al., 2011). The

250 use of the overlapping tetragrams was made to avoid randomness analysis, based on previous studies

251 that showed increased randomness can be observed when sequence of choices are restricted to two

252 or three choices (Frith and Done, 1983).

253

254 *2.8. Pavlovian fear conditioning*

255 Pavlovian fear conditioning is a behavioral task used to investigate learning and memory in

256 which animal organisms learn to predict aversive event (Maren, 2001). Zebrafish *(n =14 - 16)* were

257 individually placed in one of four lanes of a tank (25 cm length x 15 cm, 1 L of water) for 1 hour

258 (Brock et al., 2017; Cleal and Parker, 2018; Valente et al., 2012). Fish were habituated for 30-min

259 in the test environment, which comprised a half check and half grey base screen (position switched

260 every 5-min). Baseline preference was established using the last 10-min baseline preference by

261 assessing the time spent in the tank areas. Baseline was followed by a conditioning phase in which

262 a conditioned stimulus (CS+; full screen of "check" or "grey", randomized between each batch)

263 was presented for 1.5-s and followed by a brief mild shock (9 V DC, 80ms; unconditioned stimulus

264 (US)). Subsequently, an 8.5-s of inter-trial interval (ITI) of the non-CS (CS−) exemplar was

265 presented at the bottom of the tank. The CS+/US was repeated eight times. Finally, avoidance of

266 CS+ was assessed by presenting the baseline screen (CS+ and CS− simultaneously) for 1-min, and

267 switching positions after 30-s. The retention index was calculated by the following formula:

268 $retention\ index = (baseline - probe) - 1$.

269

270 *2.9. Whole-body cortisol*

271 For the analysis of cortisol levels, 4-month-old fish were killed by immersion in ice-water

272 after the behavioral protocols (except fish tested in the Pavlovian fear conditioning). The whole

273 bodies were snap-frozen using liquid nitrogen and kept in −80C until assay. Cortisol levels were

274 assessed using a human salivary cortisol ELISA kit (Salimetrics Salivary Cortisol ELISA, Stratech,

275 Cambridge, UK) as previously described (Cachat et al., 2010; Parker et al., 2012). Samples were

276 homogenized in 5 ml ice-cold PBS. 5 ml of diethyl ether was added, and samples were centrifuged

277 (3500 x g) for 30 minutes, and the organic layer (top layer) was removed. This process was repeated

278 to remove all of the cortisol-containing layer, and then the diethyl ether was evaporated overnight.

279 The resulting cortisol was reconstituted in 1 ml ice cold PBS and the ELISA was then performed in

280 96-well plates as per the manufacturer's instructions. Cortisol concentrations (ng/g^{-1}) were

281 determined from OD readings compared against manufacturer's standards. All samples were run in

282 duplicate and the inter- and intra-assay coefficients of variation were determined.

283

284 *2.10. Statistics*

285 Data from the Y-maze protocol was obtained as number of entries into each arm (1, 2, 3 and

286 middle section 4) across a 1-hour trial. To analyze the data according to left and right turns in 10-

287 minute time bins, raw data was processed using the Zantiks Y-maze Analysis Script created

288 specifically for this purpose (available from: https:/

289 /github.com/thejamesclay/ZANTIKS_YMaze_Analysis_Script). Subsequently, data were analyzed

290 in IBM SPSS® Statistics and normality of data and homogeneity of variances were analyzed by

291 Kolmogorov-Smirnov and Bartlett's tests, respectively. The results were expressed as means ±

292 standard error of the mean (S.E.M). To assess whether there were any effects of stress in FPM Y-

293 maze analysis, total turns, alternations and repetitions were analyzed two-way ANOVA with stress

294 (five levels – 0, 1, 3, 7 and 14 days of stress) and time (six levels – 10 min time bins) as the fixed

295 factors. To analyze the effects of the stress in locomotion (young and adult animals), anxiety-related

296 parameters and Pavlovian fear conditioning one-way ANOVA was used. Two-way ANOVA was

297 used to investigate sex differences in the time spent in top and dark zone using sex and stress as

298 factors. Tukey's test was used as post-hoc analysis, and results were considered significant when p

299 ≤ 0.05. Because more than 2 survival curves were investigated, the survival rate analysis was

300 assessed by using the Log-rank (Mantel-Cox) test.

301

302 **3. Results**

3.1. CUELS does not affect survival rates and changes behavioral patterns at 21 dpf

The CUELS protocol had no significant effect on the survival of larval zebrafish (Chi square= 3.432; df=4; p=0.4884) (**Fig. 3A**). To assess whether these animals had any change in locomotion or anxiety-like behavior we analyzed the behavioral patterns of 21 dpf animals in the open field for 5 minutes (**Fig. 3B**). We detected a significant effect on total distance traveled ($F_{(4, 115)}$ = 4.172; p=0.0034**) and thigmotaxis ($F_{(4, 115)}$ = 3.173; p=0.0164*). *Post-hoc* analysis showed decreased distance traveled in animals stressed for only 7 days compared to fish that has been stressed for 3 and 14 days. Finally, thigmotaxis was decreased in animals that were stressed for 7 days compared to control animals and those subjected to 14 days of UCS stress.

3.2. CUELS affects anxiety-related parameters in adult zebrafish

Regarding total distance traveled, ANOVA did not show any significant effect between groups ($F_{(4, 79)}$ = 1.601; *p*=0.1823). For time spent in top, a significant effect of CUELS was observed ($F_{(4, 79)}$ = 6.690; p****<0.0001), where animals submitted to CUELS for 7 days showed decreased time spent in top compared to control (p**=0.0093*)* and 14 days (p***=0.0002*)* of ELS. A significant decrease of time spent in top was also observed for animals submitted to 14 days of CUELS compared to 3 days of CUELS (p**=0.0022*)* (**Fig. 4A**). Similarly, a significant effect of CUELS was observed on the time spent in the dark ($F_{(4, 70)}$ = 5.237; p***<0.0010). *Post-hoc* analysis yielded a significant decrease of time spent in the dark for the 7 days ELS group compared to control (p*=0.0464) and 14 days (p***=0.0004). 14 days of CUELS also significantly increased time spent in the dark compartment compared to 3 days (p*=0.0412) (**Fig. 4B**). Two-way ANOVA, using sex and stress as factors, showed a significant effect for stress time spent in top ($F_{(4, 74)}$ = 29.90; p****<0.0001) and time spent in dark zone ($F_{(4, 65)}$ = 23.16; p***=0.006). Although no sex difference was observed ($F_{(1, 74)}$ = 0.8056; p=0.3386; $F_{(1, 65)}$ = 2.052; p=0.1646) a significant interaction between *stress*sex* was observed for time spent in top ($F_{(4, 74)}$ = 9.054; p=0.0424). No post-hoc significant difference was found when comparing female *vs.* male of each stressor group.

330   *3.3. Social behavior and cognitive-related tasks are not changed after CUELS*

331   To investigate whether CUELS affected social behavior or learning and cognitive-related

332   tasks, we submitted the animals to the shoal behavior task, FPM Y-maze and Pavlovian fear

333   conditioning (**Fig. 5**). No significant effect of stress or interaction *stress*time* were observed for

334   total turns ($F_{(4, 63)}$ = 2.028; p=0.101 and $F_{(20, 315)}$ = 1.189; p=0.262), alternations ($F_{(4, 63)}$ = 0.688;

335   p=0.603 and $F_{(20, 315)}$ = 0.743; p=0.781) or repetitions ($F_{(4, 63)}$ = 1.105; p=0.362 and $F_{(20, 315)}$ = 0.921;

336   p=0.560) in the FPM Y-maze. However, two-way ANOVA showed a significant time effect for

337   alternations (rlrl + lrlr) ($F_{(5, 315)}$ = 5.768; p****<0.0001) and repetitions (rrrr + llll)) ($F_{(5, 315)}$ =

338   2.990; p=0.012) patterns indicating a variation in the alternation profile across time that was not

339   dependent on the ELS exposure **(Fig. 5A).** Furthermore, in the fear conditioning task, no significant

340   effect ($F_{(4, 71)}$ = 1.466; p=0.2218) was observed (**Fig. 5B**). When looking at social behavior, neither

341   shoal area **($F_{(4, 35)}$ = 1.245; p=0.3111)** nor inter-fish distance **($F_{(4, 35)}$ = 0.7491; p=0.5657)** was

342   affected in adult animals submitted to CUELS (**Fig. 5C**).

343

344   *3.4 Basal cortisol levels of adult zebrafish submitted to CUELS*

345   To assess if CUELS caused any hypothalamic pituitary adrenal (HPA) axis-related changes

346   in adult animals, cortisol levels were measured. Although significant changes were observed for

347   anxiety-related responses, no significant difference for cortisol levels were observed between

348   groups ($F_{(4, 33)}$ = 2.036; p=0.1121) (**Fig. 6**).

349

350   **4. Discussion**

351   For the first time, we have created a CUELS protocol that can be applied to zebrafish larvae

352   that does not increase mortality, but significantly decreased anxiety-like behavior and exploratory

353   activity in animals stressed for 7 days. We also find that CUELS exposure affects behavior later in

354   life by decreasing anxiety-like behavior in animals exposed for 7 days, but has no effect on social

355   interactions, memory and cognition. When looking at adult physiological responses, no change was

356 observed for cortisol levels which suggests that the reduced anxiety displayed following CUELS is

357 not necessarily caused by changes in stress axis activity.

358   Early adverse experiences can enhance stress responsiveness and lead to greater

359 susceptibility to psychopathology in adulthood such as anxiety-related disorders and depression

360 (Carr et al., 2013; Heim et al., 2008; Syed and Nemeroff, 2017). However, the impact of ELS in

361 adulthood follows an inverted U-shape response, where mild/moderate stressors can impact in a

362 beneficial way building resilience and severe acute or prolonged chronic exposure can lead to

363 negative outcomes (Russo et al., 2012; Sapolsky, 2015). Thus, the behavioral and physiological

364 effects of ELS will depend on intensity and duration. We previously investigated the role of a 3

365 days mild stress protocol of young zebrafish (6 weeks old) and its impact on 4-month-old adult

366 animals (Fontana et al 2020). Mild ELS improved adult zebrafish working memory, thus apparently

367 building resilience; however, no change in anxiety levels were previously observed. Based on these

368 findings, we adapted an unpredictable chronic stress protocol (Piato et al. X), that has been shown

369 to have a negative impact upon adult animals after 7 or 14 days of application, aiming to observe

370 any negative effects of early adverse experiences.

371   We found that in larvae, the CUELS protocol decreased anxiety after 7 days of stress at both

372 periods of life: 21 dpf and 4 months old without cause changes in the cortisol levels. Indeed, early-

373 life adversities can prepare animals to better cope with a challenging adult environment without

374 necessarily affecting glucocorticoid receptors and the functionality of the hypothalamic-pituitary-

375 adrenal axis (Santarelli et al., 2017). Usually, anxiety-related tasks in zebrafish evaluate the

376 behavioral responses of animals in a novel environment (Stewart et al., 2012); animals that are less

377 anxious tend to explore more "dangerous" areas such as the top part of the tank and lit areas. Here

378 animals exposed to 7 days of CUELS significantly decreased the time spent in the dark zone and

379 significantly explored more the top zone compared to both control and 14 days stressed animals.

380 These data indicate that those animals had decreased anxiety-levels While decreased anxiety could

381 be regarded as a positive effect, from a translational perspective (e.g. in presence of a psychiatric

382 condition or in everyday life challenges), the increased exploration of "dangerous" areas can, in

fact, negatively affect animal survival in nature by increasing their chances of being predated. From a translational perspective, increased risk-taking is related to several psychiatric conditions such as addiction, ADHD and other impulse-control or compulsive disorders (Dayan et al., 2010; Humphreys et al., 2011). In both novel tank and light-dark preference task no eminent danger is presented, therefore future studies should be performed to evaluate if this lower anxious phenotype is associated to increased risk-taking in dangerous situations. In addition, it would be worthwhile to examine other behavioral domains, such as impulse control and drug seeking, to examine the extent effects of ELS on these parameters.

Sex differences are known to have a role in behavior of several species (Mowrey and Portman, 2012; Rose and Rudolph, 2006), affecting phenotypes and levels of expression for a variety of psychiatric disorders (Bekker and van Mens-Verhulst, 2007; Laurin et al., 2007; Mandy et al., 2012). We have previously shown that female zebrafish have increased anxiety-like phenotypes (Fontana et al., 2019b), and here we investigated if sex had a role in the anxiety-related outputs. Although no overall sex difference (female *vs* male) was observed, the analysis was likely to be underpowered due to the small size of following splitting the stress groups. Interestingly a significant interaction (*stress*sex*) effect was observed for time spent in top suggesting that there may be differential effects of male/female in this context, although follow up tests were inconclusive. Future studies should be performed to analyze if sex is differently affecting the decreased anxious phenotype observed for animals exposed for 7 days of CUELS.

Regarding working memory, our previous work demonstrated that mild ELS positively affected zebrafish working memory (Fontana et al., 2020); however, here, we found no effects of CUELS on working memory performance. In our previous work the ELS was applied to 6-week-old animals (for 3 days) and here we use 1-week old animals. Although both forms of early life stress may be indicative of increased resilience, the lack of replication of differences across the behavioral domains further demonstrates that there is a difference in the way ELS can build resilience depending on zebrafish age and time of stress, which was previously described in other species (Heim and Binder, 2012).

410   The negative impacts of ELS are widely investigated, and it is known that ELS can increase

411   vulnerability to the development of psychiatric (Carr et al., 2013) and metabolic disorders (Vargas

412   et al., 2016). However, in two independent studies applying ELS to zebrafish we have found that

413   the protocol either improved working memory or decreased anxiety, rather than having a clear

414   negative impact. One possible hypothesis as to why ELS having positive impacts in zebrafish is that

415   positive and negative adaptation occurs as a result of the animal's growing environment. Thus, if

416   the environment is neutral or positive animals will improve its ability to cope with stressful

417   situations building resilience (Clark et al., 1997; Grissom and Bhatnagar, 2009; Kant et al., 1985).

418   Meanwhile, if adaptation is inadequate, maladaptive, or future environment differs from the

419   naturalistic phenotype, animals will be more likely to develop vulnerability to diseases (Daskalakis

420   et al., 2013; Gluckman et al., 2007; Maniam et al., 2014; McEwen, 1998; Schmidt et al., 2011).

421   Interestingly, animals exposed to 14 days of CUELS had the lowest exploration to the top and spend

422   most of their time in the dark zone. Although there is no significant difference between this group

423   and the control for anxiety-related parameters, there is a tendency for these animals have higher

424   anxiety levels. This raises the possibility that an even longer exposure to the CUELS may have

425   continued the trend towards negative effects. Further studies should be performed increasing

426   CUELS duration and considering adverse environments (*e.g.* abnormal water conditions) to

427   evaluate whether this protocol can have a negative impact upon the behavioral phenotype of

428   zebrafish.

429   Regarding physiology, cortisol levels were measured to observe whether the CUELS

430   protocol was affected stress axis activity in adulthood. Previous work have shown that after acute

431   and chronic stress protocols, young and adult animals have significantly higher cortisol levels

432   (Fontana et al., 2020; Ghisleni et al., 2012; Piato et al., 2011; Ramsay et al., 2009). ELS causing

433   resilience is linked to different metabolic adaptations, such as different cortisol reactivity levels. For

434   example, patients that experienced ELS had blunted cortisol reactivity but a greater total cortisol

435   production (Goldman-Mellor et al., 2012). Here no cortisol differences were observed when looking

436   at their adult baseline levels and the cortisol reactivity was not assessed. Indeed, the investigation

of stress reactivity of CUELS exposed adult zebrafish could reveal a different pattern of behavior and physiological aspects. However, we suggest that the low anxiety levels observed for animals exposed to CUELS for 7 days are not linked to changes in cortisol levels.

5. Conclusion

In summary, here we present a novel CUELS protocol for larval zebrafish which has an impact on adult behavior, decreasing their anxiety in the novel tank diving test. No differences in the baseline cortisol levels were observed. The pathway between ELS causing resilience or vulnerability to disease was previous linked to the environment in which the organism is growing after the stress. Thus, here animals grown in naturalistic environment and CUELS decreased anxiety in adult zebrafish. In the future, the CUELS protocol can be a useful model to study resilience in zebrafish and the mechanisms underlying it, however further studies are necessary to investigate if CUELS decreasing anxiety is associated to increased risk-taking. Moreover, ELS can also be used to investigate the combined effect of environment and genotype in zebrafish models of psychiatric and neurodevelopmental disorders. The CUELS protocol may also have the potential to investigate the negative impact of ELS on adult animals if applied for a longer period. Similarly, the role of environment after CUELS could also be investigated.

Data accessibility statement

All raw data from this study are openly available in the University of Portsmouth Pure repository, at research.port.ac.uk/portal/

Conflict of Interest

The authors declare no conflict of interest exists.

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Table 1

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

Table 1. Procedure of the unpredictable chronic early life stress protocol in zebrafish larvae (7 dpf).
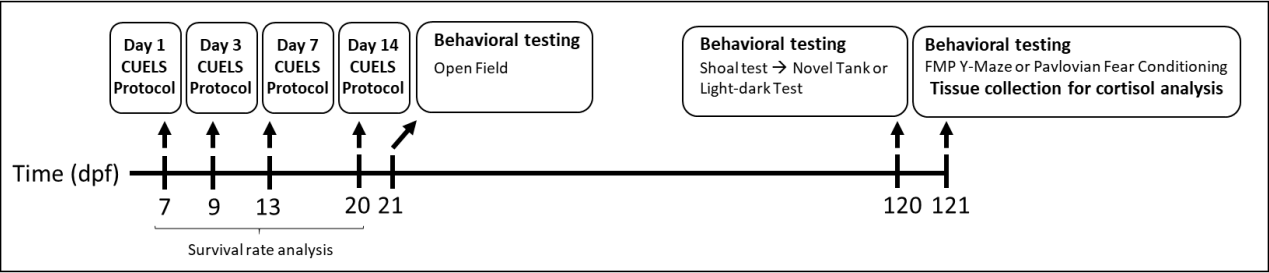
Figures

Figure 1. Timeline showing the CUELS protocol. Zebrafish larvae were exposed to different durations of CUELS protocol depending on the group. Open field was tested at 21 dpf. At 4 months old, adult zebrafish were tested for shoaling (social behavior) followed by the novel tank diving task or light-dark preference test. One day later, animals were exposed to either the FMP Y-maze or Pavlovian fear conditioning task. After all behavioral protocols, whole-body was collect for further cortisol analysis.
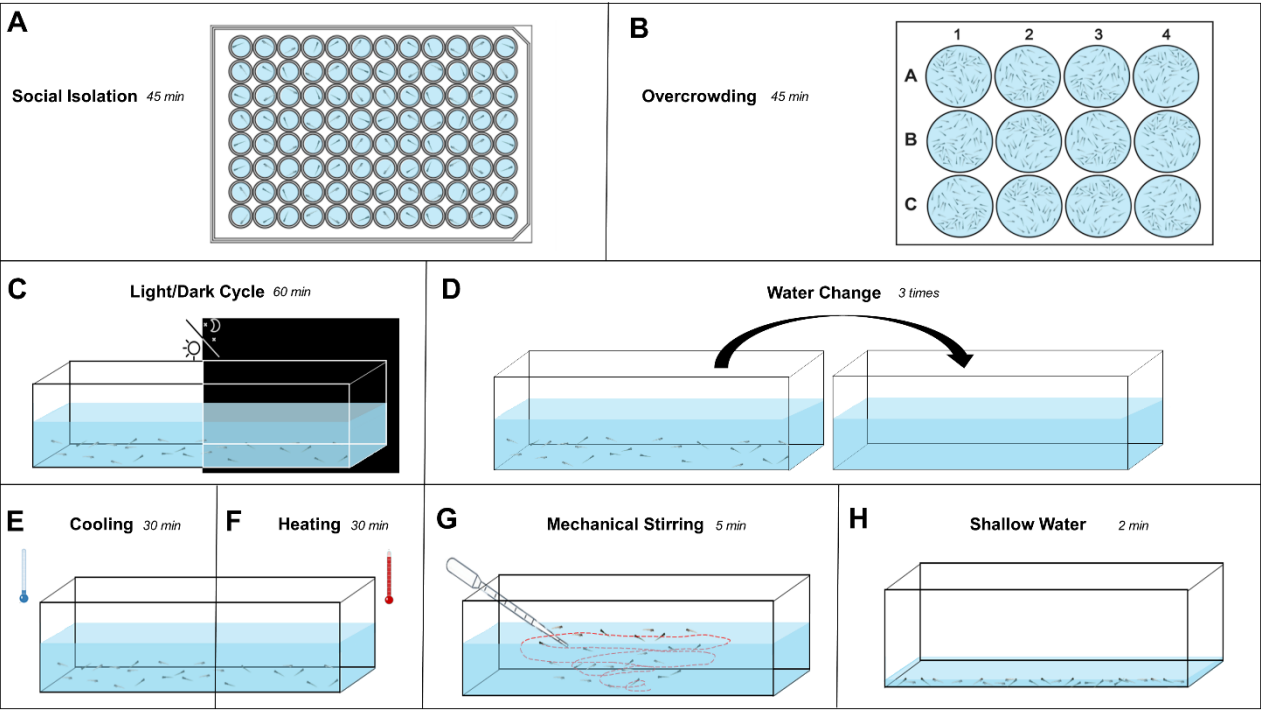
Figure 2. Schematic representation of the CUELS stressors. **(A)** Social isolation, animals individually transferred to well in a white 96-well plate for 45 min. **(B)** Overcrowding, 40 zebrafish larvae transferred to a well in a 12-well plate for 45 minutes. **(C)** Light/dark cycle changes change animals' tanks for a totally dark or light environment depending on the time of the day. **(D)** Water change, change animals to a new environment 3 times. **(E)** Cooling, transfer tanks to an incubator

until the water reaches 23 ºC for 30 minutes. **(F)** Heating, transfer tanks to an incubator until the water reaches 33 ºC for 30 minutes. **(G)** Mechanical stirring, stir the water with a Pasteur pipette for 5 minutes. **(H)** Shallow water, remove water from the tank until body exposure and leave in the condition for 2 minutes.
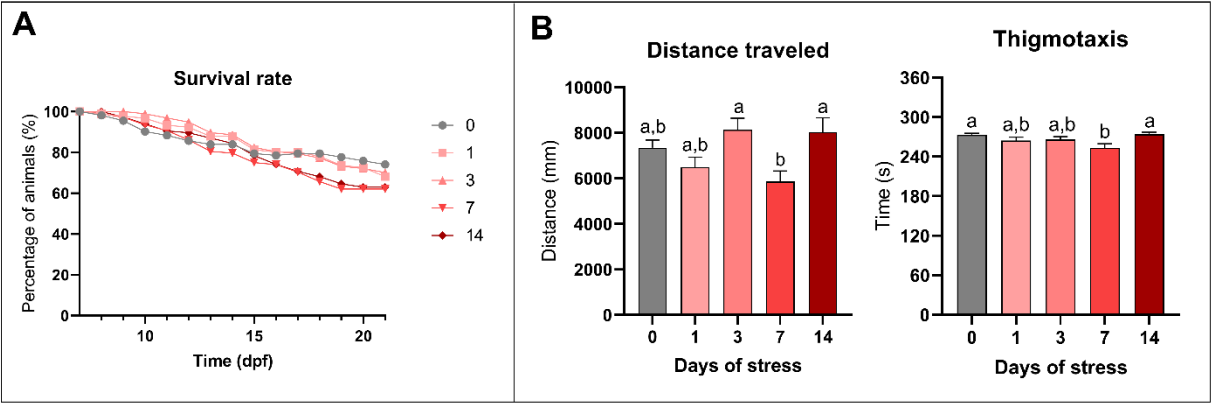

Figure 3. Effects of CUELS at early stages. **(A)** Survival rates are not affected by CUELS protocol. **(B)** Locomotion and thigmotaxis are decreased in animals submitted to 7 days of CUELS at early stages (22 dpf). Data were represented as mean ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test multiple comparison test. Different letters indicate statistical differences between groups (*$p < 0.05$; *n = 23 - 24*).
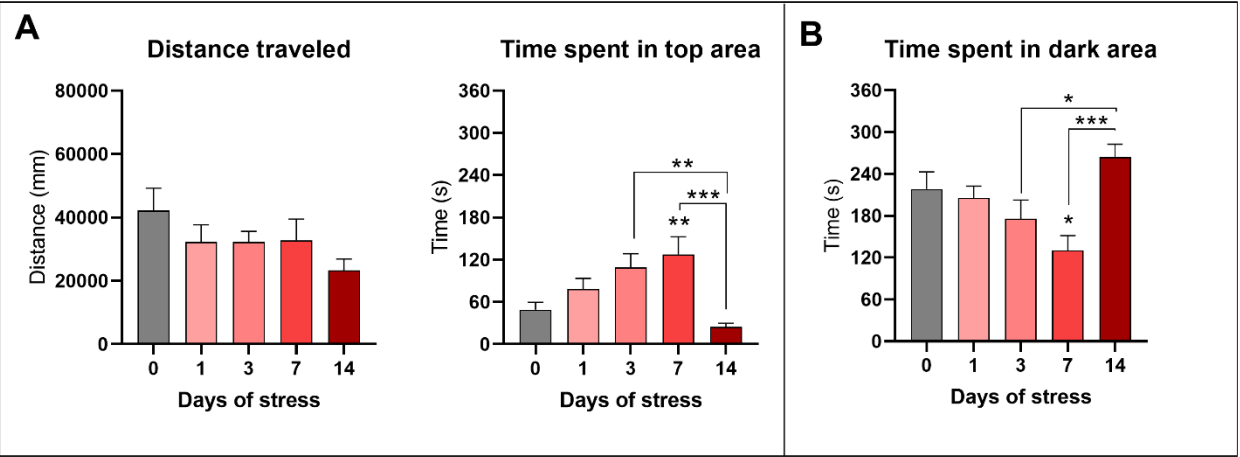

Figure 4. Effects of CUELS in anxiety-like behavior of adult zebrafish. **(A)** Time spent in top zone was increased in animals exposed to 7 days of CUELS (*n = 16 – 17 per group)*. **(B)** Animals submitted to a CUELS protocol when larvae showed a decreased time spent in the dark zone when

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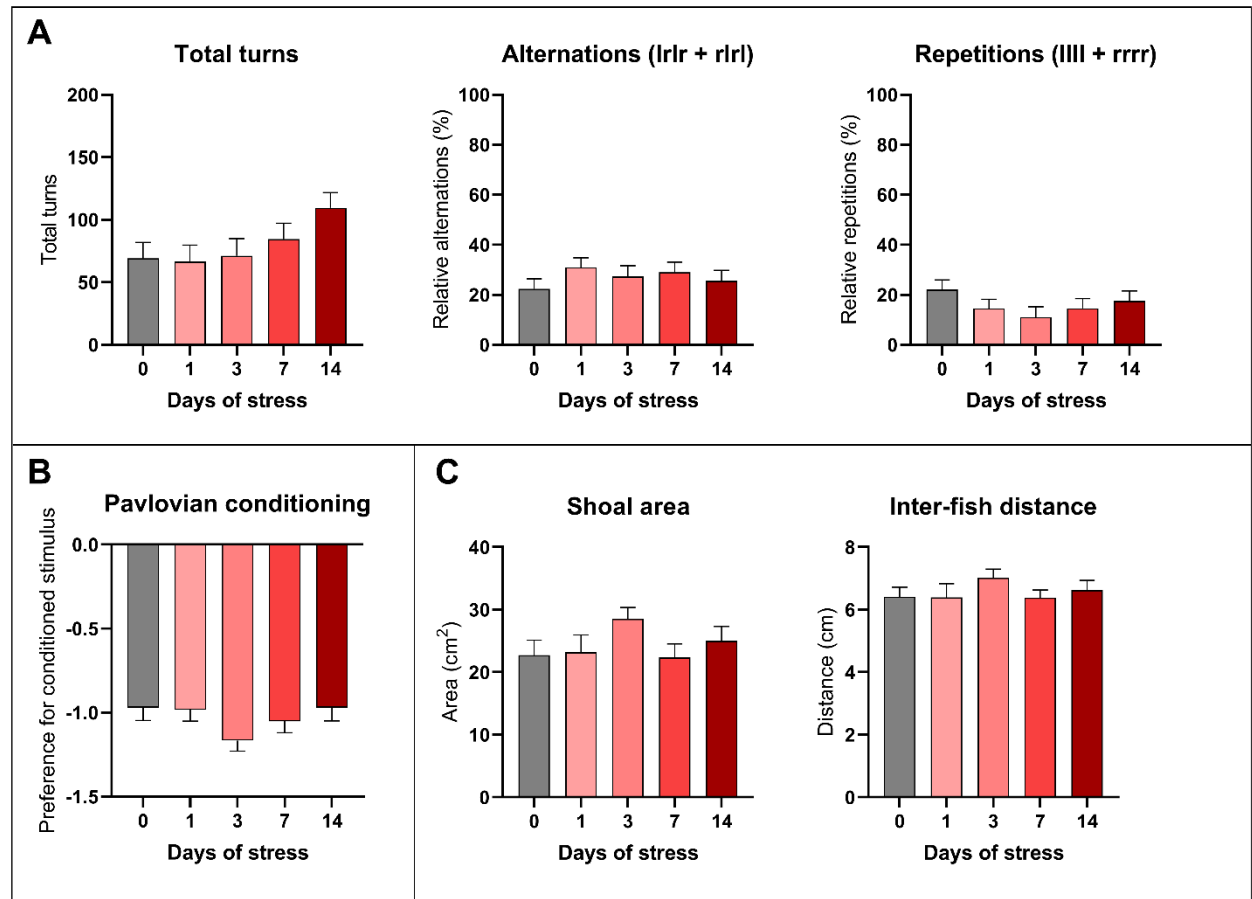
Figure 5. Effects of CUELS in memory and cognition, and social behavior. **(A)** CUELS do not change FMP Y-maze patterns (*n = 12 per group*). **(B)** Preference for the conditioned stimulus was not affected by CUELS (*n = 14 – 16 per group*). **(C)** Social behavior parameters, such as shoal area and inter-fish distance were not changed by CUELS (*n = 7 - 8 shoals per group*). Data were represented as mean ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test multiple comparison test.

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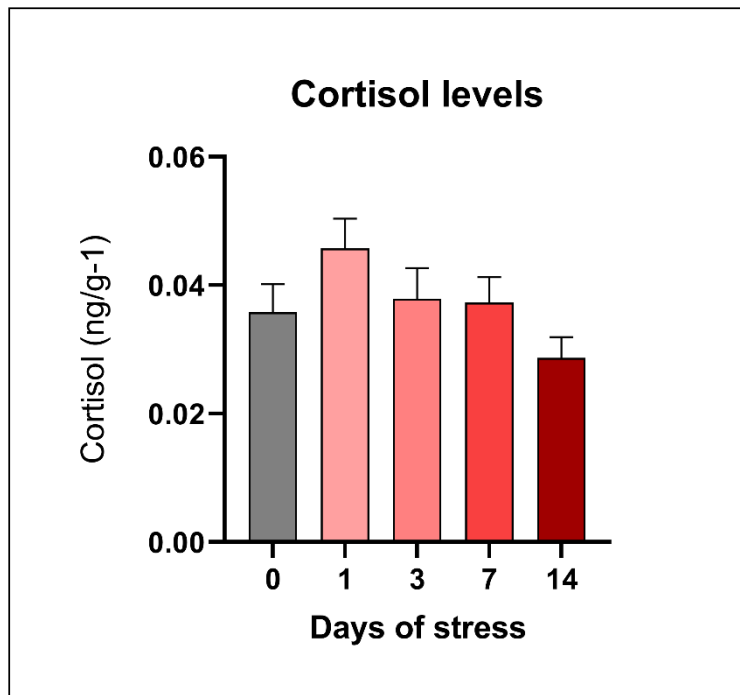
Cortisol levels

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710   **Figure 6.** Baseline cortisol levels for adult animals previous exposed to CUELS protocol. Data were

711   represented as mean ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test multiple

712   comparison test (*n = 7 − 8 per group*).

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