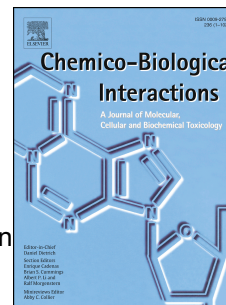


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Daphnia magna responses to fish kairomone and chlorpromazine exposures

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Authors statement

GDA – Conceptualization; Methodology; Formal analysis; Investigation; Visualization;
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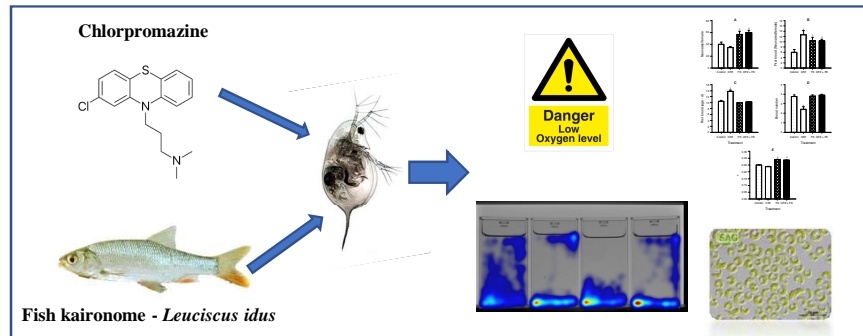
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acquisition



1 ***Daphnia magna* responses to fish kairomone and chlorpromazine**
2 **exposures**

3
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27 **Abstract**

28 To avoid being preyed, organisms must be able to identify predatory threats by sensing
29 molecules released by predators (kairomones), and to employ effective strategies to
30 avoid detection by predators. Furthermore, in the wild, organisms are also exposed to
31 chemicals that may alter their behavioral traits, such as neuroactive pharmaceuticals.
32 Considering the co-occurrence of both types of chemicals, their possible interaction
33 needs to be studied. To address this topic, the aim of this study was to verify the effects
34 of fish kairomone (FK – a chemical associated to putative predation by fish) and
35 chlorpromazine (CPZ - neuroactive pharmaceutical drug, environmental contaminant),
36 isolated and in combination, in different functional endpoints of *Daphnia magna*, such
37 as oxygen consumption, feeding rate, behavior and reproduction. Among these
38 endpoints, oxygen consumption was only affected by the combination of compounds
39 (FK + CPZ). On the other hand, feeding rate was affected by all treatments, being
40 smaller than control. For life history traits and phototactic behavior, the effects of FK
41 predominated over the ones caused by CPZ exposure, incrementing the reproductive
42 output of females, leading to greater population growth rates and decreasing swimming
43 behaviour.

44 **Keywords:** Predation; pharmaceutical effects; oxygen consumption; feeding rate;
45 reproduction; behavior swimming.

46

47 **Introduction**

48 Among aquatic animals, chemical signals can mediate many intra- and inter-specific
49 interactions (DeMile et al., 2016) and even visual species respond strongly to such cues
50 (Hay, 2009). In aquatic systems, chemical cues are recognized as being highly

51 important for the detection of predators, thereby determining prey survival (Mirza and
52 Chivers, 2000). Alarm cues and predatory cues (i.e. kairomones) are the first two
53 primary stimuli extracted from the sensory landscape as information about the potential
54 predation (Schoeppner and Reylea, 2009). In addition, many predator-prey
55 relationships are mediated by the release and detection of kairomones. The importance
56 of these agents as chemical mediators is extreme, since they are responsible for the
57 induction of different responses, such as modifications in behavior, feeding, and
58 reproduction (Lima and Dill, 1990).

59 A biological interaction during which one organism kills and feeds on another organism
60 is one of predation. This relationship not only shapes natural ecosystems but is also
61 likely to occur in anthropogenically impacted environments (Birkhofer et al., 2017). To
62 avoid becoming someone's meal, an organism must be able to identify predatory threats
63 and employ effective strategies to avoid being detected by predators. In the event that
64 such avoidance fails, animals can trigger behaviors to prevent predators from attacking,
65 and also deploy tactics that will increase their chances of surviving the attack, in the
66 case if it does occur (Rosier and Langkilde, 2011). This can be achieved by avoiding the
67 same habitat that is occupied by predators, or by being active at different times of the
68 day or altering the behavioral activity in response to changes in predatory risk (Rosier
69 and Langkilde, 2011), among others. Besides these behavioral alterations, predator-
70 induced responses are important aspects of the population and even community ecology
71 of freshwater zooplankton (Dodson, 1989). This happens since a good perception of
72 danger and a correct anti-predator strategy should increase the chances of survival at the
73 population level, regardless the species-specific reaction (Šmejkal et al., 2018).

74 Furthermore, in the wild individuals interact with other individuals of the same (or of
75 other species) and are exposed to chemicals. Among the array of environmental

76 pollutants, one may find pharmaceutical compounds, which can alter population
77 dynamics, animal behavior, reproduction, health and other features (Daniel et al., 2018;
78 Falcão et al., 2019). In terms of behavioral alterations, especially those drugs with
79 neuroactive properties are particularly troublesome. Pharmaceutical drugs are a global
80 concern in aquatic environments, since the number of these chemicals that are found in
81 the wild is ever increasing (aus der Beek et al., 2016). One of these pharmaceuticals is
82 chlorpromazine (CPZ), a low-cost aliphatic phenothiazine, widely available and used in
83 treatments of psychiatric disorders worldwide, including schizophrenia (Adams et al.,
84 2014). Its primary antipsychotic activity is due to the blockade of dopamine (D2)
85 receptors in the mesolimbic pathway of the brain (Bryan, 2011). This pharmaceutical
86 has been shown to cause toxicological effects in non-target organisms, such as
87 macroinvertebrates (Oliveira et al., 2015a), macrophytes (Alkimin et al., 2019), and fish
88 (Li et al., 2008), and it has been found in different aquatic compartments in
89 concentrations ranging from 1 to 364 ng/L (Roberts and Bersuder, 2006; Yuan et al.,
90 2013).

91 The toxic effects of environmental chemicals needs a better and more realistic
92 comprehension about their consequences and their impact; to attain this purpose, the
93 organisms must be analyzed in more complex interaction scenarios, mimicking the
94 actual and more realistic conditions of the ecosystem in which organisms live in
95 (Beklioglu et al., 2010). Ecotoxicological studies focusing on the crosstalk between
96 predation avoidance (and the effects of chemicals that act as chemical signals during
97 this intercourse) and pharmaceuticals are adequate to study the joint contribution of
98 natural and anthropogenic stressors. Despite the increasing number of studies dealing
99 with the effects on living organisms caused by a large set of both natural and
100 anthropogenic stressors, the characterization of these two combined factors is still

101 scarce, despite being recognized as very important to understand how environmental
102 contamination can affect the natural responses of aquatic animals to predation. *Daphnia*
103 species are sensitive to a large number of substances and can be easily cultured under
104 laboratory conditions, being therefore very useful in ecotoxicology as a model
105 organisms (Bownik, 2017). In addition, *Daphnia* species seem particularly sensitive to
106 chemicals that are released by their predators, and somehow involved in their predation.
107 The adaptive responses induced by predator kairomones are a key element in the
108 modulation of individual fitness, which is the result of the contribution of both survival
109 and reproduction success. Therefore, the success of a population is the consequence of
110 individual survival and fitness, consequently, for the maintenance of *Daphnia*
111 populations in aquatic ecosystems, the protection conferred by kairomone-mediated
112 antipredator defenses is vital (Barry, 2002).

113 *Daphnia* species are ecologically important due to their role in many aquatic food webs.
114 *Daphnia* act as primary consumer, and thereby control populations of primary
115 producers; in addition, they also provides a food source for many secondary consumers
116 (DeMille et al., 2016). Consequently, the responses of *Daphnia* towards aquatic
117 contamination and/or kairomones may affect the entire ecosystem. To address this topic,
118 the aim of this paper was to verify the effects of fish kairomone (indicative of predation)
119 and chlorpromazine (environmental contaminant), alone and in combination, in
120 different functional endpoints in *Daphnia magna*.

121

122 **Material and methods**

123 **Chemicals**

124 Chlorpromazine hydrochloride (CAS: 69-09-0) was purchased from Sigma-Aldrich
125 (USA) with 98% of purity. All other used reagents had analytical purity.

126

127 ***Daphnia* culture and maintenance**

128 Clone P₁32,85 (used in all definitive experiments) was obtained from two generations of
129 intracloonal mixes within clone P₁, isolated from a small pond which contained fish
130 (located at Driehoeksvijver, Heusden, The Netherlands; animals were isolated in August
131 1986). According to De Meester (1993) organisms from this clone, in the presence of
132 fish kairomones, became negatively phototactic. Cultures of 10 animals/L were
133 maintained in ASTM hard water (ASTM, 1994) and fed every other day with
134 5×10^5 cells/mL of the freshwater algae of the species *Raphidocelis subcapitata*. Three
135 times a week the culture medium was renewed and a set up to of 14h:10h light:dark
136 cycle and 20 ± 2 °C was used as photoperiod and temperature, respectively.

137 **Chemical stability experiment**

138 The stability of the studied chemicals in ASTM water was assessed measuring the
139 concentration of 1000 µg/L of the tested chemicals in water in freshly prepared and old
140 (24, 48 h) test solutions by liquid chromatography connected to a triple quadrupole
141 detector (Xevo TQS, Acquity Waters, Mildford, USA) (LC-MS/MS). The analysis was
142 performed in selected reaction monitoring (SRM) mode using two transitions from de
143 precursor ion to the product ion to identify each compound. The system and data
144 management were processed using MassLynx v4.1 software package.

145 **Obtaining Fish kairomone**

146 To obtain fish kairomone (FK), two 8 cm juvenile fish (*Leuciscus idus*) were allowed to
147 swim in a 12 L aquarium, filled with ASTM hard water medium for 24 h, after which the
148 medium was filtered (0.045 μm). This procedure was repeated for every experiment, to
149 use a fresh FK solution for every experiment. Only for the reproduction tests, a single
150 FK preparation was used in three media changes. This method is routinely used to
151 simulate fish predation risk (De Meester and Cousyn, 1997; Jansen et al. 2013). The
152 kairomone dilution was roughly to 1 fish in 50 L.

153

154 **Experimental conditions**

155 The experimental conditions for *D. magna* exposures were defined as control, FK (fish-
156 kairomone conditioned water); CPZ (10 $\mu\text{g/L}$ of CPZ); and the combination of both
157 treatments (FK + CPZ). These conditions are designated along this study as Control,
158 FK, CPZ, and FK + CPZ respectively. The dilution of 1:8 of FK was chosen in order to
159 avoid interference of ammonia on the toxic effects elicited on *D. magna*, as
160 demonstrated by previous experiments performed by our research group; and the
161 concentration of 10 $\mu\text{g/L}$ of CPZ was chosen after the analysis of the results obtained
162 for the behavior preliminary tests (data not shown).

163

164 **Respiration assay – oxygen consumption**

165 Respiration rate was assessed by measuring the oxygen consumption through the
166 difference between the initial and final oxygen concentrations and converted to
167 proportional oxygen (%) relative to control. To attain this purpose, five replicates with
168 four 15 d old synchronized daphnids (Clone P_{132,85}) per experimental treatment were
169 used. The animals were maintained during 24 h in the dark at 20 ± 2 °C in 50 mL

170 gastight syringes (Hamilton, USA – final volume of 30 mL of test-solution). Oxygen
171 interface (model 928, Strathkelvin Instruments, Glasgow, UK) was used to measure
172 initial and final oxygen concentration. Media with no daphnids (blank controls) for each
173 treatment were used to correct natural oxygen depletion and to avoid the interference by
174 algae oxygen consumption no food was added.

175

176 **Feeding rate (F)**

177 The test-chambers (five replicates per treatment) consisted of 100 mL glass flasks filled
178 with test-solution, each replicate was composed by five 15 d old daphnids (Clone
179 P_{132,85}). Briefly, the animals were submitted during 24 h, in the dark, to all
180 experimental conditions, with 5×10^5 cells/mL of *R. subcapitata* in the test-chambers.
181 Afterwards, the animals were transferred to a new test-chamber (clean media), also in
182 the dark, for 4 h with the same algal concentration (post-exposure period) to evaluate
183 the recovery from exposure to toxicants. Blank controls (media with no daphnids) were
184 included in both cases. At the end of each period, the algal concentrations were
185 spectrophotometrically measured (Du[®] 730) at 440 nm (Allen et al., 1995). Finally, the
186 feeding rate was calculated converting the obtained values to proportional algae
187 consumption (%) relative to control.

188

189 **Swimming behaviour assay**

190 The swimming tracks of the 15 d old females from clone P_{132,85} pre-exposed for 24 h
191 to the four studied treatments were assessed using the arena video tracking system
192 described by Simão et al. (2019). The pre-exposure period was chosen taking into
193 account that after 3 h of FK exposure, it is possible to detect changes in behaviour of

194 daphnids, according to De Meester and Cousyn (1997). Groups of five adults were pre-
195 exposed to the tested treatments in 100 mL media containing food (*R. subcapitata*,
196 5×10^5 cells/mL) for 24 h prior to behavioral tests, each with five replications. Five
197 behavioral trials were performed per treatment. In each trial, groups of five organisms
198 from two different randomized treatments were distributed among the two arenas filled
199 with 50 mL of test solution. Animals were then acclimated in the dark (only infrared
200 light on) for 5 min before video recording. For the behavioral analysis, animals were
201 initially subjected to a dark period (5 min), and then to a moderate light intensity period
202 (10 lux, 15 min.). The software EthoVision XT12 video tracking (video-recording at 20
203 frames per s) was used as a tool to analyze the position changes of each animal. The
204 first step of this procedure was of dividing the arenas in three virtual zones: bottom,
205 middle and top, to simulate a water column. Then, the social interaction module was
206 used to analyze, individually, tracks of 5 organisms and to determine time spent in the
207 top of water column (virtual zone determined in the arena; %), total distance moved
208 (cm) and the distance inter-organisms (aggregation) measured by the average distance
209 (cm) among individual animals in the arena. For statistical analysis three periods were
210 considered: the last two minutes of the dark period, the first two and the last ten minutes
211 of light period, hereafter referred as dark, first and late light, respectively. Values were
212 determined per min. In addition, the used software measures the complete tracking in
213 the two dimensions. So, it also includes the different types of organisms' behavior, with
214 the exception of the rotational behavior of animals (turning around, without
215 displacement in the x-y axis). Besides, the use of 15 d old organisms was necessary to
216 ensure that test organisms had a minimum size to be reliably detected by the hardware +
217 software. This age was also chosen, since it corresponds to the complete development of
218 phototaxis. Finally, animals with the same age were used in the feeding and oxygen

219 consumption tests, to allow the possibility of comparing data from all these endpoints,
220 and, in our laboratory conditions, 15 d old organisms have an average size of 2.76 ± 0.2
221 mm ($N = 56$, Mean \pm SD).

222 **Reproduction assay**

223 Reproduction test was conducted according to OECD recommendations (OECD, 2012).
224 Organisms less than 24-h old were exposed, individually, in 100 mL glass flasks, to
225 each experimental condition and a total of 10 replicates per condition was used. The
226 organisms were maintained and fed as previously described in *Daphnia* culture and
227 maintenance section and every other day the medium was renewed. Throughout the 21-
228 d assay period the individuals were monitored daily and the endpoints evaluated were:
229 total number of neonates, brood number, time to first brood, neonates in the first brood
230 and Intrinsic rate of population increase (r), this last one calculated using the Euler's
231 ($1 = \sum_{x=0}^n e^{-rx} l_x m_x$) where r is the population increase rate (per day), x is the age in days
232 ($0 \dots n$), l_x is the survival probability in the age x , and m_x is the fecundity in the age x .
233 Pseudo-values from replicates were generated using the jackknife procedure (Meyer
234 et al., 1986).

235

236 **Data analysis**

237 Treatment effects on life-history and behavioral traits, proportional oxygen
238 consumption and feeding rates were compared against the control by one-way ANOVA
239 followed by post-hoc Dunnett's test. Prior to analysis, data were checked for ANOVA
240 assumptions of normality and variance homoscedasticity. After transformation, not
241 normally distributed data were compared using a Kruskal-Wallis test followed by

242 Wilcoxon and Wilcox test (Zar, 1996). These analyzes were performed using the
243 statistical software SigmaPlot 14.0 and the adopted significance was $p < 0.05$.

244

245 **Results**

246 Obtained results indicated that the tested compound was stable in ASTM water since
247 concentrations in freshly prepared solutions ($N = 4$, Mean \pm SE, $1092.8 \pm 10.4 \mu\text{g/L}$)
248 were similar to those aged for 24 ($1073.5 \pm 5.6 \mu\text{g/L}$) and 48 h ($1063.2 \pm 11.5 \mu\text{g/L}$).

249 The oxygen consumption was smaller ($F_{3;12} = 4.091$, $p < 0.05$) than control in the
250 combination (FK + CPZ) (fig. 1). On the other hand, feeding rates (fig. 2) were
251 significantly smaller ($p < 0.05$) in all treatments, during the exposure ($F_{3;16} = 4.825$) and
252 post-exposure ($F_{3;15} = 5.444$) periods, relative to controls.

253 Figure 3 includes the behavioral responses per min in the left panel graphs (fig. 3A, C,
254 E) and grouped by the three analyzed periods (right panel graphs, Fig 3B, D, F).
255 Females exposed to fish waterborne kairomone of fish (FK) alone or with CPZ were
256 situated closer to the bottom (fig. 3A) when light was present, and those exposed to FK
257 and CPZ moved less and aggregated less when light was on (fig 3C, E). Consequently,
258 there were significant differences in the % of time that animals swam close to the
259 surface, between FK and FK+CPZ treatments and controls ($F_{3;87} = 17.660$, $p < 0.001$, fig.
260 3B). The combination of CPZ+FK was able to decrease significantly the moved
261 distance in all analyzed periods (dark – $F_{3;81} = 8.945$, $p < 0.001$; first light – $H_{3\text{df}} = 18.359$,
262 $p < 0.001$; late light – $F_{3;79} = 12.934$, $p < 0.001$) (fig. 3D). In addition, FK exposure caused
263 a significant decrease ($H_{3\text{df}} = 13.051$, $p = 0.005$) in the inter-organismic distance in the
264 dark, while CPZ+FK caused an increase ($H_{3\text{df}} = 22.015$, $p < 0.001$) in a late light period
265 (fig. 3F).

266 The cumulative number of neonates per female (fig. 4A) was significantly higher (H_{3df}
267 = 16.037, $p < 0.001$) in females exposed to FK treatments (FK, CPZ+FK), whereas the
268 clutch size of the first brood was larger in all exposed females ($F_{3;34} = 4.592$, $p < 0.01$,
269 fig. 4B) in comparison to control. CPZ, alone, was capable of causing a delay ($H_{3df} =$
270 25.497, $p < 0.001$; fig. 4C), approximately of 4 days, in the age of the first brood. This
271 pharmaceutical was also responsible for a decrease ($H_{3df} = 18.145$, $p < 0.001$) in the total
272 number of broods (fig. 4D). Finally, the population growth rate (fig. 4E) was
273 significantly higher ($H_{3df} = 36.018$, $p < 0.001$) in organisms exposed to FK (FK,
274 FK+CPZ).

275

276 Discussion

277 Zooplanktonic organisms, and in particular, *Daphnia* species, have been shown to
278 exhibit responses to predator-specific chemicals (i.e. kairomones) in different
279 parameters, as life history and behavioral traits (Larsson and Dodson, 1993). However,
280 the association of this natural stressor with anthropogenic ones (i.e. pharmaceuticals) is
281 less studied, and this study brings some perspectives about this potential association.
282 The here-adopted strategy is a comprehensive framework that includes a series of
283 multiple endpoints with high physiological importance, whose impairment may result in
284 strong population effects.

285 As a sensitive metabolic biomarker and a high potential for toxicity screening,
286 monitoring oxygen consumption can be an advantageous tool when compared to
287 mortality (Zitova et al., 2009). In *Daphnia* spp. the oxygen consumption, in the absence
288 of food, is an important tool to provide estimates about costs of physiological
289 maintenance, which include swimming/ventilation and basal metabolism, activities that
290 are essential to life (Campos et al., 2012). In this study, the oxygen consumption was

291 smaller when the organisms were exposed to the mixture, that is, less oxygen was
292 consumed. This can be an indication that anthropogenic and natural stressors can
293 interact and cause metabolic alterations in exposed animals. Although not significantly,
294 oxygen consumption in CPZ and FK alone were smaller than control, thus it is possible
295 to hypothesize that the joint effect of both stressors was additive. Concomitant with the
296 reported oxygen effects, females exposed to the combination of FK and CPZ also
297 moved less, thus, requiring lower oxygen consumption. In addition, another explanation
298 for the here observed effects is the relationship between oxygen consumption and
299 distance moved; both analyzed endpoints suffered a decrease after exposure to the
300 combination FK+CPZ. Thus, these two effects possibly indicate that impaired oxygen
301 consumption is associated with shorter distance moved, i.e. reduced swimming activity.

302 In metabolic or respiration rate, food ingestion is an energetically expensive action and
303 can represent up to 40% of this expense of energy consumption (Philippova and
304 Postnov, 1988; Barber et al., 1994). The maintenance costs may increase in the presence
305 of pollutants by increasing the rate of detoxification, a demand side effect. However,
306 more often, these compounds decrease metabolic rates inhibiting food ingestion (supply
307 side effects) when food is present (Barber et al., 1994). Beside that, a functional and
308 fitness link between food impairment and feeding behavior can be a crucial endpoint
309 measurable through the feeding performance (Loureiro et al., 2012) which is related to
310 the reproductive outputs (Pieters et al., 2005; Barata and Baird, 2000). Filter-feeding
311 zooplanktonic organisms demand movement of appendages coordinated by the nervous
312 system to assure food filtration (Villarroel et al., 1999). During this process, *Daphnia*'s
313 feeding apparatus repeats constantly these movements, but not in the same intervals,
314 making it a dynamic system (Peñalva-Arana et al., 2007). Therefore, loss of
315 coordination can be caused by toxicants affecting the nervous system and consequently

316 reducing rates of filtration, as observed in this study. This hypothesis may be
317 corroborated by Rocha et al. (2014) after exposing *D. magna* to neuroactive compounds
318 such as neostigmine and pyridostigmine, which compromised neuronal transmission by
319 cholinesterasic impairment, altering behavioral features. Additionally, fish predation
320 can represent a risk to prey animals, and this stimulus can trigger an adaptation of prey
321 defense; this should represent a balance between avoiding being preyed (benefit), and
322 reductions in fitness (cost) caused by the deployment of the defensive measures
323 (Ramcharan et al., 1992) and consequently a reduction in the feeding rate. Related to
324 post-exposure period (4 h of recovery), daphnids presented similar results, with a
325 reduction in feeding rate in all experimental conditions in comparison with the control.
326 This result shows that in the time frame here adopted, the organisms were not capable to
327 recover from the exposure in any of the tested conditions. This result is in line with data
328 obtained by Villarroel et al. (1999) and Mc William and Baird (2002) after exposing *D.*
329 *magna* to several contaminants. Overall, the here reported results for oxygen
330 consumption, feeding and swimming speed indicated that, upon the presence of fish
331 kairomone in water, females moved to a lesser extent, hence reducing their oxygen
332 consumption and feeding rates. These parameters have been investigated in other
333 researches, e.g., Pestana et al. (2013) also found a decrease in filtration after a 24 h
334 exposure to FK and to alarm cues (crushed *Daphnia*), however, the authors reported an
335 increase in oxygen consumption. These differences can be assumed by the age of
336 exposed animals and the use of different clones. Contrary to the present study Pestana et
337 al. (2013) used 4 d old juveniles, while our study was conducted with 15 d old adults,
338 and behavior differences can be related with the daphnids age and genotype (De
339 Meester, 1991, 1992). These effects, in general, result from reversible behavioral
340 responses, which can include changes in feeding behavior, increased vigilance, decrease

341 activity, alertness and change in the swimming speed (Pijanowska, 1997). All the
342 above-mentioned behaviors can alter respiration and feeding rates.

343 A phototactic response is an orientated reaction to light stimuli (Ringelberg, 1964). In
344 *D. magna* this characteristic is heritable (varying from clone to clone), and can be
345 distinguished between positively, intermediately and negatively phototactic genotypes
346 (De Meester, 1991). Phototactic-positive genotypes spend much time in the upper water
347 level, whereas phototactic-negative genotypes spend most of their time close to the
348 bottom sediments (Ebert, 2005). The behavior of juveniles is less clone-specific than
349 that of adults (De Meester, 1992). The here studied clone is a phototactic-positive
350 genotype, thereby explaining why organisms spent most of the time in the top of water
351 column. Thus, this behavior was affected by FK in late light exposure, since the animals
352 remained less time near the top of the water column. This happened because, in general,
353 the phototactic behavior may be modulated by environmental factors, such as the
354 presence of predators, temperature and food availability, which are likely to fluctuate
355 continuously in the wild (Michels et al., 1999), and the fish presence can increase the
356 phototactic negative response of organism (Eber, 2005). On the other hand, and
357 according to Barrozo et al. (2014) there are some evidences that specific dopaminergic
358 signaling pathways are involved in the decrease of *Daphnia* swimming behavior. This
359 trend confirmed after exposing these organisms to neurotoxins that specifically act on
360 such dopaminergic neurotransmission route. Nevertheless, CPZ, a dopamine antagonist,
361 was not capable of changing the phototactic behavior of daphnids, either alone or in
362 combination with the natural stressor (FK), since animals exposed to the combination
363 (CPZ+FK) presented the same behaviour was when exposed only to FK. This similar
364 trend possibly indicates that no interaction between stressors occurred, concerning this
365 specific endpoint.

366 In general, various mechanisms of action can be affected by neurotoxic substances,
367 which may result in depression or excitation of the nervous system, which in turn may
368 induce opposite effects on the cumulative moved distance (Bownik, 2017).
369 Chlorpromazine is a dopamine antagonist, mostly blocking dopamine receptors, thus,
370 blocking these receptors causes diminished neurotransmitter binding in the forebrain
371 and consequently cause a depression in the nervous system. Considering that daphnids
372 also have dopaminergic neurotransmission (Hartline and Christie, 2010), it is possible to
373 suggest a similar pathway. However, these effects seem not to have happened in this
374 study when the organisms were exposed to the pharmaceutical alone. Interestingly, only
375 the combination of CPZ with waterborne fish kairomone (and not both stressors alone)
376 decreased swimming speed; light also decreased aggregation rates. Both effects may
377 diminish the ability of *D. magna* females to escape from fish predation since most fish
378 prefer to prey on isolated prey that swim slower (Allen, 1920). It is possible that an
379 additional stress, FK in this case, can consequently increase the *D. magna* susceptibility
380 to CPZ toxicity. This type of interaction was also reported by other studies: the presence
381 of predator increased the mortality of tadpoles when exposed to carbaryl (Relyea and
382 Mills, 2001) and predation was responsible for an increase of the toxic effects provoked
383 by esfenvalerate in *Artemia* sp., resulting in a population decrease (Beketov and Liess,
384 2006). However, evaluations regarding the putative effects of xenobiotics (namely
385 chemicals whose environmental presence derives directly from anthropogenic
386 contaminant) considering simultaneously the occurrence of natural compounds (e.g.
387 FKs), are still scarce within the scope of plankton ecotoxicology (Gutierrez et al.,
388 2012). This is aggravated if one considers only studies focusing on the interaction of
389 such FKs with pharmaceutical drugs, since most of the studies conducted so far were
390 carried out with metals and pesticides (Gutierrez et al., 2012; Hanzato, 1999; Scherer et

391 al., 2013; Pestana et al., 2010). On the other hand, the prediction of potential effects of
392 these combinations, and the characterization of such interactions, can be a complicated
393 issue since the nature and origin of FKs was not fully elucidated yet, despite the efforts
394 of several work teams. Ringelberg and Van Gool (1998) suggested that FKs may in fact
395 be a bacteria present in the fish skin. In addition, Boriss et al. (1999) proposed that
396 trimethylamine, a tertiary amine produced by fish, is the compound responsible for
397 triggering the defensive behavior of *Daphnia* against predatory fish. In addition, other
398 chemicals have been identified, being extracted from fish water; these chemicals are 5-
399 hydroxy-4-decanolide (Tjossem, 1990), 6-hydroxy-5-tetradec-2-enolide (Lass et al.,
400 2001) and phenylalanine-proline diketopepirazine (Dawidowicz et al., 1990). However,
401 as previously cited, these are assumptions, and the complete nature, origin, and
402 composition of FKs are still unclear. Furthermore, the precise fundamental pathways by
403 which kairomone perception occurs in daphnids remain to be determined, which is a
404 critical step for the understanding of mechanisms, which are activated and induce
405 *Daphnia* defenses (Weiss et al., 2015a). However, these same authors showed that, in
406 *D. longicephala*, the first antennae are a location of kairomone-detecting
407 chemoreceptors, a finding in agreement with data for other cladocerans (Ekerholm and
408 Hallberg, 2002; Hallberg et al., 1992). We are thus able to hypothesize that this
409 mechanism, being conserved, may also occur in *D. magna* as one of the pathways for
410 the detection of kairomones. Considering the absence of information about the putative
411 mechanisms of action of these kairomones in freshwater crustaceans, it is impossible to
412 predict the type and extent of the interaction of kairomones with environmental
413 toxicants. However, kairomones are able to trigger neuronal responses, which are
414 responsive without any pre-existent learning process. In rodents, this chemosensing
415 system exists in specialized structures, such as the vomeronasal organ and the main

416 olfactory epithelium. These receptors are specialized in sensing kairomones from the
417 external media, sending information about their presence for central integration
418 (Stowers and Kuo. 2016). The underlying mechanisms of chemosensing of kairomones,
419 and the subsequent biological response, are still not elucidated in rodents, and are not
420 likely to have been also described in crustaceans. But it is possible to suggest that, being
421 based on neuronal networks, these processes are likely to be affected and compromised
422 by the exposure to neuroactive chemicals, including pharmaceutical drugs such as CPZ.

423 Many chemicals are able to decrease reproduction and delay first reproduction (brood)
424 (Heckmann et al., 2007; Oliveira et al., 2015b), often by impairing food acquisition
425 (Barata and Baird, 2000). There are, however, trade-offs between producing more
426 offspring and delayed reproduction (Barata and Baird, 1998). In this study, CPZ delayed
427 reproduction and increased the offspring production in the first brood, but not in
428 subsequent ones. As a result, females exposed to CPZ had equivalent levels of
429 population growth rates (r) as control ones. Additionally, predators' exudates can exert
430 a selective pressure in planktonic animals and, in response, many morphological
431 aspects, life-history and behavioural traits can suffer changes (Pijanowska and Stolpe,
432 1996). In aquatic environments size-selective predation can greatly influence both
433 community and population dynamics by altering the onset of reproduction, the size of
434 the first clutch and hence the age structure of population (Weider and Pijanowska,
435 1992). For example, fish prefer to predate on larger prey, thus *Daphnia* individuals
436 should reproduce more efficiently and earlier upon fish predation (Barata et al., 2001;
437 Riessen, 1999). Our results are in agreement with previous studies since females
438 exposed to FK alone, or to FK and CPZ, produced more offspring in the first clutch.
439 Again, CPZ did not affect life-history responses when combined with FK. Following
440 detection, predator-specific signals are transferred via independent neurosignalling

441 pathways (Mitchell et al., 2017). Responses of *D. pulex* e. g. to fish predators are
442 mediated via the suppression of the inhibitory GABAergic pathways, resulting in the
443 release of ecdysteroids (Dennis et al., 2014; Weiss et al., 2012, 2015b). Ecdysteroids
444 promote the reallocation of energy from somatic growth to reproduction, which results
445 in the life-history changes described above (Mitchell et al., 2017). The here obtained
446 results show this trend. This outcome occurred even when the exposed organism had the
447 same number of broods than control animals, confirming the reallocation of energy
448 provoked by ecdysteroids to reproduction. This trend also supports the hypothesis
449 formulated by Stibor (2002): the predator can induce life history shifts, which are not
450 the result of changes in metabolism but rather the result of changes in energy allocation
451 induced early in the development, as previously cited. In addition, the interaction
452 between CPZ and FK resulted in the alteration of some life history parameters; this
453 effect may be associated to the known CPZ mode of action, which occurs by blocking
454 dopamine (D2) receptors (Bryan, 2011). This is of critical importance, since the
455 dopaminergic systems in daphnids are involved in the recognition of predator (by
456 sensing FKs), and in triggering the defensive system. The dopaminergic system of
457 daphnids seems to be interconnected with endocrine pathways, namely in the control of
458 juvenile hormone and ecdysone levels, which are important in the physiological
459 regulation of crustacean growth, thereby causing significant alterations in the life
460 history traits of these organisms (Weiss et al., 2015b). Furthermore, Tams and co-
461 workers (2019) suggested that the life history alterations in crustaceans are not
462 necessarily related to changes in gene expression caused by exposures to predator. This
463 relationship can occur just marginally, and may also occur by additional
464 posttranslational processes, such as miRNA-mediated gene regulation or increased
465 degradation (Schwarzenberger et al., 2009). However, we must assume that all

466 mechanisms of prey-predator interactions have not been fully elucidated. Finally, the
467 non-consistent patterns of response in terms of several endpoints here analyzed may be
468 attributed to the specificity of the mechanisms of action, both of the drug and of the FK
469 itself. In addition, the mechanisms by which CPZ and FK interact remain little
470 elucidated in the literature. Thus, more studies of this nature are required to understand
471 the mode of action of isolated chemicals, and underlying mechanisms of drug+FK
472 interaction, that may serve as the basis for the here-observed modifications.

473

474 **Conclusions**

475 In summary, the tested natural and anthropogenic stressors (FK and CPZ, respectively)
476 acted jointly decreasing oxygen consumption rates, swimming speed and aggregation,
477 but operated independently on feeding. Concerning life history traits and phototactic
478 behavior, the effects of FK predominated over those of CPZ, making females reproduce
479 to a greater extent and hence having greater population growth rates and also decreasing
480 positive phototactic behavior. Finally, according to the analyzed endpoints, it is also
481 possible to assume that these two stressors are capable to interact, since some of the
482 analyzed parameters were only altered in organisms exposed to the combination of both
483 stressors. This assumption indicates the ecological importance of the here obtained data,
484 and of this type of study. Consequently, more studies with this type of combination are
485 necessary to understand how aquatic organisms can act/react when challenged by two
486 different risk sources in the environment. However, it is necessary to take into account
487 that in the environment, organisms are challenged by more risks of predation (other
488 predator organisms are present) and by more drugs. Consequently, it is difficult to
489 predict the response resulting from interactions in the natural environment.

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503

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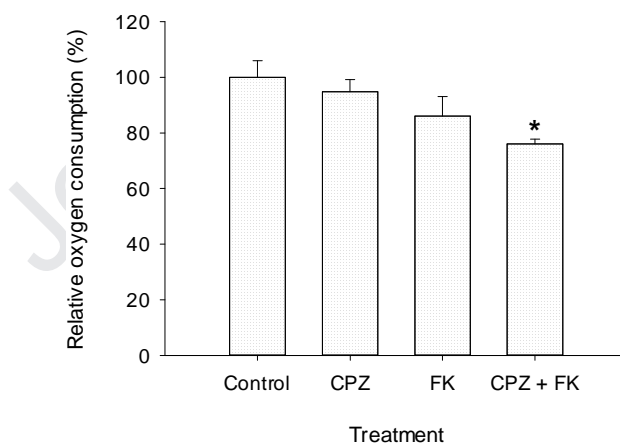
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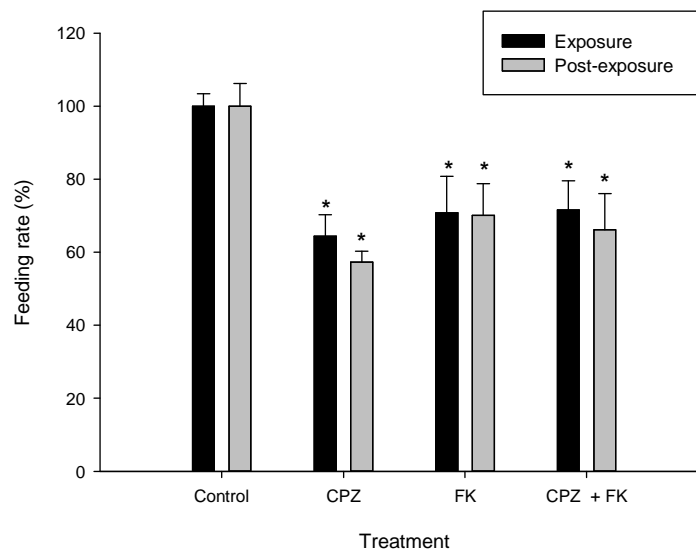
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818 **Figures**



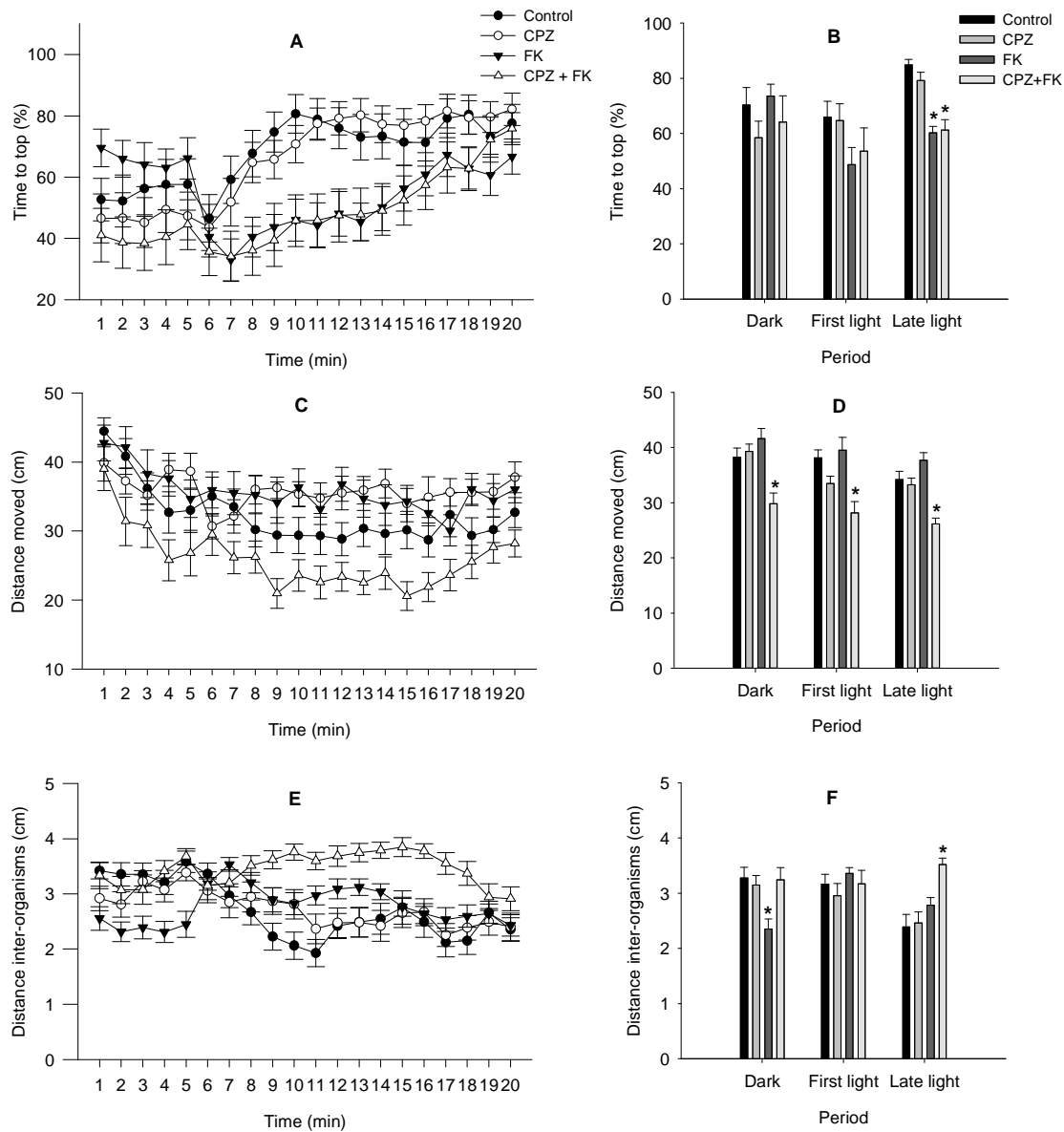
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820 Figure 1. Effects of chlorpromazine (CPZ), of fish kairomone (FK) and of the mixture (CPZ+FK) on *D.*
821 *magna* oxygen consumption. For each parameter, mean and standard error are shown. * stands for
822 statistical differences in relation to respective control following ANOVA and post-hoc test (or the
823 equivalent non-parametric test).



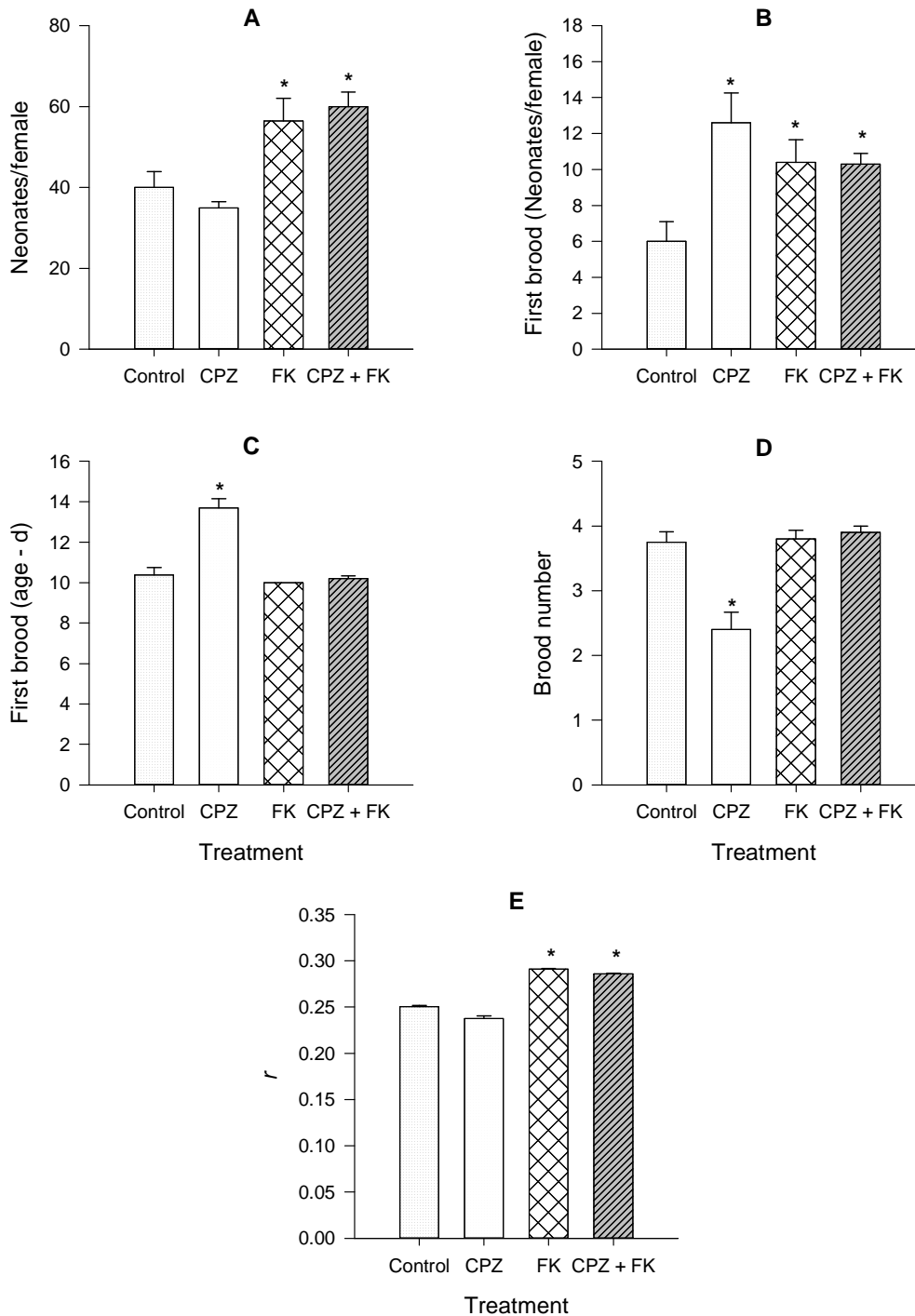
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825 Figure 2. Feeding rate (%) of *D. magna* exposed to chlorpromazine (CPZ), to fish kairomone (FK) and to
826 the mixture (CPZ+FK). For each parameter, mean and standard error are shown. * stands for statistical
827 differences in relation to respective control following ANOVA and post-hoc test (or the equivalent non-
828 parametric test).



829

830 Figure 3. Effects of chlorpromazine (CPZ), of fish kairomone (FK) and of the mixture (CPZ+FK) on *D.*
 831 *magna* behavior, plus control. (A) and (B) correspond to time to top (%), (C) and (D) to distance moved
 832 (cm) and (E) and (F) to inter-organisms distance. (A), (C) and (E) correspond to the results on the integra
 833 (all the evaluation time) and (B), (D) and (F) to the results divided in categories (dark, first light, and late
 834 light). For each parameter, mean and standard error are shown. * stands for statistical differences in
 835 relation to control following ANOVA and post-hoc test (or the equivalent non-parametric test).



836

837 Figure 4. Effects of chlorpromazine (CPZ), fish kairomone (FK) and the mixture (CPZ+FK) in *D. magna*
 838 reproductive parameters. (A) Total number of neonates per female; (B) Total number of neonates in the
 839 first brood; (C) Age of the first brood (d); (D) Total number of broods and (E) Population increase rate
 840 per day (r). For each parameter, mean and standard error are shown. * stands for statistical differences in
 841 relation to control following ANOVA and post-hoc test (or the equivalent non-parametric test).

Highlights

- Chlorpromazine (CPZ) and Fish kairomone (FK) caused functional alteration in *D. magna*;
- Feeding rate was decreased in all treatment conditions;
- Oxygen consumption was only affected in the mixture treatment (CPZ + FK);
- For life history traits and phototactic behavior the effects of FK dominated those of CPZ

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: