Exposure during embryonic development to Roundup® Power 2.0 affects lateralization, level of activity and growth, but not defensive behaviour of marsh frog tadpoles

Alessandro Bolis [a], Andrea Gazzola [a], Daniele Pellitteri-Rosa [a], Anita Colombo [b], Patrizia Bonfanti [b], Adriana Bellati [a,*]

[a] Department of Earth and Environmental Sciences, University of Pavia, Via Ferrata 9, 27100 Pavia, Italy

[b] Department of Environmental and Earth Sciences, Research Centre POLARIS, University of Milano Bicocca, P.zza della Scienza 1, 20126 Milano, Italy

[*] corresponding author: Adriana Bellati, Department of Earth and Environmental Sciences, University of Pavia, Via Ferrata 9, 27100 Pavia, Italy; tel: +39 0382986839; e-mail: adriana.bellati@unipv.it

ABSTRACT

As glyphosate-based herbicides, sold under the commercial name Roundup®, represent the most used herbicides in the world, contamination of the freshwater environment by glyphosate has become a widespread issue. In Italy, glyphosate was detected in half of the surface waters monitoring sites and its concentrations were higher than environmental quality standards in 24.5% of them. It can last from days to months in water, leading to exposure for aquatic organisms and specifically to amphibians' larvae that develop in shallow water bodies with proven effects to development and behaviour. In this study, we tested the effects of a 96h exposure during embryonic development of marsh frog's tadpoles to three ecologically relevant Roundup® Power 2.0 concentrations. As expected, given the low concentrations tested, no mortality was observed. Morphological measurements highlighted a reduction in the total length in tadpoles exposed to 7.6 mg a.e./L, while an increase was observed at lower concentrations of 0.7 and 3.1 mg a.e./L compared to control group. Tadpoles raised in 7.6 mg a.e./L also showed a smaller tail membrane than those raised in the control solution. Regarding behaviour, we tested tadpoles in two different sessions (Gosner stages 25 and 28/29) for lateralization, antipredator response and basal activity. Lower intensity of lateralization was detected in tadpoles raised at the highest Roundup® concentration in the first session of observation, while no significant difference among treatments was observed in the second one. In both sessions, effects of glyphosate embryonic exposure on antipredator response, measured as the proportional change in activity after the injection of tadpole-fed predator (*Anax imperator*) cue, were not detected. Tadpoles exposed during embryonic development to Roundup® exhibited lower basal activity than the control group, with the strongest reduction for the 7.6 mg a.e./L treatment. Our results reinforce the concern of glyphosate contamination impact on amphibians.

KEYWORDS

43   **INTRODUCTION**

44   Glyphosate-based herbicides (GBHs) are between the most sold broad-spectrum herbicides in the

45   world (Duke and Powles, 2008), whose use reaches nearly 100 million kilograms on an annual basis

46   (Grube et al., 2011). The active ingredient, glyphosate, appears in several alternative formulations,

47   altogether known under the commercial name Roundup® (Monsanto Co., St. Louis, MO, USA).

48   Glyphosate use spans different applications, from no-tillage farming and conventional agriculture to

49   non-cultivated areas, forest management and private gardening (Dill, 2005; Dill et al., 2010). Its use

50   is approved until 2022 by the European Union (European Commission, 2017), however in Italy its

51   application is restricted during harvest and threshing, and banned in public gardens and parks,

52   schools, private gardening and health facilities (Italian Ministry of Health, 2016). Although the

53   aforementioned restrictions, a recent report concerning surface waters pesticide contamination

54   highlighted the presence of this molecule in 47.4% of the Surface Waters Monitoring Sites in Italy

55   (SWMS), with its concentration exceeding the Environmental Quality Standards (EQS) in 24.5% of

56   them (ISPRA, 2018).

57   As this molecule has been engineered to kill plants, specifically post-emergent leaves and

58   grasses, glyphosate is considered to have low mammalian toxicity, being "practically non-toxic" for

59   bees, birds and most aquatic organisms, according to the World Health Organization/Food and

60   Agriculture Organization (Lajmanovich et al., 2011). Microorganism degrade the active ingredient

61   into its metabolite aminomethylphosphonic acid (AMPA), which is eventually oxidized into carbon

62   dioxide, in both soil and water. Since the rate of degradation is strictly dependent on physical (e.g.

63   temperature) and chemical (e.g. pH) environmental conditions, glyphosate's half-life can last from

64   days to months (e.g. 7–70 days in the water; Giesy et al., 2000), leading to possible chronic

65   exposures for organisms living in certain environments (Feng et al., 1990; Borggaard and Gimsing,

66   2008; Bailey et al., 2018). GBH formulations are generally more toxic than glyphosate by itself,

67   mainly due to the presence of surfactants (e.g. polyoxyethylene - POE, polyethoxylated tallow

68   amine - POEA). Previous studies showed that the toxicity of GBHs to aquatic organisms is largely

69   caused by the surfactant in the mixture (Edginton et al., 2004; Moore et al., 2012). These substances

70   may not only cause toxicity by themselves, but also facilitate the penetration of the active ingredient

71   in animal cells causing teratogenic effects, as shown in amphibian embryos and tadpoles of several

72   species (Borggaard and Gimsing, 2008; Paganelli et al., 2010; Bonfanti et al., 2018; Gill et al.,

73   2018).

74   To date, GBHs have been proved to affect amphibians' development through growth

75   retardation and weight reduction during larval stages, especially in anurans (Smith, 2001; Cauble

76   and Wagner, 2005; Lanctôt et al., 2014; Navarro-Martín et al., 2014). Furthermore, GBHs have

77   been shown to alter behaviour in aquatic organisms, like fishes and amphibians, involving

78   locomotion, memory, visual and olfactory senses and antipredator responses (Tierney et al., 2006;

79   Moore et al., 2015; Besson et al., 2017; Bridi et al., 2017; Mikò et al., 2017). In the poecilid

80   *Cnesterodon decemmaculatus*, exposure to GBHs during development has been proven to cause a

81   significant inhibitory effect on AChE (acetylcholinesterase) activity in the tail (Menéndez-Helman

82   et al., 2012). The inhibition of this enzyme, involved in the breakdown of the neurotransmitter

83   acetylcholine in muscle and nerve synapses and thus in the end of a transmission of a neural

84   impulse (Zimmerman and Soreq, 2006; Tripathi and Srivastava, 2010), leads to a possible reduction

85   of basal activity. Nonetheless, another study demonstrated that AChE activity increases in tadpoles

86   of the marsh frog *Pelophylax ridibundus*, the green toad *Bufotes viridis*, and the African clawed

87   toad *Xenopus laevis* exposed to GBH formulations (Güngördü, 2013). This finding however is still

88   unmatched by other studies, at least for anurans since glyphosate instead decreased AChE activity

89   in tadpoles of the toad *Rhinella arenarum* in a study by Lajmanovich and colleagues (2011).

90   An additional negative effect of herbicide water contamination could be lateralization

91   impairment (Besson et al., 2017). Brain lateralization is the difference between the activity of the

92   right and left hemisphere in the execution of several tasks, implying the preferential use of one body

93   portion over the other (Davidson and Hugdahl, 1996; Bisazza et al., 1998). Laterality is thought to

94   augment cognitive abilities, by optimizing the processing of information in the two-separate brain

95   hemispheres, thus enhancing the ability to make decisions when facing novel multi-sensory stimuli

96   (Vallortigara and Rogers, 2005; Salva et al., 2012). Defence against predators is a well-known

97   lateralized behaviour in vertebrates, as the recognition and escape performances seem to depend on

98   the side of appearance of a predatory threat (Siniscalchi et al., 2010; Shibasaki et al., 2014). Fishes,

99   birds, and reptiles have shown eye preference when keeping track of predators or choosing for a

100   prevalent escape direction in predatory risk conditions (Sovrano et al., 2005; Koboroff et al., 2008;

101   Martín et al., 2010; Pellitteri-Rosa and Gazzola, 2018).

102   In larval amphibians, defensive behaviour is generally activated by chemical (olfactory)

103   cues, released in the water both by injured conspecifics (alarm cue) as well as by predators

104   (kairomones). It has been shown that glyphosate negatively affects the functionality of the olfactory

105   system by inactivating the chemical cues conveying information on predation risk, thus inhibiting

106   predator recognition, or by lowering the learning ability of tadpoles (Mandrillon and Saglio, 2007a,

107   b; Moore et al., 2015). An altered olfactory system could in turn weaken defensive responses and

108   negatively affect tadpoles' survival in their natural environment.

109   Behavioural alterations may appear when tadpoles develop in the contaminated medium (i.e.

110   chronic exposure), or alternatively when adults are exposed, even briefly, to the substance (i.e.

111   acute-transitory exposure). Therefore, contaminants may affect amphibians at every life stage, with

112   negative effect on growth and reproduction, and finally survival of the single individual likewise the

113   entire population (Relyea, 2005; Gill et al., 2018). The complex life cycle of anurans, which shifts

114   their biological cycle from water to terrestrial habitats and the assumption that several

115   environmental cues experienced during embryonic development can affect the range of

116   physiological and behavioural responses available as adults (Dufty et al., 2002), makes them an

117   ideal candidate to explore the effects of pollutants during development.

118   In this study, we explored the effects of glyphosate on the morphology and some fitness-

119   related behavioural traits in tadpoles of a widely distributed European anuran, the marsh frog *P.*

120   *ridibundus*, by exposing embryos to different ecologically relevant concentrations of the

121   commercial GBH Roundup® Power 2.0 (Monsanto Co., St. Louis, MO, USA). *P. ridibundus* eggs

122   are frequently found in areas with vegetation, wetlands, agricultural areas, and urban regions where

123   the presence of pesticides could be significant. Our aims were to investigate the possible effects of

124   early (embryonic) exposure to RU-PW on: (1) total length and tail depth, to detect growth

125   retardation; (2) lateralization, as a proxy of the correct development of the nervous system of the

126   tadpole; (3) level of activity (both basal and in the presence of a predatory stimulus), to infer the

127   ability of the individual to correctly cope with important environmental pressures (i.e. predation).

128   Previous studies showed that *P. ridibundus* tadpoles are more resistant to glyphosate-based

129   herbicide than other species of anurans (Güngördü, 2013). Nonetheless, adults of this species

130   demonstrated a slight increase in the hepatosomatic index (HSI) when exposed via intraperitoneal to

131   0.138×10^{-3} mL Roundup®/g per body mass (Păunescu and Ponepal, 2011). According to previous

132   findings, we expect to find a relationship between glyphosate exposure and both tadpoles

133   morphological and behavioural traits. At the same time, we also expect these effects to only appear

134   at particular developmental stages and have little or no effect on the long-term survival of

135   individuals, as a result of general high resistance of the species to environmental stressors, like

136   xenobiotics.

137

138   **MATERIALS AND METHODS**

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140   *Animals collection and breeding*

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142   The marsh frog is an anuran endemic to Central and Eastern Europe. In Italy, native populations of

143   this taxon only occur at the edge of the Northern-Eastern region of Friuli-Venezia Giulia (Lanza et

144   al., 2006). Nonetheless, several populations of this frog had been introduced in Italy for edible

145   purposes. According to the Environmental Impact Classification of Alien Taxa (EICAT) of the

146   International Union for Conservation of Nature (IUCN), the species is considered one of the

147 invasive species with the highest potential to impact native species diversity worldwide, because of

148 its ability to occupy a wide variety of habitats on one hand, and to hybridize with native taxa, thus

149 producing viable and fertile hybrids, on the other (Kumschick et al., 2017).

150 Adult marsh frogs were collected at night on June 15th, 2018, from an artificial pond situated

151 in the municipality of Mezzolombardo (46.19°N, 11.09°E, TN, Italy). Here, previous surveys had

152 highlighted the presence of water frogs that were ascribed to *P. ridibundus* following molecular

153 analysis. Frogs were captured using fishing nets and transported to the laboratory of the Department

154 of Earth and Environmental Sciences of the University of Pavia. Until the beginning of the 96h

155 exposure, frogs were kept in plastic tanks filled with dechlorinated tap water and fed with crickets

156 (*Acheta domesticus*) ad libitum.

157 Embryos of *P. ridibundus* used in the 96h exposure were obtained through in vitro

158 fertilization following the procedure for anuran breeding provided by Pruvost (2013), and Berger et

159 al. (1994) with modifications (Bonfanti et al., 2004). Specifically, 24 h before egg collection, we

160 triggered ovulation stimulation by injecting both females and males (n = 6) with LHRH hormone

161 (BachemH-7525, Bubendorf, Switzerland) at a concentration of 2 mg in 100 mL Holtfreter's

162 solution (Holtfreter, 1944). Individuals were injected into the dorsal lymph sac 10 µl/g of body

163 mass of hormone.

164 The male selected for the crossing was euthanized in a MS-222 solution (Sigma A-5040, St.

165 Gallen, Switzerland) at 2 mg/L. Testes were removed, put in 2 mL of cold DBT solution (Tris-HCL

166 10 m, pH 7.5) and minced to obtain a sperm suspension. Egg groups, obtained by gently massaging

167 the abdominal region of the females, were collected in 90-mm plastic Petri dishes and immediately

168 inseminated with sperm suspension; after 2 min, 30 ml of Holtfreter's solution were added to each

169 Petri dish. Successful insemination was detected when after 30 min of incubation at 25 °C all the

170 eggs were oriented with the dark side (animal pole) up. Embryos (Gosner stages 4-5) were selected

171 under a stereomicroscope and the jelly coat was removed by swirling the embryos for 1-2 min in a

172 2.25% L cysteine solution (pH 8.1). All the fertilizations were performed on June 18th, 2018.

173   We collected five dragonfly larvae (*Anax imperator*) from a small pond situated in the

174   botanical garden in Pavia (45.18°N, 9.15°E, Italy) on July 2nd, 2018. They were individually kept in

175   800 mL cups filled with 500 mL of aged tap water and wooden sticks as perching sites. These

176   animals were later used for behavioural trials.

177

178   *Roundup® Power 2.0 solutions*

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180   Roundup® Power 2.0 (Monsanto Italia S.P.A.), referred to as RU-PW, was formulated with a

181   guarantee of 360 g glyphosate acid equivalent (a.e.) per litre present as the potassium salt (CAS RN

182   70901-12-1), six percent by volume of ethoxylated ether alkyl ammine (CAS RN 68478-96-6) and

183   58.5% water and other ingredients not specified by the producer.

184   A stock solution of RU-PW at nominal concentration of 100 mg a.e./L using FETAX

185   solution was prepared. In according to previous experiments, we opted to use FETAX solution as

186   control, since it is the optimal solution for the development of water frogs. The composition in

187   mg/L was: NaCl 625, $NaHCO_3$ 96, KCl 30, $CaCl_2$ 15, $CaSO_4$-$2H_2O$ 60, and $MgSO_4$ 70, pH 7.5–8.5

188   (Dawson and Bantle, 1987).

189

190   *96h exposure test*

191

192   Normally-cleaved embryos at the midblastula stage (Gosner stage 8; Gosner, 1960) five hours post

193   fertilization (hpf), were selected from each female (n = 2), assigned to the experimental groups (n =

194   15 per group) and placed in covered Petri dishes containing 40 mL of control (FETAX) or RU-PW

195   solution. For each treatment (three RU-PW concentrations and control) and for each female, five

196   replicas were performed for a total of 300 embryos per female.

197   RU-PW concentrations used for the 96h exposure were 0.7 mg a.e./L, 3.1 mg a.e./L and 7.6

198   mg a.e./L.; control treatment was represented by FETAX solution. Each experimental concentration

199 corresponded to one of the following three different scenarios: environmental concentration of the

200 herbicide without intervention (0.7 mg a.e./L), concentration occurring shortly after the application

201 of the herbicide (3.1 mg a.e./L), and concentration estimated in worst-case scenarios (e.g. direct

202 spraying in a flooded field, 7.6 mg a.e./L) (Wagner, 2013). Embryos were incubated in a

203 thermostatic chamber at 25 ± 0.5 °C and exposure solutions were renewed every 24 h (semi-static

204 conditions). The selected concentrations should not cause different mortality rates with respect to

205 the control group, since the estimated LC50 for RU-PW is 24.75 mg a.e./L for *Xenopus laevis*

206 (Bonfanti et al., 2018), a species considered more sensitive to Roundup than marsh frogs

207 (Güngordü, 2013).

208

209 *Morphological measurements*

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211 At the end of the exposure period (96 hpf) we randomly selected a total of 220 tadpoles equally

212 balanced both for treatment and female. They were euthanized and formalin fixed and photographed

213 through a stereomicroscope equipped with a camera (AxioCam ERc5s) to estimate growth

214 retardation by measuring the total length and maximum tail height (Altig, 2017), using the

215 digitizing software AxioVision. Remaining individuals were reared in 16 plastic tanks (2 tanks per

216 treatment and female) filled with dechlorinated tap water (8 L), under natural light conditions. After

217 hatching, tadpoles were fed with rabbit food, and reunited in four plastic tanks according to

218 treatment (control, 0.7 mg a.e./L, 3.1 mg a.e./L, 7.60 mg a.e./L solutions) on July 2nd.

219

220 *Behavioural trials: rotational preference, antipredator response and basal activity*

221

222 We began the behavioural trials when tadpoles reached Gosner developmental stage 25 on July 3rd.

223 We recorded the activity of 200 tadpoles (n = 50 from each treatment) in the span of three days,

224 from 9:00 am to 4:00 pm. The tests consisted in recording the activity of individuals for 15 min,

225   while measuring three distinct behavioural variables: rotational preference (i.e. lateralization),

226   antipredator response and basal activity.

227   Before starting the trials, each individual was placed in 100×20 mm Petri dishes filled with

228   60 ml of well-aged tap water for a period of acclimation (15 min). To measure rotational preference,

229   we recorded the time spent swimming in both clockwise and counterclockwise direction during the

230   first 10 min of the test. This is a well-established method for assessing lateralization in tadpoles

231   (Vandenberg and Levin, 2013; Lucon-Xiccato et al., 2016). Only clockwise and counterclockwise

232   movements made in the outer portion of the Petri dishes were measured. We recorded lateralization

233   only when tadpoles' distance from the centre of the Petri dishes was at least 3 cm. Tadpoles that did

234   not move for the whole test were excluded from the analysis (12 tadpoles in the first session and no

235   tadpoles in the second one).

236   Tadpoles of many frog species are known to reduce level of activity after being exposed to

237   predatory cues, in particular when chemicals are produced by a familiar predator preying upon

238   conspecific prey (Ferrari and Chivers, 2009). Accordingly, for each trial we collected 1 mL of water

239   from four different plastic tanks (500 ml) containing *Anax imperator* larvae, which were fed on

240   conspecific tadpoles (one per dragonfly larvae) at least one hour before the beginning of the

241   recording sessions. This procedure allowed us to have freshly produced odours, and to use them as a

242   reliable olfactory signal consisting in predatory kairomones released by predators, and tadpoles'

243   alarm cue (Hettyey et al., 2015).

244   The antipredator response was estimated by recording the amount of time (in seconds) the

245   tadpoles were active in a five minutes time span both before and after the injection of the stimulus,

246   which consisted in 1 mL of either water (blank) or alarm cue. In order to detect possible differences

247   within tadpoles from different developmental treatment in antipredator responses, the movement

248   after and before the injection were compared. We considered the activity recorded before the

249   injection of water or alarm cue, measured in a 5 min period, as the basal activity level.

250   Three weeks later, when tadpoles reached Gosner stage 28/29, we repeated the experiment

251   using the same experimental protocol as explained above and recorded the activity of 80 tadpoles (n

252   = 20, from each developmental treatment).

253

254   *Statistical analysis*

255

256   We applied the non-parametric test Kruskall-Wallis to investigate differences in the total length,

257   and Dunn's test of multiple comparisons with p-values adjusted with the Benjamini-Hochberg

258   method to compare control with other treatments. We then compared maximum tail height in

259   tadpoles exposed to RU-PW to those raised in the control solution with a robust bootstrap version

260   of ANCOVA for trimmed means with the total length as covariate (bootstrap = 10,000, 20%

261   trimmed means) using the R package WRS2, function: *ancboot* (Wilcox and Mair, 2016). This test

262   compares trimmed means at different points along the covariate and finds five points where the

263   slopes are roughly the same, then it compares the trimmed means at these points and explore the

264   possible differences. Since this analysis can be performed only when comparing two groups, the

265   control group was compared to the three RU-PW treatments in three different analysis.

266   Rotational preference was analysed through two parameters often used to study

267   lateralization (Cantalupo et al., 1995). Lateralization directionality (L_R index) was calculated with

268   the following formula: (clockwise swimming time – counterclockwise swimming time) / (clockwise

269   swimming time + counterclockwise swimming time) $\times$ 100. Moreover, the intensity of lateralization

270   (L_A index) equals to the absolute value of L_R ($L_A = |L_R|$). We compared both indexes between

271   tadpoles from different developmental treatments using a bootstrap version of one-way ANOVA for

272   trimmed means (R package: WRS, function: *t1waybt*), both for the first and second session

273   (bootstrap = 10,000, 20% trimmed means; Wilcox, 2011). We then compared indexes between the

274   two experimental sessions with a two-way ANOVA for trimmed means (R package: WRS2,

275   function: *t2way*). Post-hoc tests were executed with *lincomb* function (R package: WRS).

276   Regarding the antipredator response, we calculated change in activity using the following

277   formula: (movement after – movement before) / (movement before) the injection of the stimulus. In

278   order to explore the disturbance effect of the injection procedure we used a non-parametric

279   Wilcoxon rank sum test to compare the tadpoles mean level of activity before and after the water

280   injection for each treatment in both experimental sessions. A two-way ANOVA for trimmed means

281   was used to compare change in activity between groups and experimental session (R package:

282   WRS2, function: *t2way*). For each session, we used one-way ANOVA for trimmed means to

283   explore differences among embryonic treatments for the level of activity.

284   Finally, we used a linear model (*lm*) to explore differences in the basal activity level and

285   included embryonic treatment, experimental session and their interaction as main predictors.

286   Planned comparisons with control group in each session were extracted from the model with

287   "emmeans" R-package and no *p*-value correction (Lenth, 2018).

288

289   **RESULTS**

290

291   *Morphology*

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293   We detected significant differences between treatments regarding the measured morphological

294   parameters (Fig. 1). The total length was different between embryonic treatments ($X^2 = 50.0$, $P <$

295   0.001). Tadpoles exposed to low concentrations RU-PW (0.7 and 3.1 mg a.e./L) showed a higher

296   total length than those raised in the control solution ($Z = -3.01$, $P = 0.003$; $Z = -3.27$, $P = 0.002$

297   respectively). On the contrary, tadpoles which developed in the highest concentration (7.6 mg

298   a.e./L) showed a lower total length compared to the control group ($Z = 2.26$, $P = 0.008$). Results

299   from robust bootstrap version of ANCOVA for trimmed means showed that significative

300   differences in the maximum tail height emerged only for the highest glyphosate treatment (7.6 mg

301   a.e./L) in comparison to the control group (Table 1).

302

Lateralization

304

In the first behavioural session, carried out at Gosner stage 25, L_A index was not equal among developmental treatments (F = 3.34, P = 0.024, d = 0.28; Fig. 3). The L_A index was significantly higher in the control group compared to tadpoles raised in the highest treatment concentration tested (7.6 mg a.e./L; Ψ= 22.22, P = 0.003), however no significant difference was detected between control and both 0.7 and 3.1 mg a.e/L (lower p-value: Ψ = 13.25, P = 0.07). In the second session (Gosner stage 28/29), statistically significant differences among treatments were no longer observed (F = 1.66, P = 0.20, d = 0.33). The global analysis for trimmed means showed a significant effect for the embryonic treatment (F = 10.48, P = 0.02) and for the experimental session (F = 62.32, P = 0.001), but not for the interaction of the two (F = 4.76, P = 0.20; Fig. 2). In all four groups L_A index values decreased from the first to the second session (Fig. 2).

Tadpoles did not exhibit directional preference for L_R index, and we did not observe differences between the treatment groups in both sessions (Session 1: F = 0.46, P = 0.69, d = 0.11; Session 2: F = 0.27, P = 0.83, d = 0.15).

318

Antipredator response

320

In both sessions (Gosner stages 25 and 28/29), the level of activity before and after the injection of water stimulus was not significantly different for all treatments (highest difference: W = 210, P = 0.11; Fig. 3a and 3c), thus showing a negligible disturbance effect of the injection procedure. The sole exception was represented by 3.1 mg a.e./L treatment in the second session, which significantly reduced activity level after the water stimulus injection (W = 21, P = 0.02; Fig. 3c). Concerning the antipredator response, we did not detect any significant difference in tadpoles' activity change among embryonic treatments after the injection of the stimulus (either water or predatory cue; P =

328   0.75; Fig. 3b and 3d). Nevertheless, chemical stimulus and experimental session seemed to have a

329   significant effect for the proportional change in the level of activity (value= 1087.24, $P < 0.001$ and

330   value=5.36, $P = 0.02$, respectively). All groups showed a strong significant decrease in the level of

331   activity after the injection of predatory cue in comparison to water injection (Session 1: F = 979.22,

332    $P < 0.001$; Session 2: F = 1721.90, $P < 0.001$). No significant interaction was detected between

333   embryonic treatment and the type of stimulus injected (Session 1: F = 1.45, $P = 0.70$; Session 2: F =

334   2.43, $P = 0.53$).

335

336   *Basal activity*

337

338   The global linear model highlighted a significant interaction between embryonic treatment and

339   experimental session (F = 3.23, $P = 0.02$; Fig. 4). In the first session (Gosner stage 25) we observed

340   that basal activity, measured in a 5 min time span, was significantly higher in tadpoles raised in the

341   control solution than in those exposed to the herbicide (F = 5.62, $P = 0.001$). In the first session, the

342   intermediate RU-PW concentration groups (0.7 and 3.1 mg a.e./L) showed a significant reduction in

343   the basal activity compared to the control (t.ratio = -2.54, $P = 0.01$ and t.ratio = -2.04, $P = 0.04$,

344   respectively). Tadpoles exposed to the highest concentration of the herbicide (7.6 mg a.e./L)

345   exhibited the strongest reduction in the basal level of activity (t.ratio = - 4.23, $P < 0.001$) when

346   matched to the control group. In the second session (Gosner stage 28/29), tadpoles raised with RU-

347   PW were overall more active than the control group but no significant difference was detected

348   (highest difference: t.ratio = -1.79, $P = 0.07$).

349

350   **DISCUSSION**

351

352   Our study clearly supports the hypothesis that GBHs in freshwater habitat could affect the life cycle

353   of marsh frog's tadpoles in terms of development, lateralization and activity level, all factors closely

354   related to the ability of the larvae to cope with environmental pressures. As a general consideration,

355   the absence of mortality rate in the different groups tested (both control and exposed) indicates a

356   good quality of the experimental conditions and confirms the sub-lethal concentrations of GBHs.

357   Similarly, no mortality has been observed in experiments using tadpoles exposed to ecologically

358   relevant concentrations of several herbicides (Dornelles and Oliveira, 2014; Wilkens et al., 2019).

359

360   *Effects of RU-PW on morphological traits*

361

362   Analysis of the morphological parameters revealed that growth of marsh frog tadpoles was affected

363   by ecologically relevant glyphosate concentrations after the 96h laboratory exposure. Tadpoles

364   exposed to low and intermediate concentrations seemed longer than control, pointing to a growth

365   hastening in a low pollution levels scenario, while those exposed to the highest concentration

366   appeared significantly shorter. This result could be interpreted as a growth retardation in highly

367   polluted environments. This hormetic trend in tadpoles' development has already been observed for

368   different pesticides and heavy metals (James and Little, 2003; Smith et al., 2004; Nations et al.,

369   2011, 2015).

370   In the first scenario, we can assume that the environmental stressor represented by the

371   pollutant bursts tadpole's development in terms of adaptive stress response, increasing length,

372   which would eventually favour movement away from the unsuitable – polluted – medium. On the

373   other hand, an indirect effect of low GBHs water contamination could consist in a faster

374   development and an early metamorphosis at smaller size, with obvious negative outcomes in terms

375   of higher predation risk and possible lower reproductive success in later-life stages (Altwegg and

376   Reyer, 2003; Cauble and Wagner, 2005).

377   RU-PW caused a general growth retardation for tadpoles raised in 7.6 mg a.e./L, for both

378   total length and maximum tail height, thus producing shorter individuals with thinner tails (Fig. 5).

379   This could be due to the energetic cost of detoxification, reducing the amount of available energy

380   for growing and metamorphosis. Wilkens and colleagues (2019) recently demonstrated an effect of

381   two xenobiotics (sulfentrazone and glyphosate) and their blend on metabolic rates, oxidative stress

382   and plasma corticosterone circulating level in tadpoles of the bullfrog *Lithobates catesbeiana*. The

383   authors conclude that tolerance to herbicide is associated with an increase in the energy demand to

384   keep the homeostasis and ensure the animal's survival. A significant reduction in the embryo total

385   length, a sensitive parameter of the teratogenesis assay in *Xenopus* (FETAX), was also observed in

386   *Xenopus* embryos starting from the RU-PW concentration of 5mg a.e./L (Bonfanti et al., 2018).

387   Growth retardation may impair tadpoles' ability to swim efficiently at hatching and

388   therefore negatively influence their chances to cope with potential predation threats. Although in

389   our study growth retardation was only detected at 7.6 mg a.e./L, which is the worst-case scenario

390   concentration predicted in shallow water bodies, similar effects were found in mesocosm

391   experiments at lower concentrations (Relyea, 2005). Nonetheless, in previous studies there seems to

392   be differences in the effects on development and survivability of tadpoles exposed to GBHs, with

393   lower impacts generally observed in mesocosms experiments even when testing POEA containing

394   herbicides, the most toxic surfactant for amphibians (Mikó et al., 2015).

395

396   *Lateralization impairment at early developmental stages*

397

398   Among the behavioural traits investigated in this study, the intensity of lateralization seemed to be

399   affected by exposure to glyphosate, as significant higher values were observed for the control group

400   when compared to the experimental group raised in 7.6 mg a.e./L RU-PW. At Gosner stage 25, we

401   discovered that lateralization index (L_A) was lower in exposed tadpoles compared to those raised in

402   the control solution. Laterality is linked to anti-predatory behaviour and, therefore, an alteration

403   caused by environmental factors could lead to a decreased efficacy of defensive responses and

404   higher larvae mortality rates.

405   To date, no study has investigated how glyphosate exposure during development might

406   affect lateralization in early vs later life stages of amphibians' tadpoles. As Roundup® seems to

407   activate the physiological pathway of developmental plasticity linked to anti-predator responses in

408   tadpoles of the wood frog (*Lithobates sylvatica*), the leopard frog (*L. pipiens*) and the agile frog

409   (*Rana dalmatina*) (Relyea, 2012; Mikó et al., 2017), and given that a higher predation risk

410   environment during early ontogeny has been proven to lead to a higher L_A index in tadpoles in later

411   life stages (Lucon-Xiccato et al., 2017), a higher L_A index in tadpoles treated with RU-PW during

412   early ontogeny could have been a plausible developmental plastic response. Nonetheless, this was

413   not the case in our experiment. RU-PW did not seem to induce behavioural and morphological

414   adaptations linked to antipredator responses. Indeed, tadpoles raised in 7.6 mg a.e./L had a smaller

415   tail membrane than those raised in the control solution, rather than a deeper tail membrane which is

416   a typical morphological anti-predator change (Van Buskirk, 2001).

417   In the second session, when tadpoles reached Gosner stage 28/29, no significant differences

418   between groups were detected although laterality was lower in all four groups with respect to the

419   first session (Gosner 25; Fig. 2). So, it seems that the effects of RU-PW were only detectable

420   shortly after hatching, and our results support the hypothesis that behavioural changes may be

421   influenced by the developmental stages in which it falls (Mikò et al., 2017). To our knowledge, no

422   study has yet explored how lateralization varies during tadpoles' development and how it can be

423   affected by environmental factors during ontogeny. If we consider the modification of the L_A index

424   in the control group through development, as the natural occurring variation of the intensity of

425   lateralization, we could hypothesize that lateralization index is higher just after hatching and then

426   decreases at later life stages. A higher L_A index at earlier life stages could help tadpoles to cope

427   with the higher early predation threats (Dadda et al., 2010), however it seems that experience is

428   required in order to develop a certain level of behavioural lateralization (Lucon-Xiccato et al.,

429   2017).

430

431   *RU-PW and antipredator response*

432

433   The capacity of larval anurans to detect water borne cues produced by predators and properly alter

434   their behaviour (e.g. hiding or reducing activity) is crucial for survival: tadpoles of many frog

435   species are known to reduce level of activity after being exposed to predatory cues, in particular

436   when chemicals are produced by a familiar predator preying upon conspecific prey (Moore et al.,

437   2015). In our study, we did not detect any effect of the exposure during development regarding

438   antipredator response both in the first and second trial sessions. This means that tadpoles raised in

439   RU-PW solution had the ability to detect predatory and conspecific cues in a way that apparently

440   resembles the ability of individuals raised in the control solution. Nonetheless, we cannot rule out

441   that given the high concentration of predator and conspecific cue, any possible alternative responses

442   of the distinct treatments could have been concealed by the triggering of a general strong decrease

443   in the tadpole activity level. Another possibility is that the larvae of *P. ridibundus* are able to cope

444   with the negative effect of the herbicide toxicity in ecological relevant concentrations, and

445   particularly do not suffer shortcomings of the exposure at the sensory and nervous level. However,

446   the observed differences in lateralization and basal activity (see section *Lateralization impairment*

447   *at early developmental stages*) seem to support the first scenario (i.e. excess of stimulus).

448

449   *Lower basal activity in exposed tadpoles*

450

451   The dramatic decrease in basal activity observed in tadpoles exposed to RU-PW, compared to those

452   raised in the control solution in the first experimental session, may have consequences in the

453   survivability and ability to forage of tadpoles. Indeed, foraging is crucial for survival and tadpoles

454   need to efficiently balance the risk of starving and the risk of predation by adjusting their activity

455   levels (Werner and Anholt, 1996). In case of general lower basal activity, tadpoles may be impaired

456   in the foraging activity, which translates in less energy acquisition and growth and prolonged

457   exposure to predators and water pollutants. This may result in a positive feedback cycle: higher

458   Roundup® levels during development decreases basal activity, which may decrease foraging

459   activity and result in lower growth rates that ultimately lead to prolonged time of exposure to water

460   pollutants.

461   Bridges (1999) noted that tadpoles of *Hyla versicolor* exposed to carbaryl had significantly

462   lower activity levels, even when no predator were present, than tadpoles not exposed to the

463   herbicide. The consequence is a longer time spent in environments with less food availability since

464   spending too much time resting may lower predation risk, but at the same time decreases energy

465   acquisition.

466   In shallow ephemeral ponds, glyphosate concentrations are generally higher due to being located

467   near Roundup® application and due to lack of use restrictions since they are not considered water

468   bodies (Battaglin et al., 2009). In these environments, the reduced growth rate caused by the

469   herbicide may increase the risk of not achieving metamorphosis before the water dries out (Bridges,

470   1999). Furthermore, as water stratification does not occur in shallow water bodies, a phenomenon

471   which causes glyphosate and surfactants to concentrate near the surface, the risk of exposure

472   increases even more for the offspring of amphibians that breed in ephemeral ponds (Jones et al.,

473   2010).

474   At Gosner stage 28/29, we observed a reduction in the basal activity of tadpoles from the

475   control group, but no significant variation was observed among all the treatments. Despite this

476   significant drop in activity for the control group, tadpoles raised in 7.6 mg a.e./L still showed

477   similar activity levels to the control group in the second session. One possible explanation for the

478   observed shift in the basal activity of tadpoles exposed to RU-PW is that the exposed tadpoles may

479   have increased their activity to compensate the initial negative effect of the herbicide.

480   Alternatively, the control group may have decreased its basal activity to optimize their growth and

481   to storage the required amount of energy for further metamorphosis.

482

483   **CONCLUSIONS**

484

485   Amphibians are experiencing a decline on a global scale, mainly due to human activity (Carey and

486   Bryant, 1995; Stuart et al., 2008). One of the main factors considered to negatively impact

487   amphibians' conservation is the environmental pollution through pesticides employment, which are

488   often directly applied to the soil and may contaminate the aquatic environment through leaching,

489   wind or transported by runoff waters (Collins and Storfer, 2003; Saunders and Pezeshki, 2015).

490   Our study demonstrates that tadpoles of *P. ridibundus* are sensitive to a glyphosate-based

491   herbicide in terms of both morphological and behavioural modifications. Tadpoles exposed

492   developed faster at low concentrations (0.7 and 3.1 mg a.e./L), while were affected by overall

493   growth retardation at the highest concentration tested (7.6 mg a.e./L). Lateralization and basal

494   activity were affected by embryonic exposure to glyphosate, however we could not detect any effect

495   on antipredator behavioural responses. Some of the observed modifications may be attributed to

496   altered or reduced brain development, or to the inflammation and consequent infiltration of

497   eosinophilic granule cells/mast cells in neuronal bodies, as demonstrated by previous laboratory

498   tests involving both non-model or model organisms (Ramírez-Duarte et al., 2008; Bonfanti et al.,

499   2018). Although our study reports the results of acute exposure to glyphosate, it can be assumed

500   that, after prolonged exposure, the observed behavioural alterations would only worsen, making

501   tadpoles less responsive to stressful synergetic situations such as habitat fragmentation, UV

502   radiation, pollutants, pathogenic agents, invasive species, and predators. All these factors, among

503   others, would contribute to the decrease of populations of this and other similar species.

504   To the best of our knowledge, this is the first study describing variation in lateralization in

505   tadpoles of the genus *Pelophylax*, which comprise nearly 20 taxa spread throughout Europe and

506   Asia. Noteworthy, *P. ridibundus* has been widely translocated among European countries, and it is

507   known to hybridize with local taxa with unknown ecological consequences. In Northern Italy,

508   where this species has undergone multiple release, hybridization with *P. lessonae* (the pool frog)

509 and particularly *P. esculentus* (the edible frog, which carries the genome of the latter two species as

510 a result of hybridogenetic mechanisms; Berger, 1973), may eventually impact the species-specific

511 response of native species to environmental pressures like predation in polluted environments. *P.*

512 *ridibundus* is overall less sensitive to disturbance than the native taxa, particularly *P. lessonae*,

513 therefore it can be assumed that observed effects of herbicide would be more evident in less tolerant

514 tadpoles.

515 Nonetheless, the latter statement needs to be supported by further studies, in order to clarify if

516 marsh frogs may be further advantaged on native taxa via pollutant resistance.

517 Finally, since aquatic environments are essential to both the life cycle of amphibians and

518 their reproductive success, in this study we supported the hypothesis that water contamination may

519 greatly impair the survivability of amphibian populations.

520

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522

526

527 **References**

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Table 1: Results from Robust ANCOVA analysis (20% trimmed means, bootstrap = 10.000). The *design points* are total length (covariate) values (μm) for which the relationship between total length and maximum tail height are comparable in the compared groups. *N1* and *N2* are the number of individuals that have a covariate value close to the *design points*. The *difference* column shows the difference in the trimmed means for the maximum tail height between the two groups and the test statistic values are stored in the *F* column (*difference/*S.E.) with the corresponding *p*-values in the *P* column. 95% confidence intervals are in the *LCI95%* (lower) and *UCI95%* (upper).

|	design points	N1	N2	difference	LCI 95%	UCI 95%	F	P
	5612.60	32	14	-53.97	-127.37	19.43	-2.17	< 0.05
	5736.65	36	22	-58.16	-128.33	12.00	-2.44	< 0.05
C – T1	5958.68	40	33	-59.99	-136.11	16.12	-2.32	< 0.05
	6221.33	28	42	-25.74	-107.76	56.28	-0.93	0.37
	6734.02	13	16	-3.42	-104.86	98.02	-0.09	0.92
	5690.44	33	12	-86.17	-149.54	-22.80	-3.86	<0.001
	5854.84	42	23	-54.98	-115.72	5.75	-2.57	< 0.05
C – T2	6018.72	40	35	-43.00	-114.78	28.77	-1.70	0.10
	6265.36	27	35	12.17	-69.08	93.43	0.43	0.67
	6691.35	13	13	11.71	-92.27	115.68	0.32	0.75
	5512.82	27	20	272.15	200.15	345.05	10.58	**< 0.001**
	5631.46	33	32	283.85	223.67	344.03	13.27	**< 0.001**
C – T3	5735.77	36	36	287.59	225.15	350.03	12.95	**< 0.001**
	5894.17	43	25	301.28	234.04	368.51	12.61	**< 0.001**
	6018.72	40	17	303.48	233.18	373.78	12.14	**< 0.001**

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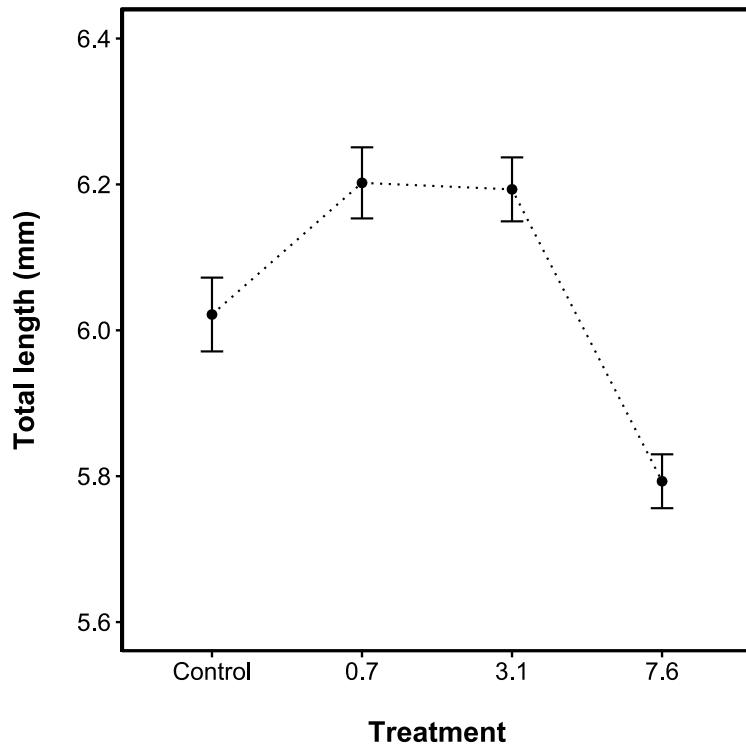
Figure 1: Mean ± S.E. of total length in the four treatment groups. Dashed line highlights the

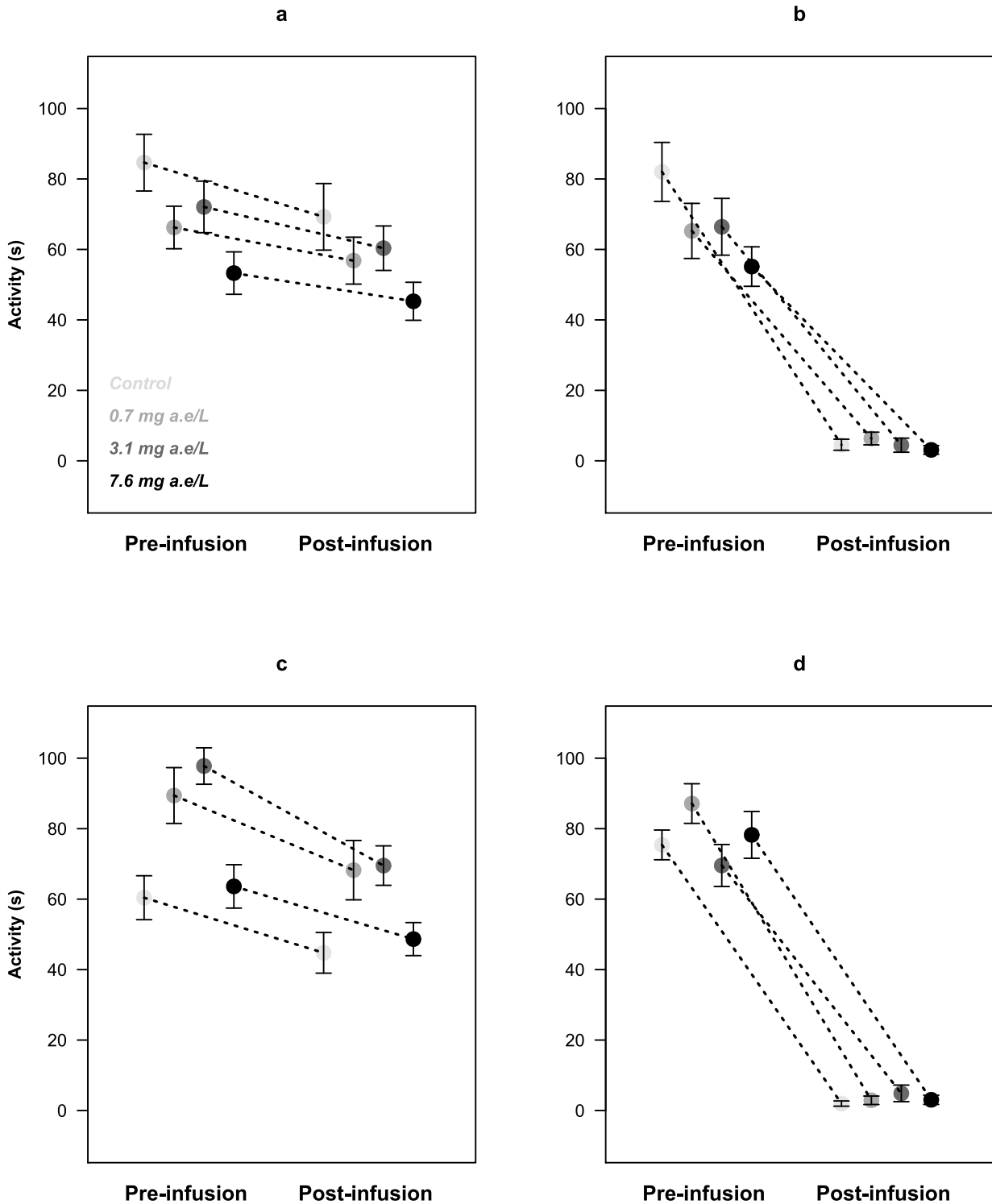hormetic effect of RU-PW on the growth rate. Concentrations are reported in mg a.e./L.

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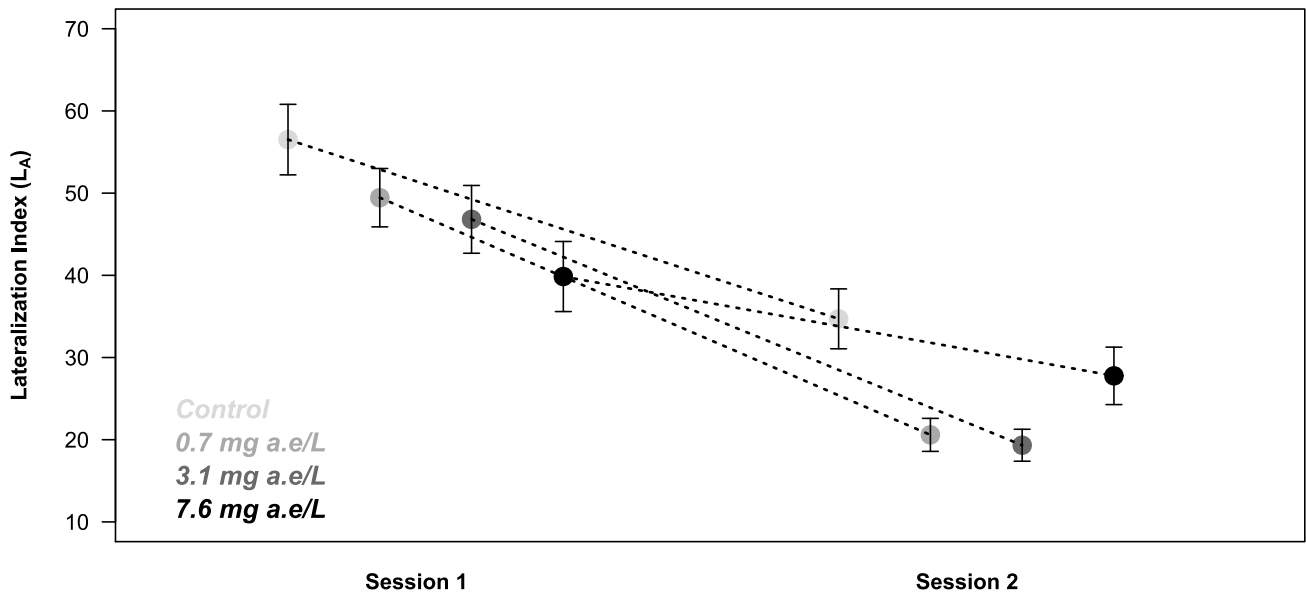
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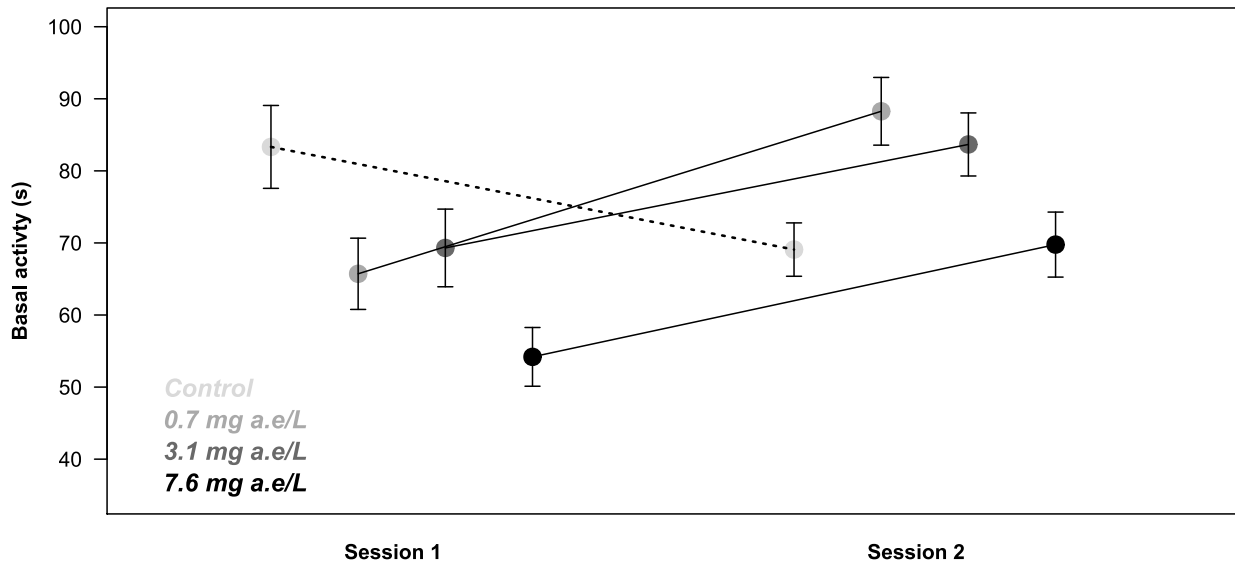780 **Figure 2:** L_A index for all groups in the first and second trial session. Values are mean $\pm$ S.E.

Figure 3: Activity before and after injection of water (**a, c**), predatory and conspecific cue (**b, d**) in the first (**a, b**) and the second session (**c, d**).
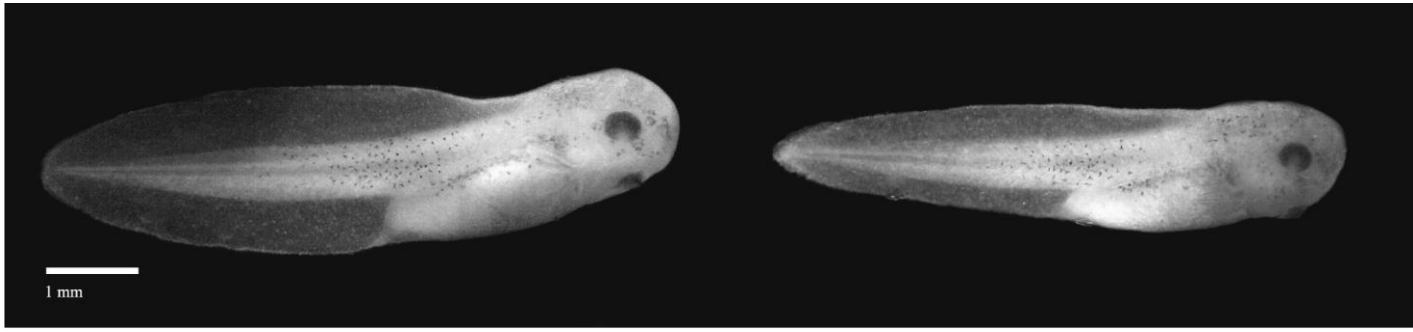
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787 **Figure 4:** Basal activity levels (in seconds) for both sessions. Values are mean ± S.E.

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Figure 5: Lateral view of tadpoles after the 96h exposure from the control group (left) and RU-PW

7.6 mg a.e./L (right) showing the difference in maximum tail height.