Anxiolytic and anti-stress effects of acute administration of acetyl-L-carnitine in zebrafish

Lais Pancotto [1], Ricieri Mocelin [2], Matheus Marcon [2], Ana P Herrmann [1], Angelo Piato [Corresp. 1, 2, 3]

[1] Programa de Pós-Graduação em Farmacologia e Terapêutica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Programa de Pós-Graduação em Neurociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[3] Zebrafish Neuroscience Research Consortium (ZNRC), Los Angeles, United States

Corresponding Author: Angelo Piato
Email address: angelopiato@ufrgs.br

Studies have suggested that oxidative stress may contribute to the pathogenesis of mental disorders. In this context, molecules with antioxidant activity may be promising agents in the treatment of these deleterious conditions. Acetyl-L-carnitine (ALC) is a multi-target molecule that modulates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, acetylcholine production, protein, and membrane phospholipid synthesis, capable of promoting neurogenesis in case of neuronal death. Moreover, neurochemical effects of ALC include modulation of brain energy and synaptic transmission of multiple neurotransmitters, including expression of type 2 metabotropic glutamate (mGlu2) receptors. The aim of this study was to investigate the effects of ALC in zebrafish by examining behavioral and biochemical parameters relevant to anxiety and mood disorders in zebrafish. ALC presented anxiolytic effects in both novel tank and light/dark tests and prevented the anxiety-like behavior induced by an acute stressor (net chasing). Furthermore, ALC was able to prevent the lipid peroxidation induced by acute stress in the zebrafish brain. The data presented here warrant further investigation of ALC as a potential agent in the treatment of neuropsychiatric disorders. Its good tolerability also subsidizes the additional studies necessary to assess its therapeutic potential in clinical settings.

1   **Anxiolytic and anti-stress effects of acute administration of acetyl-L-carnitine in zebrafish**

2   Lais Pancotto[1#], Ricieri Mocelin[2#], Matheus Marcon[2], Ana P. Herrmann[1], Angelo Piato[1,2,3*]

3   [1]Programa de Pós-Graduação em Farmacologia e Terapêutica, Universidade Federal do Rio

4   Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

5   [2]Programa de Pós-Graduação em Neurociências, Universidade Federal do Rio Grande do Sul

6   (UFRGS), Porto Alegre, RS, Brazil.

7   [3]Zebrafish Neuroscience Research Consortium (ZNRC), Slidell, LA, USA.

8   [#]Both authors contributed equally to this article.

10 *Correspondence to:

11 Angelo Piato, Ph.D.

12 Programa de Pós-Graduação em Farmacologia e Terapêutica, ICBS, Universidade Federal do

13 Rio Grande do Sul. Av. Sarmento Leite 500/305, Porto Alegre, RS, Brazil.

14 90050-170; Phone/Fax: +55 51 33083121; E-mail address: angelopiato@ufrgs.br

15   **ABSTRACT**

16   Studies have suggested that oxidative stress may contribute to the pathogenesis of mental

17   disorders. In this context, molecules with antioxidant activity may be promising agents in the

18   treatment of these deleterious conditions. Acetyl-L-carnitine (ALC) is a multi-target molecule

19   that modulates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation,

20   acetylcholine production, protein, and membrane phospholipid synthesis, capable of promoting

21   neurogenesis in case of neuronal death. Moreover, neurochemical effects of ALC include

22   modulation of brain energy and synaptic transmission of multiple neurotransmitters, including

23   expression of type 2 metabotropic glutamate (mGlu2) receptors. The aim of this study was to

24   investigate the effects of ALC in zebrafish by examining behavioral and biochemical parameters

25   relevant to anxiety and mood disorders in zebrafish. ALC presented anxiolytic effects in both

26   novel tank and light/dark tests and prevented the anxiety-like behavior induced by an acute

27   stressor (net chasing). Furthermore, ALC was able to prevent the lipid peroxidation induced by

28   acute stress in the zebrafish brain. The data presented here warrant further investigation of ALC

29   as a potential agent in the treatment of neuropsychiatric disorders. Its good tolerability also

30   subsidizes the additional studies necessary to assess its therapeutic potential in clinical settings.

INTRODUCTION

Acetyl-L-carnitine (ALC) facilitates the movement of acetyl-CoA into the mitochondria during the oxidation of fatty acids in mammals (Chapela et al., 2009). Moreover, this molecule is widely consumed as a dietary supplement for physical exercise (Ribas, Vargas & Wajner, 2014; Nicassio et al., 2017). Recently, preclinical and clinical studies have demonstrated the effects of ALC on parameters relevant to anxiety, schizophrenia, and mood disorders; with onset of action faster than antidepressant drug and exert neuroprotective, neurotrophic, and analgesic effects (Levine et al., 2005; Wang et al., 2015; Traina, 2016; Singh et al., 2017; Nasca et al., 2017; Chiechio, Canonico & Grilli, 2017).

A growing body of evidence suggests that psychiatric disorders such as anxiety and depression are associated with oxidative damage (Ortiz et al., 2017; Niedzielska et al., 2016; Schiavone, Colaianna & Curtis, 2015; Cobb & Cole, 2015; Ng et al., 2008), since a decrease in antioxidant capacity can impair the organism's protection against reactive oxygen species and cause damage to fatty acids, proteins, and DNA (Maes et al., 2011). Superoxide and hydroxyl radical (free radicals) or hydrogen peroxide and their derivatives (non-radical molecules) called reactive oxygen species (ROS) are responsible for causing oxidative damage (Smaga et al., 2015). The antioxidant defense mechanism they are the non-enzymatic (i.g. glutathione) and enzymatic antioxidants (i.g. superoxide dismutase and catalase) which show a trend to decrease in neuropsychiatric diseases (Ozcan et al., 2004; Hassan et al., 2016). Preclinical and clinical research has evaluated antioxidant compounds (i.g. N-acetylcysteine, resveratrol and curcumin) in the treatment of psychiatric disorders, and it has been reported that these compounds are able to protect against oxidative stress-induced neuronal damage, preventing lipid peroxidation and

58 behavioral changes (Mecocci & Polidori, 2012; Berk et al., 2014; Wang et al., 2014; Mocelin et

59 al., 2015; Patel, 2016; Santos et al., 2017).

60   With simple, rapid and cheaper tests when compared with rodents, zebrafish have been

61 used as a powerful complementary model for the study of a variety of neuropsychiatric diseases

62 through behavioral and biochemical parameters (Stewart et al., 2015; Mocelin et al., 2015;

63 Marcon et al., 2016, 2018; Khan et al., 2017). There are several behavioral protocols extensively

64 used and described for this species, such as the novel tank and light/dark tests. The novel tank

65 diving test is based on an anti-predatory defense mechanism that induces fish to swim at the

66 bottom of the tank, whereas the light/dark test evaluates anxiety based on the innate preference

67 of adult zebrafish to dark over light areas (Levin, Bencan & Cerutti, 2007; Gebauer et al., 2011;

68 Khan et al., 2017; Pittman & Piato, 2017).

69   In addition to its role in lipid metabolism, ALC also possesses free radical scavenging

70 properties, and may thus protect the cells from oxidative damage by acting as an antioxidant

71 (Gülçin, 2006; Sepand et al., 2016). Therefore, the aim of this study was to investigate the effects

72 of ALC in zebrafish by examining behavioral and biochemical parameters relevant to anxiety

73 and mood disorders in zebrafish.

74

75 **MATERIALS AND METHODS**

76 **Animals**

77   240 adult zebrafish (*Danio rerio,* F. Hamilton 1822) wild-type short fin strain (6-month-

78 old, 3-4 cm long) 50:50 male/female ratio were purchased from Delphis aquariums (Porto

79 Alegre, Brazil). The fish were kept for 15 days in a closed acclimation tank system of 16 L (40 x

80 20 x 24 cm) identical to the experimental tanks. Housing conditions consisted only of a tank with

81   water, heater, filter and aeration system, and were maintained as previously described in Marcon

82   et al. (2016). The tanks contained non-chlorinated, aerated tap water (pH 7.0 ± 0.3; temperature

83    $26 \pm 1°C$; total ammonia at <0.01 mg/L; nitrite < 0.01 mg/L; dissolved oxygen at 7.0 ± 0.4 mg/L;

84   alkalinity at 22 mg/L $CaCO_3$ and total hardness at 5.8 mg/L), with a light/dark cycle of 14/10 h

85   (lights on at 06:00 am). The fish were fed twice a day with a commercial flake fish food (Alcon

86   BASIC®, Alcon, Brazil). On the experimental days, all the fish were only fed early in the

87   morning before behavioral testing began. The order of testing was counterbalanced so that

88   fasting time was randomized across experimental groups. All experiments were approved by the

89   Ethics Committee of Universidade Federal do Rio Grande do Sul (#30992/2015).

90

91   **Drug and experimental design**

92   O-Acetyl-L-carnitine hydrochloride (ALC, CAS number 5080-50-2) was acquired from

93   Sigma-Aldrich (St Louis, Missouri, USA). In all experimental protocols (novel tank, light/dark,

94   and acute chasing stress tests), the animals were treated or not with ALC (0.1, 1.0 and 10.0

95   mg/L) in a beaker for 10 minutes. In the first protocol, immediately after the treatment, the

96   animals were placed in the novel tank test (NTT) for 6 minutes. In the second protocol, after the

97   treatments, the animals were placed in the light/dark test (LDT) for 5 minutes. Finally, in the

98   third protocol, the animals were treated as previously and then chased with a net for 2 minutes.

99   Then, the animals were placed in the NTT. The biochemical analyses were performed in animals

100   submitted to this last protocol. A control group was submitted to the same experimental

101   conditions (stressed or not) but without treatment. Different sets of animals were used in each

102   experimental protocol. The experimental design is shown in Figure 1 and was based on the

103   previously published study by Mocelin et al. (2015). All behavioral tests were performed

104   between 09:00 am and 16:00 pm. The researchers who performed the behavioral tests and

105   analyzed the data were unaware of the allocation of animals to the experimental groups. The

106   concentrations were based on previous studies with another antioxidant compound (N-

107   acetylcysteine) and pilot studies with a wider concentration range. The same concentrations were

108   used in a chronic study with ALC (manuscript in preparation). We do not attempt to extrapolate

109   the drug concentrations we used in a fish study to human dosage since there is not a

110   straightforward calculation to be done. Since the half-life and other pharmacokinetic parameters

111   of ALC in zebrafish are not known, it is difficult to precisely compare the concentration range

112   that we observed here with the dose range for humans.

113

114   **Novel tank test (NTT)**

115   The novel tank test followed the protocol already described in Mocelin et al. (2015).

116   Briefly, the animals were separately moved to the apparatus (2.7-L tank, 24 x 8 x 20 cm,

117   virtually divided into three equal horizontal zones and filled with standard tank water up to 15

118   cm) and video recorded for 6 min to be later analyzed by the ANY-maze™ software (Stoelting

119   Co., USA). To evaluate exploratory behavior and locomotion we measured the parameters: total

120   distance moved (m), number of transitions between zones, time spent in the upper and bottom

121   zones of the tank, and number of transitions to the upper zone. Total distance and crossings were

122   used as an indicator of overall locomotor activity. The upper zone of the tank corresponds in rats

123   and mice protocols to the periphery region of the open-field test. Alterations in time spent and

124   number of crossings to this zone are frequently used as a parameter of anxiety in zebrafish

125   (Mocelin et al., 2015; Giacomini et al., 2016; Marcon et al., 2016; Mocelin et al., 2017; Marcon

126   et al., 2018).

127

Light/dark test (LDT)

129   The light/dark test followed the protocol already reported by Gebauer et al. (2011).

130 Specifically, the apparatus consisted of a glass tank (18 x 9 x 7 cm) divided by a raised glass into

131 a dark and a white compartment of equal sizes, with the water level set at 3 cm and the partition

132 raised 1 cm above the tank floor. One fish at a time was positioned in the white zone of the

133 apparatus immediately after treatment. We recorded the number of crossings and the time spent

134 in the white compartment for 5 min. Zebrafish have a natural preference for dark environments

135 and the white compartment is very anxiogenic for this species; anxiolytics increase the time

136 spent in the white compartment (Maximino et al., 2010; Mocelin et al., 2015).

137

Acute chasing stress test (ACS)

139   The acute stress protocol was performed according to the previous study published by

140 Mocelin et al. (2015). Briefly, the animals were treated for 12 minutes and then chased for the

141 last 2 minutes with a net before being moved to the novel tank, where they were recorded for 6

142 minutes. The behavioral parameters were quantified as described above for the NTT.

143

Tissue preparation

145   Samples were collected and prepared as previously reported by Mocelin et al. (2018).

146 Specifically, after the ACS fish were anesthetized by immersion in cold water and euthanized by

147 decapitation. Each independent sample was then obtained by pooling four brains, which were

148 homogenized on ice in 600 μL phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich®). The

149 homogenate was centrifuged at 10,000 g for 10 min at 4 ºC in a cooling centrifuge, and the

150 supernatant was packed in microtubes for further assays.

151

152 **Protein determination**

153 Protein was determined by the Coomassie blue method described in detail by Bradford

154 (1976). Specifically, we used bovine serum albumin (Sigma-Aldrich®) as standard and the

155 absorbance of samples was measured at 595 nm.

156

157 **Lipid peroxidation (TBARS)**

158 Lipid peroxidation was measured by the quantification of thiobarbituric acid reactive

159 species (TBARS) production according to the method reported by (Draper & Hadley, 1990).

160 More specifically, we followed the protocol described by Mocelin et al. (2018), in which 50 μL

161 of the sample (80-100 μg protein) was mixed with 75 μL of trichloroacetic acid (TCA 10%,

162 Sigma-Aldrich®) and centrifuged at 6000 rpm for 5 min at 4 ºC in a cooling centrifuge. In the

163 supernatants were added to 75 μL thiobarbituric acid (TBA 0.67%, Sigma-Aldrich®), then

164 homogenized in a vortex for 5s and heated at 100 ºC for 30 min. TBARS levels were measured

165 by absorbance (532 nm) in a microplate reader, using malondialdehyde (MDA, Sigma-Aldrich®)

166 as a standard, and results were expressed as nmol MDA/mg protein.

167

168 **Reduced thiol (SH) and Non-protein thiols levels (NPSH)**

169 SH and NPSH levels were determined and measured at 412 nm in a microplate reader

170 according to the method described by Ellman (1959). More specifically, we followed the steps

171 described by Mocelin et al. (2018). Briefly, for SH the samples (60-80 μg protein) were added to

172   10 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) dissolved in ethanol, developing yellow color

173   after 1 h. The NPSH were similarly assessed, except that the sample was mixed with equal

174   volumes of the 10% trichloroacetic acid (TCA) and centrifuged (6000 rpm, 5 min). The

175   supernatant was used for the biochemical assay. Results were expressed as μmol SH/mg protein.

176

177   **Superoxide dismutase (SOD) and catalase (CAT) activities**

178   SOD and CAT activities were determined according to the method reported by Misra &

179   Fridovich (1972) and Aebi (1984), respectively. The protocol followed the more specific details

180   described by Dal Santo et al. (2014). Specifically, SOD activity was quantified in a microplate

181   reader (480 nm) by testing the inhibition of radical superoxide reaction of the sample ($20 - 30\ \mu g$

182   protein) in the presence of adrenalin, monitoring adrenochrome formation in a medium

183   containing a glycine-NaOH buffer (pH 10) and adrenaline (1 mM). CAT activity was assessed

184   by measuring the decrease in H_2O_2 absorbance in a microplate reader (240 nm). The assay

185   mixture consisted of sample ($20 - 30\ \mu g$ protein), phosphate buffered saline (pH 7.4), and 5 μL

186   H_2O_2 (0.3 M). Results were expressed as units/mg protein.

187

188   **Statistics**

189   Normality and homogeneity of variance of the data were checked by D'Agostino-Pearson

190   and Levene tests, respectively. Results were analyzed by one- or two-way ANOVA followed by

191   Tukey's post hoc test. Two-way ANOVA was used to identify the main effects of stress and

192   treatment, as well as their interactions. Data are expressed as a mean + standard error of the

193   mean (S.E.M.). The level of significance was set at $p < 0.05$.

194

195   **RESULTS**

196   **Behavioral parameters**

197   Figure 2 shows the effects of ALC (0.1, 1.0 and 10.0 mg/L) on the novel tank test in

198   zebrafish. ALC significantly increased the time spent in the top (0.1 and 1.0 mg/L, Fig. 2D) and

199   decreased the time spent the bottom (0.1 mg/L, Fig. 2E) zone of the tank ($F_{3,77} = 8.0$, p=0.0001

200   and $F_{3,77} = 5.6$, p=0.0016, respectively). Locomotor parameters of groups treated with ALC (0.1,

201   1.0, and 10.0 mg/L) did not differ from control (Fig. 2A and 2B).

202   In the light/dark test, ALC (0.1 and 10.0 mg/L) significantly increased the time spent in

203   the lit side of the tank when compared to control ($F_{3,92} = 3.6$, p=0.0161, Fig. 3B). The number of

204   crossings between the light and dark compartments was not altered by any of the concentrations

205   ($F_{3,92} = 0.9$, p=0.4284, Fig. 3A).

206   Figure 4 shows the effects of ALC in the acute chasing stress (ACS) in zebrafish and

207   *Table 1* summarizes the two-way ANOVA analysis. As expected, ACS decreased the distance

208   total traveled, crossings, entries and time in the top area (Fig. 4A, 4B, 4C, and 4D, respectively)

209   and increased the time in the bottom area (Fig. 4E). ALC (0.1, 1.0 and 10.0 mg/L) prevented the

210   effects of ACS on the time in the top and bottom areas in the novel tank test (Fig. 4D and 4E).

211   Also, ALC (0.1 mg/L) prevented the effects of ACS on the total distance traveled.

212

213   **Biochemical parameters**

214   Figure 5 shows the effects of ALC (0.1, 1.0 and 10.0 mg/L) on oxidative status. ACS

215   significantly increased lipid peroxidation (TBARS), non-protein sulfhydryl (NPSH) and

216   superoxide dismutase (SOD) activity (Fig. 5A, 5C, and 5D, respectively), but did not alter

217   sulfhydryl (SH) content and catalase (CAT) activity (Fig. 5B and 5E, respectively). Treatment

218   with ALC (0.1, 1.0 and 10.0 mg/L) prevented oxidative damage as measured by TBARS. ALC

219   also prevented the increase of antioxidant defenses as measured by NPSH (0.1 mg/L) and SOD

220   (0.1, 1.0 and 10.0 mg/L). Two-way ANOVA analyses were summarized in *Table 2*.

221

222   **DISCUSSION**

223   Here, we showed for the first time that ALC presents anxiolytic effects in both novel tank

224   and light/dark tests in zebrafish. Moreover, ALC was able to prevent the anxiogenic effects and

225   lipid peroxidation induced by an acute stress protocol. These results indicate a potential use of

226   ALC in mental disorders related to stress.

227   ALC increased the time spent in the upper as well as decreased the time spent in the

228   bottom zones of the tank. Previous studies have shown that anxiolytic drugs such as buspirone,

229   fluoxetine, diazepam, and ethanol increase the time spent in this zone (Bencan, Sledge & Levin,

230   2009; Egan et al., 2009; Gebauer et al., 2011). In the light/dark test, ALC increased the time

231   spent in the lit side of the tank. This effect was observed with other drugs as clonazepam,

232   bromazepam, diazepam, buspirone, and ethanol (Gebauer et al., 2011). Additionally, multi-target

233   drugs other than ALC, for instance, N-acetylcysteine (NAC) and taurine, also increase the time

234   in the lit side in the LTD in zebrafish (Mocelin et al., 2015; Mezzomo et al., 2015). In both NTT

235   and LDT, ALC presented biphasic response. We can only speculate that different mechanisms of

236   action may be involved in the effects of low versus high dose, but lower and higher

237   concentrations would have to be tested for us to have a bigger picture of the dose-response

238   relationship. ALC also prevented the locomotor impairment and anxiogenic behavior induced by

239   the chasing stress protocol. Recently, our group has shown that fluoxetine, diazepam, and NAC

240   prevented the effects of a similar stress protocol in zebrafish (Mocelin et al., 2015; Giacomini et

241   al., 2016).

242   The anxiolytic and antidepressant effects of ALC have been already reported in rodents

243   (Levine et al., 2005; Wang et al., 2015; Lau et al., 2017). ALC modulates the cholinergic system

244   by increasing acetyl-CoA content and choline acetyltransferase activity. Moreover, it modulates

245   GABAergic, dopaminergic and glutamatergic neurotransmitter systems (Chapela et al., 2009;

246   Nasca et al., 2013; Wang et al., 2014; Singh et al., 2016; Chiechio, Canonico & Grilli, 2017). In

247   rats, ALC decreased the immobility time in the forced swim test and increased sucrose

248   preference in 3 days of treatment, whereas 14 days were necessary to obtain the same effects

249   with clomipramine (Nasca et al., 2013).

250   Under normal conditions, damage by reactive oxygen species (ROS) is kept in control by

251   efficient antioxidant systems, such as SOD and CAT enzymes, as well as non-enzymatic

252   scavengers (Schiavone et al., 2013; Schiavone, Colaianna & Curtis, 2015; Sandi & Haller, 2015).

253   Studies have demonstrated that ALC protects cells against lipid peroxidation and membrane

254   breakdown through hydrogen peroxide scavenging (Kumaran et al., 2003; Gülçin, 2006), and can

255   promote the expression of antioxidant enzymes such as SOD and CAT (Augustyniak &

256   Skrzydlewska, 2010; Li et al., 2012).

257   Even though the ACS protocol increased antioxidant defenses (NPSH and SOD), it also

258   caused lipid peroxidation (TBARS), which may indicate a possible adaptive response to ROS

259   production during stressful conditions. Similar results were observed in zebrafish and reported in

260   a previous study from our group using acute restraint stress (Dal Santo et al., 2014). Even though

261   detection of MDA levels by HPLC would be a more specific indicator of lipid peroxidation, the

262   TBARS assay we used in this study has been reported by many previous articles using samples

263   from zebrafish and other animals (Mihara & Uchiyama, 1978; Sunderman et al., 1985;

264   Armstrong & Browne, 1994; Yagi, 1998; Kim et al., 2011; Basu et al., 2014; Yavuzer et al.,

265 2016). The association of these factors could be related to the prevented effects of ALC, and that

266 our results indicate a deficit in antioxidant defenses against lipid peroxidation in zebrafish

267 submitted to the ACS protocol, providing further evidence for the hypothesis of an association

268 between behavior and ROS with the pathophysiology of mental disorders stress-related and their

269 prevention by ALC.

270

271 **CONCLUSION**

272   ALC is already widely used as supplementation for people who want to lose weight/fat

273 burner, but only a few studies assessed its effects on stress-related outcomes. In addition to its

274 antioxidant actions, ALC is also able to restore mitochondrial function, which is relevant to

275 combat the dysregulation of fatty acid metabolism in the mitochondria-associated with

276 psychiatric disorders. Furthermore, there is evidence that ALC increases expression of

277 metabotropic glutamate receptors via epigenetic mechanisms (Nasca et al., 2013), which is also

278 relevant for the pathophysiology of depression and other stress-related disorders.

279   Our study adds to a growing body of literature demonstrating the role of antioxidants in

280 modulating behavior and oxidative homeostasis. The data presented here thus warrants further

281 investigation of ALC as a potential agent in the treatment of neuropsychiatric illness. Its novel

282 mechanism of action and good tolerability also subsidize the additional studies necessary to

283 assess its therapeutic potential in clinical settings.

284

285

286

287

288 **REFERENCES**

289 de Abreu MS., Koakoski G., Ferreira D., Oliveira TA., da Rosa JGS., Gusso D., Giacomini

290   ACV., Piato AL., Barcellos LJG. 2014. Diazepam and Fluoxetine Decrease the Stress

291   Response in Zebrafish. *PLoS ONE* 9. DOI: 10.1371/journal.pone.0103232.

292 Armstrong D., Browne R. 1994. The analysis of free radicals, lipid peroxides, antioxidant

293   enzymes and compounds related to oxidative stress as applied to the clinical chemistry

294   laboratory. *Advances in Experimental Medicine and Biology* 366:43–58.

295 Augustyniak A., Skrzydlewska E. 2010. The influence of L-carnitine suplementation on the

296   antioxidative abilities of serum and the central nervous system of ethanol-induced rats.

297   *Metabolic Brain Disease* 25:381–389. DOI: 10.1007/s11011-010-9217-7.

298 Basu S., De D., Dev Khanna H., Kumar A. 2014. Lipid peroxidation, DNA damage and total

299   antioxidant status in neonatal hyperbilirubinemia. *Journal of Perinatology: Official*

300   *Journal of the California Perinatal Association* 34:519–523. DOI: 10.1038/jp.2014.45.

301 Bencan Z., Sledge D., Levin ED. 2009. Buspirone, chlordiazepoxide and diazepam effects in a

302   zebrafish model of anxiety. *Pharmacology, Biochemistry, and Behavior* 94:75–80. DOI:

303   10.1016/j.pbb.2009.07.009.

304 Berk M., Dean OM., Cotton SM., Jeavons S., Tanious M., Kohlmann K., Hewitt K., Moss K.,

305   Allwang C., Schapkaitz I., Robbins J., Cobb H., Ng F., Dodd S., Bush AI., Malhi GS.

306   2014. The efficacy of adjunctive N-acetylcysteine in major depressive disorder: a double-

307   blind, randomized, placebo-controlled trial. *The Journal of Clinical Psychiatry* 75:628–

308   636. DOI: 10.4088/JCP.13m08454.

309 Chapela SP., Kriguer N., Fernández EH., Stella CA. 2009. Involvement of L-carnitine in cellular

310   metabolism: beyond Acyl-CoA transport. *Mini Reviews in Medicinal Chemistry* 9:1518–

311   1526.

312 Chiechio S., Canonico PL., Grilli M. 2017. l-Acetylcarnitine: A Mechanistically Distinctive and

313   Potentially Rapid-Acting Antidepressant Drug. *International Journal of Molecular*

314   *Sciences* 19. DOI: 10.3390/ijms19010011.

315 Cobb CA., Cole MP. 2015. Oxidative and nitrative stress in neurodegeneration. *Neurobiology of*

316   *Disease* 84:4–21. DOI: 10.1016/j.nbd.2015.04.020.

317 Dal Santo G., Conterato GMM., Barcellos LJG., Rosemberg DB., Piato AL. 2014. Acute

318   restraint stress induces an imbalance in the oxidative status of the zebrafish brain.

319   *Neuroscience Letters* 558:103–108. DOI: 10.1016/j.neulet.2013.11.011.

320 Draper HH., Hadley M. 1990. Malondialdehyde determination as index of lipid peroxidation.

321   *Methods in Enzymology* 186:421–431.

322 Egan RJ., Bergner CL., Hart PC., Cachat JM., Canavello PR., Elegante MF., Elkhayat SI.,

323   Bartels BK., Tien AK., Tien DH., Mohnot S., Beeson E., Glasgow E., Amri H.,

324   Zukowska Z., Kalueff AV. 2009. Understanding behavioral and physiological

325   phenotypes of stress and anxiety in zebrafish. *Behavioural brain research* 205:38–44.

326   DOI: 10.1016/j.bbr.2009.06.022.

327 Gebauer DL., Pagnussat N., Piato AL., Schaefer IC., Bonan CD., Lara DR. 2011. Effects of

328   anxiolytics in zebrafish: similarities and differences between benzodiazepines, buspirone

329   and ethanol. *Pharmacology, Biochemistry, and Behavior* 99:480–486. DOI:

330   10.1016/j.pbb.2011.04.021.

331 Giacomini ACVV., Abreu MS., Giacomini LV., Siebel AM., Zimerman FF., Rambo CL.,

332   Mocelin R., Bonan CD., Piato AL., Barcellos LJG. 2016. Fluoxetine and diazepam

333   acutely modulate stress induced-behavior. *Behavioural Brain Research* 296:301–310.

334   DOI: 10.1016/j.bbr.2015.09.027.

335 Gülçin I. 2006. Antioxidant and antiradical activities of L-carnitine. *Life Sciences* 78:803–811.

336   DOI: 10.1016/j.lfs.2005.05.103.

337 Hassan W., Noreen H., Castro-Gomes V., Mohammadzai I., da Rocha JBT., Landeira-Fernandez

338   J. 2016. Association of Oxidative Stress with Psychiatric Disorders. *Current*

339   *Pharmaceutical Design* 22:2960–2974.

340 Khan KM., Collier AD., Meshalkina DA., Kysil EV., Khatsko SL., Kolesnikova T., Morzherin

341   YY., Warnick JE., Kalueff AV., Echevarria DJ. 2017. Zebrafish models in

342   neuropsychopharmacology and CNS drug discovery. *British Journal of Pharmacology*

343   174:1925–1944. DOI: 10.1111/bph.13754.

344 Kim H., Lee SW., Baek KM., Park JS., Min JH. 2011. Continuous hypoxia attenuates paraquat-

345   induced cytotoxicity in the human A549 lung carcinoma cell line. *Experimental &*

346   *Molecular Medicine* 43:494–500. DOI: 10.3858/emm.2011.43.9.056.

347 Kumaran S., Deepak B., Naveen B., Panneerselvam C. 2003. Effects of levocarnitine on

348   mitochondrial antioxidant systems and oxidative stress in aged rats. *Drugs in R&D*

349   4:141–147.

350 Lau T., Bigio B., Zelli D., McEwen BS., Nasca C. 2017. Stress-induced structural plasticity of

351   medial amygdala stellate neurons and rapid prevention by a candidate antidepressant.

352   *Molecular Psychiatry* 22:227–234. DOI: 10.1038/mp.2016.68.

353   Levin ED., Bencan Z., Cerutti DT. 2007. Anxiolytic effects of nicotine in zebrafish. *Physiology*
354   *& Behavior* 90:54–58. DOI: 10.1016/j.physbeh.2006.08.026.

355   Levine J., Kaplan Z., Pettegrew JW., McClure RJ., Gershon S., Buriakovsky I., Cohen H. 2005.
356   Effect of intraperitoneal acetyl-L-carnitine (ALCAR) on anxiety-like behaviours in rats.
357   *The International Journal of Neuropsychopharmacology* 8:65–74. DOI:
358   10.1017/S1461145704004596.

359   Li J-L., Wang Q-Y., Luan H-Y., Kang Z-C., Wang C-B. 2012. Effects of L-carnitine against
360   oxidative stress in human hepatocytes: involvement of peroxisome proliferator-activated
361   receptor alpha. *Journal of Biomedical Science* 19:32. DOI: 10.1186/1423-0127-19-32.

362   Maes M., Galecki P., Chang YS., Berk M. 2011. A review on the oxidative and nitrosative stress
363   (O&NS) pathways in major depression and their possible contribution to the
364   (neuro)degenerative processes in that illness. *Progress in neuro-psychopharmacology &*
365   *biological psychiatry* 35:676–692. DOI: 10.1016/j.pnpbp.2010.05.004.

366   Marcon M., Herrmann AP., Mocelin R., Rambo CL., Koakoski G., Abreu MS., Conterato
367   GMM., Kist LW., Bogo MR., Zanatta L., Barcellos LJG., Piato AL. 2016. Prevention of
368   unpredictable chronic stress-related phenomena in zebrafish exposed to bromazepam,
369   fluoxetine and nortriptyline. *Psychopharmacology* Oct;233(21-22):3815-3824. DOI:
370   10.1007/s00213-016-4408-5.

371   Marcon M., Mocelin R., Benvenutti R., Costa T., Herrmann AP., de Oliveira DL., Koakoski G.,
372   Barcellos LJG., Piato A. 2018. Environmental enrichment modulates the response to
373   chronic stress in zebrafish. *The Journal of Experimental Biology* Feb 22;221(1-7, Pt 4).
374   DOI: 10.1242/jeb.176735.

375 Maximino C., Marques de Brito T., Dias CAG de M., Gouveia A., Morato S. 2010. Scototaxis as

376   anxiety-like behavior in fish. *Nature Protocols* 5:209–216. DOI: 10.1038/nprot.2009.225.

377 Mecocci P., Polidori MC. 2012. Antioxidant clinical trials in mild cognitive impairment and

378   Alzheimer's disease. *Biochimica et biophysica acta* 1822:631–638. DOI:

379   10.1016/j.bbadis.2011.10.006.

380 Mezzomo NJ., Silveira A., Giuliani GS., Quadros VA., Rosemberg DB. 2015. The role of taurine

381   on anxiety-like behaviors in zebrafish: A comparative study using the novel tank and the

382   light-dark tasks. *Neuroscience Letters* Feb 2;613:19-24. DOI:

383   10.1016/j.neulet.2015.12.037.

384 Mihara M., Uchiyama M. 1978. Determination of malonaldehyde precursor in tissues by

385   thiobarbituric acid test. *Analytical Biochemistry* 86:271–278.

386 Misra HP., Fridovich I. 1972. The role of superoxide anion in the autoxidation of epinephrine

387   and a simple assay for superoxide dismutase. *The Journal of Biological Chemistry*

388   247:3170–3175.

389 Mocelin R., Herrmann AP., Marcon M., Rambo CL., Rohden A., Bevilaqua F., de Abreu MS.,

390   Zanatta L., Elisabetsky E., Barcellos LJG., Lara DR., Piato AL. 2015. N-acetylcysteine

391   prevents stress-induced anxiety behavior in zebrafish. *Pharmacology, Biochemistry, and*

392   *Behavior* Dec;139 Pt B:121-6. DOI: 10.1016/j.pbb.2015.08.006.

393 Mocelin R., Marcon M., D'ambros S., Herrmann AP., Araujo AS da R., Piato A. 2017.

394   Behavioral and Biochemical Effects of N-Acetylcysteine in Zebrafish Acutely Exposed

395   to Ethanol. *Neurochemical Research* Feb;43(2):458-464. DOI: 10.1007/s11064-017-

396   2442-2.

397   Nasca C., Bigio B., Zelli D., de Angelis P., Lau T., Okamoto M., Soya H., Ni J., Brichta L.,

398   Greengard P., Neve RL., Lee FS., McEwen BS. 2017. Role of the Astroglial Glutamate

399   Exchanger xCT in Ventral Hippocampus in Resilience to Stress. *Neuron* 96:402-413.e5.

400   DOI: 10.1016/j.neuron.2017.09.020.

401   Nasca C., Xenos D., Barone Y., Caruso A., Scaccianoce S., Matrisciano F., Battaglia G., Mathé

402   AA., Pittaluga A., Lionetto L., Simmaco M., Nicoletti F. 2013. L-acetylcarnitine causes

403   rapid antidepressant effects through the epigenetic induction of mGlu2 receptors.

404   *Proceedings of the National Academy of Sciences of the United States of America*

405   110:4804–4809. DOI: 10.1073/pnas.1216100110.

406   Ng F., Berk M., Dean O., Bush AI. 2008. Oxidative stress in psychiatric disorders: evidence base

407   and therapeutic implications. *The International Journal of Neuropsychopharmacology*

408   11:851–876. DOI: 10.1017/S1461145707008401.

409   Nicassio L., Fracasso F., Sirago G., Musicco C., Picca A., Marzetti E., Calvani R., Cantatore P.,

410   Gadaleta MN., Pesce V. 2017. Dietary supplementation with acetyl-l-carnitine

411   counteracts age-related alterations of mitochondrial biogenesis, dynamics and antioxidant

412   defenses in brain of old rats. *Experimental Gerontology* 98:99–109. DOI:

413   10.1016/j.exger.2017.08.017.

414   Niedzielska E., Smaga I., Gawlik M., Moniczewski A., Stankowicz P., Pera J., Filip M. 2016.

415   Oxidative Stress in Neurodegenerative Diseases. *Molecular Neurobiology* 53:4094–4125.

416   DOI: 10.1007/s12035-015-9337-5.

417   Ortiz GG., Pacheco Moisés FP., Mireles-Ramírez M., Flores-Alvarado LJ., González-Usigli H.,

418   Sánchez-González VJ., Sánchez-López AL., Sánchez-Romero L., Díaz-Barba EI.,

419   Santoscoy-Gutiérrez JF., Rivero-Moragrega P. 2017. Oxidative Stress: Love and Hate

420   History in Central Nervous System. *Advances in Protein Chemistry and Structural*

421   *Biology* 108:1–31. DOI: 10.1016/bs.apcsb.2017.01.003.

422 Ozcan ME., Gulec M., Ozerol E., Polat R., Akyol O. 2004. Antioxidant enzyme activities and

423   oxidative stress in affective disorders. *International Clinical Psychopharmacology*

424   19:89–95.

425 Patel M. 2016. Targeting Oxidative Stress in Central Nervous System Disorders. *Trends in*

426   *Pharmacological Sciences* 37:768–778. DOI: 10.1016/j.tips.2016.06.007.

427 Pittman J., Piato A. 2017. Developing Zebrafish Depression-Related Models. In: Kalueff AV ed.

428   *The rights and wrongs of zebrafish: Behavioral phenotyping of zebrafish*. Cham: Springer

429   International Publishing, 33–43. DOI: 10.1007/978-3-319-33774-6_2.

430 Ribas GS., Vargas CR., Wajner M. 2014. L-carnitine supplementation as a potential antioxidant

431   therapy for inherited neurometabolic disorders. *Gene* 533:469–476. DOI:

432   10.1016/j.gene.2013.10.017.

433 Sandi C., Haller J. 2015. Stress and the social brain: behavioural effects and neurobiological

434   mechanisms. *Nature Reviews Neuroscience* 16:290–304. DOI: 10.1038/nrn3918.

435 Santos P., Herrmann AP., Benvenutti R., Noetzold G., Giongo F., Gama CS., Piato AL.,

436   Elisabetsky E. 2017. Anxiolytic properties of N-acetylcysteine in mice. *Behavioural*

437   *Brain Research* 317:461–469. DOI: 10.1016/j.bbr.2016.10.010.

438 Schiavone S., Colaianna M., Curtis L. 2015. Impact of early life stress on the pathogenesis of

439   mental disorders: relation to brain oxidative stress. *Current Pharmaceutical Design*

440   21:1404–1412.

441 Schiavone S., Jaquet V., Trabace L., Krause K-H. 2013. Severe life stress and oxidative stress in

442   the brain: from animal models to human pathology. *Antioxidants & Redox Signaling*

443   18:1475–1490. DOI: 10.1089/ars.2012.4720.

444 Sepand MR., Razavi-Azarkhiavi K., Omidi A., Zirak MR., Sabzevari S., Kazemi AR., Sabzevari

445   O. 2016. Effect of Acetyl-L-Carnitine on Antioxidant Status, Lipid Peroxidation, and

446   Oxidative Damage of Arsenic in Rat. *Biological Trace Element Research* 171:107–115.

447   DOI: 10.1007/s12011-015-0436-y.

448 Singh S., Mishra A., Mishra SK., Shukla S. 2017. ALCAR promote adult hippocampal

449   neurogenesis by regulating cell-survival and cell death-related signals in rat model of

450   Parkinson's disease like-phenotypes. *Neurochemistry International* 108:388–396. DOI:

451   10.1016/j.neuint.2017.05.017.

452 Singh S., Mishra A., Srivastava N., Shukla R., Shukla S. 2016. Acetyl-L-Carnitine via

453   Upegulating Dopamine D1 Receptor and Attenuating Microglial Activation Prevents

454   Neuronal Loss and Improves Memory Functions in Parkinsonian Rats. *Molecular*

455   *Neurobiology* Jan;55(1):583-602. DOI: 10.1007/s12035-016-0293-5.

456 Smaga I., Niedzielska E., Gawlik M., Moniczewski A., Krzek J., Przegaliński E., Pera J., Filip

457   M. 2015. Oxidative stress as an etiological factor and a potential treatment target of

458   psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism.

459   *Pharmacological reports: PR* 67:569–580. DOI: 10.1016/j.pharep.2014.12.015.

460 Stewart AM., Ullmann JFP., Norton WHJ., Parker MO., Brennan CH., Gerlai R., Kalueff AV.

461   2015. Molecular psychiatry of zebrafish. *Molecular Psychiatry* 20:2–17. DOI:

462   10.1038/mp.2014.128.

463   Sunderman FW., Marzouk A., Hopfer SM., Zaharia O., Reid MC. 1985. Increased lipid

464   peroxidation in tissues of nickel chloride-treated rats. *Annals of Clinical and Laboratory*

465   *Science* 15:229–236.

466   Traina G. 2016. The neurobiology of acetyl-L-carnitine. *Frontiers in Bioscience (Landmark*

467   *Edition)* 21:1314–1329.

468   Wang S-M., Han C., Lee S-J., Patkar AA., Masand PS., Pae C-U. 2014. A review of current

469   evidence for acetyl-l-carnitine in the treatment of depression. *Journal of Psychiatric*

470   *Research* 53:30–37. DOI: 10.1016/j.jpsychires.2014.02.005.

471   Wang W., Lu Y., Xue Z., Li C., Wang C., Zhao X., Zhang J., Wei X., Chen X., Cui W., Wang

472   Q., Zhou W. 2015. Rapid-acting antidepressant-like effects of acetyl-l-carnitine mediated

473   by PI3K/AKT/BDNF/VGF signaling pathway in mice. *Neuroscience* 285:281–291. DOI:

474   10.1016/j.neuroscience.2014.11.025.

475   Yagi K. 1998. Simple assay for the level of total lipid peroxides in serum or plasma. *Methods in*

476   *Molecular Biology (Clifton, N.J.)* 108:101–106. DOI: 10.1385/0-89603-472-0:101.

477   Yavuzer H., Yavuzer S., Cengiz M., Erman H., Doventas A., Balci H., Erdincler DS., Uzun H.

478   2016. Biomarkers of lipid peroxidation related to hypertension in aging. *Hypertension*

479   *Research: Official Journal of the Japanese Society of Hypertension* 39:342–348. DOI:

480   10.1038/hr.2015.156.

481

482   **Figure captions**

483   **Figure 1.** Schematic representation of the experimental protocol. Novel tank test (A),

484   light/dark test (B), and acute chasing stress and biochemical assays (C).

485

486   **Figure 2.** Effects of ALC (0.1, 1.0 and 10.0 mg/L) on the distance traveled (A), the number of

487   crossings (B), entries (C) and time (D) in the upper zone, and time in the bottom zone (E) in the

488   novel tank test in zebrafish. The data are presented as the mean + S.E.M. One-way ANOVA

489   followed by Tukey post hoc test. n=15-23. *p<0.05, **p<0.01, ***p<0.001 vs. control group.

490

491   **Figure 3.** Effects of ALC (0.1, 1.0 and 10.0 mg/L) on the number of crossings (A) and time in

492   the lit side (B) in the light/dark test in zebrafish. The data are presented as the mean + S.E.M. One-

493   way ANOVA followed by Tukey post hoc test. n=18-27. *p<0.05 vs. control group.

494

495   **Figure 4.** Effects of ALC pretreatment against stress-induced changes in behavioral

496   parameters in zebrafish. Distance traveled (A), number of crossings (B), entries (C) and time (D) in

497   the upper zone, and time in the bottom zone (E). The data are presented as the mean + S.E.M. Two-

498   way ANOVA followed by Bonferroni's test. n=10-12. *p<0.05, ***p<0.001, ****p<0.0001 vs.

499   control group (S-); †p<0.05, †††p<0.001, ††††p<0.0001 vs. stressed control group (S+).

500

501   **Figure 5.** Effects of ALC pretreatment against stress-induced changes in biochemical

502   parameters in zebrafish. Thiobarbituric acid reactive substances (A), sulfhydryl (B), non-protein

503   sulphydryl (C), superoxide dismutase (D), and catalase, (E). The data are presented as the mean +

504   S.E.M. Two-way ANOVA followed by Bonferroni's test. n=3-4. *p<0.05, ***p<0.001 vs. control

505   group (S-); †p<0.05, †††p<0.001, ††††p<0.0001 vs. stressed control group (S+).

506

507   **Table captions**

508

509   **Table 1.** Results of two-way analysis of variance (ANOVA) of behavioral analysis and the

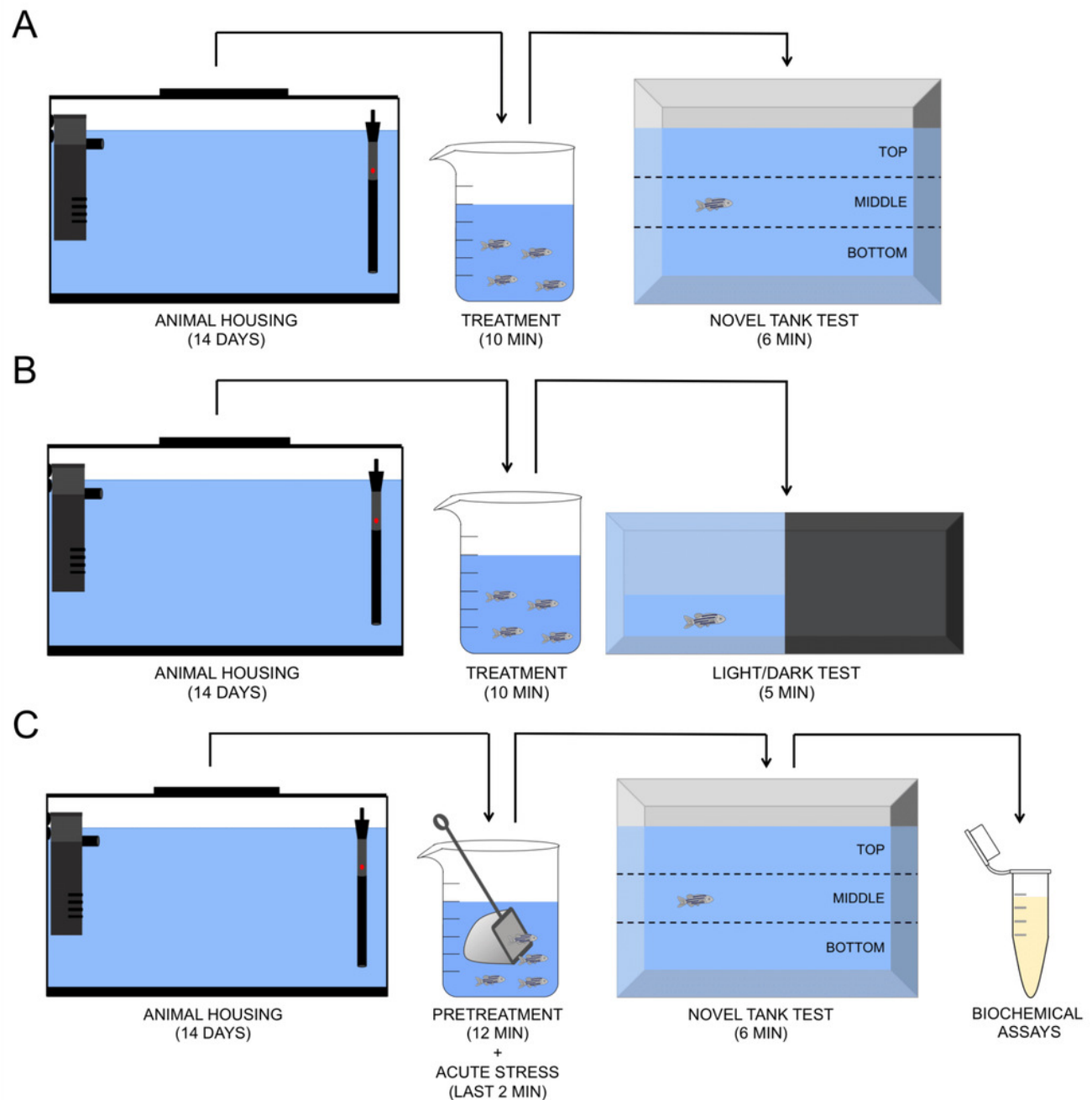
510   interaction between treatment with ALC and acute chasing stress.

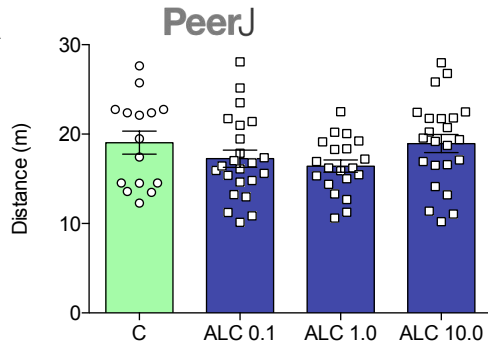
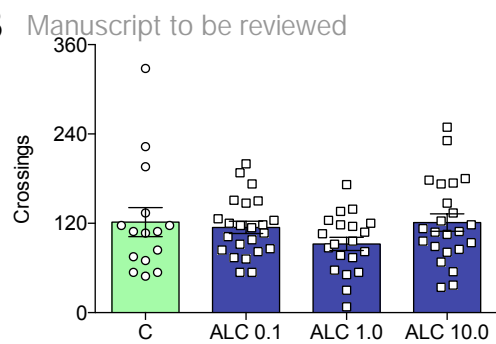
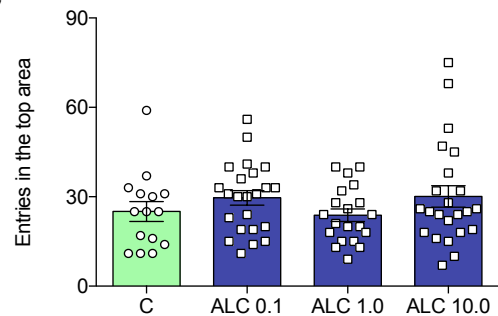
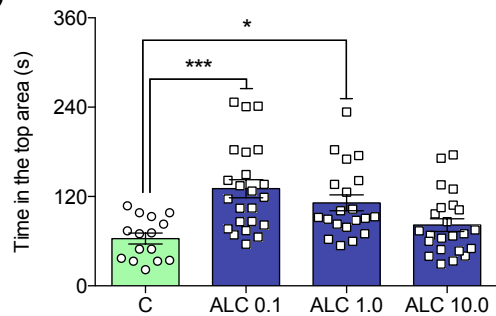
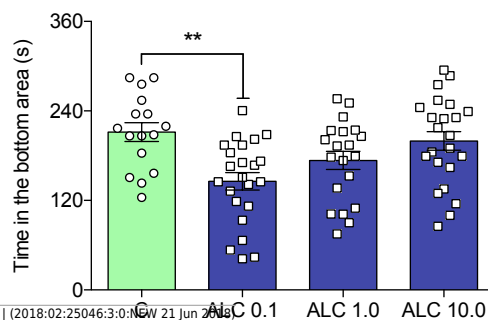
511

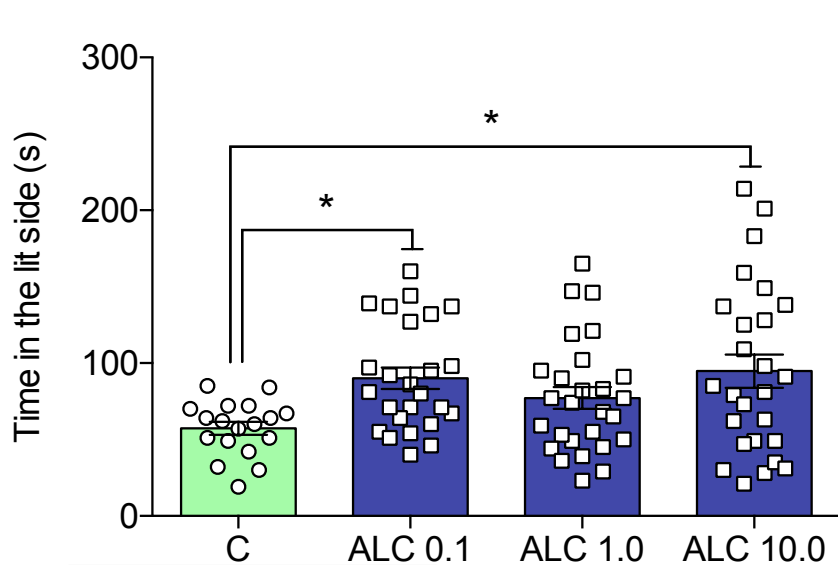
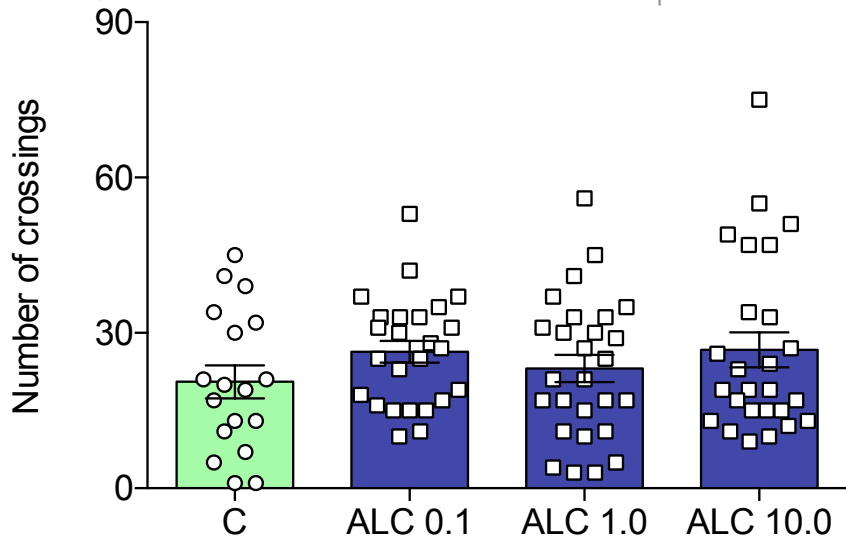
512   **Table 2.** Results of two-way analysis of variance (ANOVA) of biochemical analysis and the

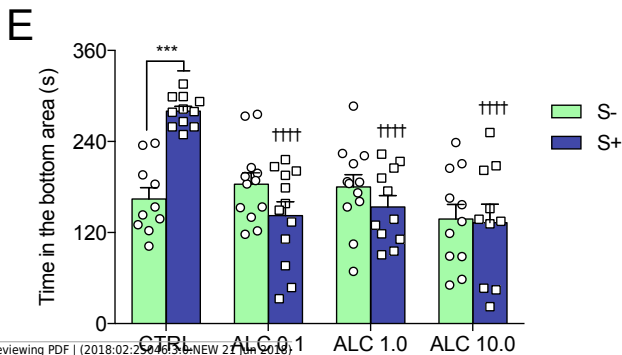
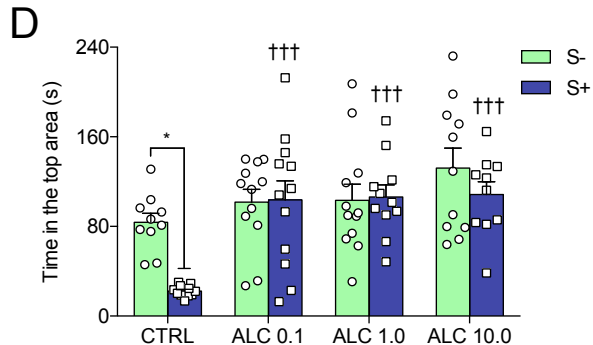
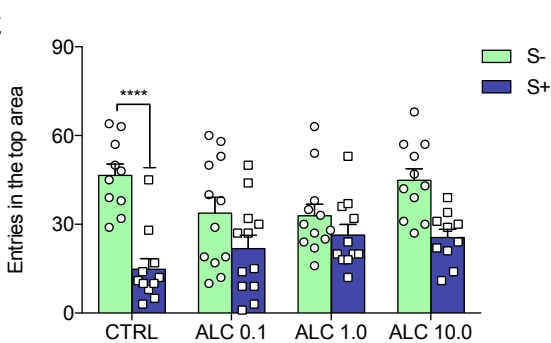
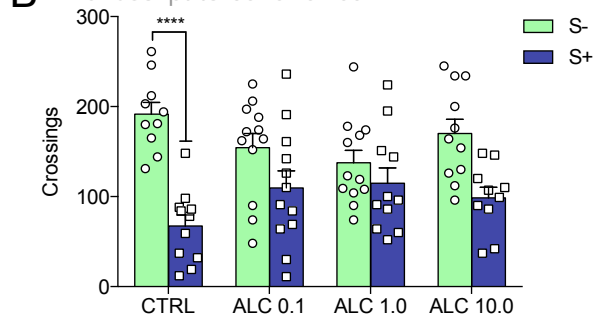
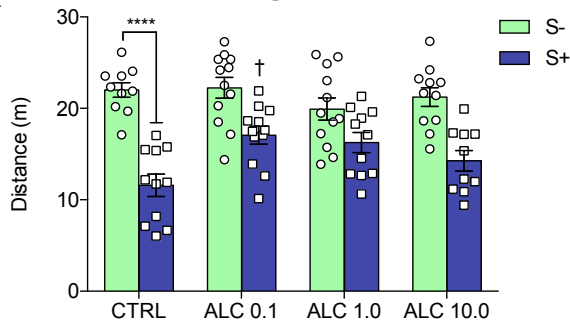
513   interaction between treatment with ALC and acute chasing stress.

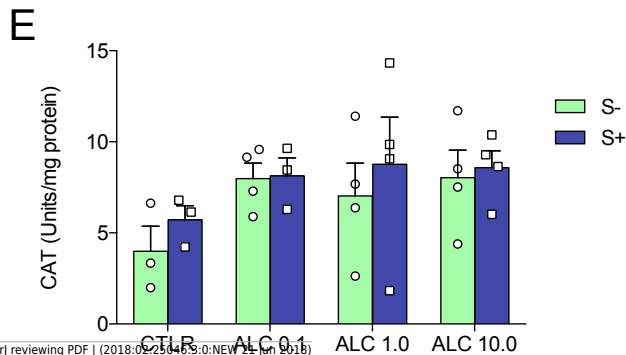
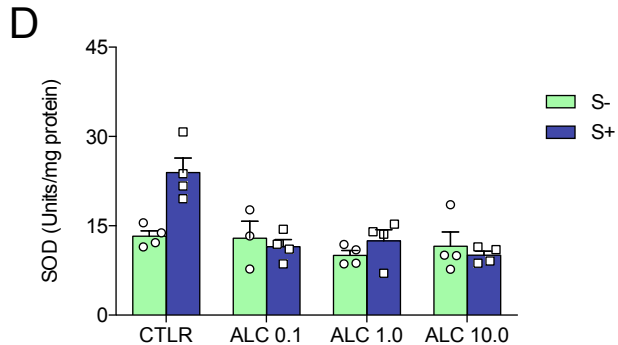
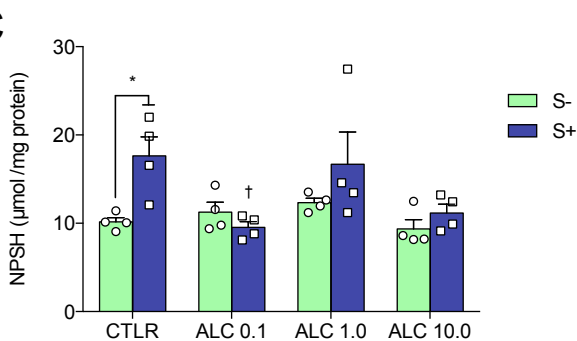
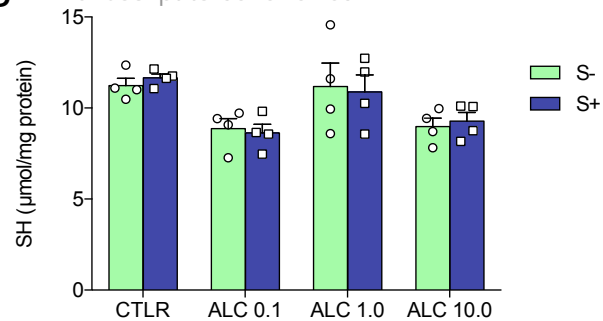
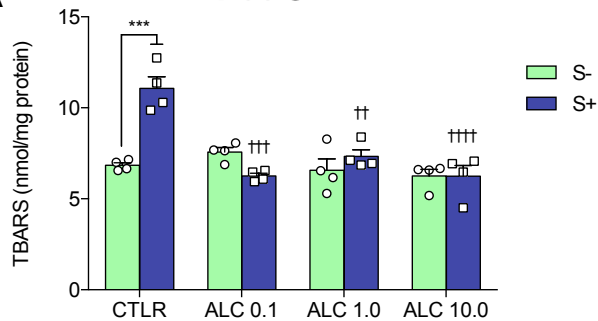
Figure 1

Schematic representation of the experimental protocol. Novel tank test (A), light/dark test (B), and acute chasing stress and biochemical assays (C).

Effects of ALC (0.1, 1.0 and 10.0 mg/L) behavioral parameters in zebrafish submitted to the novel tank test.

(A) distance traveled, (B) number of crossings, (C) entries and (D) time in the upper zone, and (E) time in the bottom zone. The data are presented as the mean + S.E.M. One-way ANOVA followed by Tukey post hoc test. n=15-23. *p<0.05, **p<0.01, ***p<0.001 vs. control group.

Effects of ALC (0.1, 1.0 and 10.0 mg/L) in the light/dark test in zebrafish.

(A) number of crossings and (B) time in the lit side. The data are presented as the mean +
S.E.M. One-way ANOVA followed by Tukey post hoc test. n=18-27. *p<0.05 vs. control group.

Effects of ALC pretreatment against stress-induced changes in behavioral parameters in zebrafish.

(A) distance traveled, (B) number of crossings, (C) entries and (D) time in the upper zone, and (E) time in the bottom zone. The data are presented as the mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. n=10-12. *p<0.05, ***p<0.001, ****p<0.0001 vs. control group (S-); †p<0.05, †††p<0.001, ††††p<0.0001 vs. stressed control group (S+).

Effects of ALC pretreatment against stress-induced changes in biochemical parameters in zebrafish.

(A) thiobarbituric acid reactive substances, (B) sulfhydryl, (C) non-protein sulphydryl, (D) superoxide dismutase, and (E) catalase. The data are presented as the mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. n=3-4. *$p<0.05$, ***$p<0.001$ vs. control group (S-); †$p<0.05$, †††$p<0.001$, ††††$p<0.0001$ vs. stressed control group (S+).

Results of two-way analysis of variance (ANOVA) of behavioral analysis and the interaction between treatment with ALC and acute chasing stress.

DF = degrees of freedom. Significant effects (p<0.05) are given in bold font.

1 **Table 1:**

2 Results of two-way analysis of variance (ANOVA) of behavioral analysis and the interaction

3 between treatment with ALC and acute chasing stress.

Dependent variable	Effects	F-value	DF	P-value
Total distance	Interaction	3.46	3,81	**0.0201**
	ALC	2.39	3,81	0.0745
	Stress	71.34	3,81	**0.0001**
Crossings	Interaction	4.04	3,81	**0.0099**
	ALC	0.10	3,81	0.9583
	Stress	37.11	3,81	**0.0001**
Entries in the top	Interaction	3.47	3,81	**0.0198**
	ALC	1.18	3,81	0.3215
	Stress	36.10	3,81	**0.0001**
Time in the top	Interaction	2.72	3,81	**0.0499**
	ALC	9.81	3,81	**0.0001**
	Stress	4.86	3,81	**0.0303**
Time in the bottom	Interaction	9.02	3,81	**0.0001**
	ALC	9.03	3,81	**0.0001**
	Stress	0.84	3,81	0.3613

5 DF = degrees of freedom. Significant effects (p<0.05) are given in bold font.

Results of two-way analysis of variance (ANOVA) of biochemical analysis and the interaction between treatment with ALC and acute chasing stress.

DF = degrees of freedom. Significant effects (p<0.05) are given in bold font.

1   **Table 2:**
2   Results of two-way analysis of variance (ANOVA) of biochemical analysis and the interaction
3   between treatment with ALC and acute chasing stress.

Dependent variable	Effects	F-value	DF	P-value
Lipid peroxidation (TBARS)	Interaction	14.70	3,24	**0.0001**
	ALC	14.39	3,24	**0.0001**
	Stress	8.80	1,24	**0.0067**
Sulfhydryl (SH)	Interaction	0.14	3,24	0.9339
	ALC	7.80	3,24	**0.0008**
	Stress	0.01	1,24	0.9289
Non-protein thiol (NPSH)	Interaction	2.73	3,24	0.0665
	ALC	3.63	3,24	**0.0273**
	Stress	6.35	1,24	**0.0188**
Superoxide dismutase (SOD)	Interaction	5.46	3,23	**0.0055**
	ALC	9.93	3,23	**0.0004**
	Stress	4.26	1,23	0.0504
Catalase (CAT)	Interaction	0.13	3,21	0.9393
	ALC	1.89	3,21	0.1626
	Stress	0.87	1,21	0.3606

5   DF = degrees of freedom. Significant effects ($p<0.05$) are given in bold font.