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Predator–prey interactions in a changing world: humic stress disrupts predator threat evasion in copepods

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2

3 **TITLE: Predator-prey interactions in a changing world: humic stress disrupts predator**
4 **threat evasion in copepods**

5

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27 **AUTHOR CONTRIBUTIONS**

28 MS originally formulated the idea. MS, MOG, LM and ES conceived and designed the
29 experiments. MS and LM performed the experiments. MS, ES and LM analyzed the data. MS,
30 LM, ES and MOG wrote the manuscript.

31

32 **ABSTRACT**

33 Increasing inputs of colored dissolved organic matter (cDOM), which is mainly composed
34 of humic substances (HS), are a widespread phenomenon of environmental change in aquatic
35 ecosystems. This process of brownification alters the chemical conditions of the environment,
36 but knowledge is lacking of whether elevated cDOM and HS levels interfere with the ability of
37 prey species to evade chemical predator cues and thus affect predator-prey interactions. We
38 assessed the effects of acute and prolonged exposure to HS at increasing concentrations on the
39 ability of freshwater zooplankton to avoid predator threat (imposed by fish kairomones) in
40 laboratory trials with two calanoid copepods (*Eudiatomus gracilis* and *Heterocope*
41 *appendiculata*). Populations of both species clearly avoided water containing fish kairomones.
42 However, the avoidance behavior weakened with increasing HS concentration, suggesting that
43 HS affected the ability of copepods to perceive or respond to the predator cue. The behavioral
44 responses of the two copepod populations to increasing HS concentrations differed, with *H.*
45 *appendiculata* being more sensitive than *E. gracilis* in an acute exposure scenario, whereas *E.*
46 *gracilis* responded more strongly after prolonged exposure. Both showed similar physiological
47 impairment after prolonged exposure, as revealed by their oxidative balance as a stress
48 indicator, but mortality increased more strongly for *H. appendiculata* when the HS
49 concentration increased. These results indicate that reduced predator threat evasion in the
50 presence of cDOM could make copepods more susceptible to predation in future, with variation
51 in the strength of responses among populations leading to changes in zooplankton communities
52 and lake food-web structure.

53

54 **KEYWORDS**

55 Brownification; chemical ecology; global change; zooplankton behavior; humic substances

56

57 **INTRODUCTION**

58 It has become increasingly clear during the past few decades that ecosystems are threatened
59 by global environmental change (Millennium Ecosystem Assessment 2005). Lakes have been
60 recognized as sentinels of this change, because they are sensitive to climate, respond rapidly to
61 shifts in environmental conditions, and integrate information about changes occurring in their
62 catchments (Adrian et al. 2009). As predator-prey interactions are a primary structuring force
63 in ecosystems (Schmitz 2005), any alteration of these interactions is likely to shift competition
64 among species, population dynamics, community structure, and ecosystem processes (Folke et
65 al. 2004; Frank et al. 2005). Thus, understanding the effects of environmental conditions on
66 predator-prey interactions is important to predict ecosystem responses to global change.

67 One prominent facet of global environmental change in lakes is the widely observed
68 increase of colored dissolved organic matter (cDOM) (Hongve et al. 2004; Erlandsson et al.
69 2008), which is mainly composed of humic substances (HS) derived from the surrounding
70 terrestrial environment (Kullberg et al. 1993; Williamson et al. 1999; Brothers et al. 2014). This
71 phenomenon is referred to as “browning” or “brownification” (Roulet and Moore 2006;
72 Kritzberg and Ekström 2012; Solomon et al. 2015). It leads to a yellowish-brownish coloration
73 of lakes, resulting in light regimes and water chemistry that can greatly differ from conditions
74 in clear waters, and effects on species and species interactions (Granéli et al. 1996; Monteith et
75 al. 2007; Robidoux et al. 2015; Solomon et al. 2015).

76 Many freshwater fishes have well-developed visual senses that are used as their primary
77 source of information (Guthrie and Muntz 1993). Therefore, a significant body of research has
78 addressed the consequences of changes in water optical properties by cDOM or HS on the

79 interactions between piscivorous fish, such as pike (*Esox lucius*) and pikeperch (*Sander*
80 *lucioperca*), and their prey, such as perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) (Ranaker
81 et al. 2012, 2014; Jonsson et al. 2013). Very less attention has been given to interactions
82 between planktivorous fishes and their zooplankton prey, and the focus of those studies has
83 been on foraging efficiency (Estlander et al. 2010; Horppila et al. 2011; Jonsson et al. 2012).
84 Given the central role of zooplankton in lake food webs (Thorp and Covich 2010), it is
85 important also to understand to what extent brownification can affect this invertebrate prey,
86 either directly, or indirectly via chemically induced behavior. Previous studies found that HS
87 can have direct physiological effects on zooplankton (e.g. Meems et al. 2004; Steinberg et al.
88 2006) and could be an important factor determining zooplankton species composition and
89 abundance in northern temperate lakes (Shurin et al. 2010; Robidoux et al. 2015). However, to
90 our knowledge, there have been no previous attempts to evaluate whether increased
91 concentrations of cDOM or HS alter the ability of zooplankton to evade predator threat by
92 planktivorous fishes.

93 One of the dominant groups of freshwater zooplankton is copepods (Balian et al. 2008;
94 Heuschele and Selander 2014). In contrast to fish, most copepod species have limited visual
95 abilities and are instead often restricted to chemosensory and hydro-mechanical information
96 (Folt and Goldman 1981; Heuschele and Selander 2014). Therefore, any alteration of their
97 olfactory system or chemical cues in lake water could reduce the ability of copepods to detect
98 and avoid predators. Thus, HS could disrupt chemical signaling pathways between organisms
99 in aquatic ecosystems with potential consequences on individual fitness, species interactions,
100 and zooplankton community structure (Ferrari et al. 2010). In addition, HS could impair the
101 ability of prey to respond to predator cues, which could have the same ecological effect.

102 The objective of the present study was to evaluate the effect of HS on the ability of
103 copepods to respond to fish scent. We performed olfactory trials with single individuals of two
104 different copepod species in a two-armed choice flume to assess the avoidance response of

105 copepods exposed to water containing both HS at increasing concentrations and olfactory cues
106 released by fish (kairomones). We assessed the ability of the copepods to respond to the fish
107 cues after acute and prolonged exposure to HS and also determined the oxidative balance of the
108 copepods as a physiological stress marker to assess potential negative effects on fitness.

109

110 **MATERIALS AND METHODS**

111 *Plankton collection and maintenance*

112 The study was conducted in August 2015 with two species of calanoid copepods:
113 *Eudiaptomus gracilis* (ovigerous females; mean length \pm SD, 1.01 \pm 0.04 mm) and *Heterocope*
114 *appendiculata* (females and males; 1.71 \pm 0.05 mm). The animals were collected with a
115 plankton net (250 μ m mesh size) on one occasion in Lake Stechlin, a deep clear-water lake in
116 northeastern Germany (53° 8' 35'' N, 13° 1' 41'' E), and kept separately at constant
117 temperature (18 \pm 1 °C) in gently aerated 10-L aquaria containing filtered lake water. The
118 photoperiod of 16 h light: 8 h darkness reflected summer conditions in the field. The copepods
119 were fed a *Cryptomonas* culture (strain SAG 26.80, Culture Collection of Algae at Göttingen
120 University, Germany) every 3 days (density 6.5 \pm 1.9 \times 10³ cells mL⁻¹, ~0.35 mg C L⁻¹). The
121 algae were kept in semi-continuous culture in aerated 1-L flasks containing 500 mL of WC
122 medium (Guillard and Lorenzen 1972) modified by using TES buffer (0.5 mM) instead of Tris.
123 Exposure to low light conditions and frequent dilution ensured that the copepods were supplied
124 with a highly nutritious food.

125

126 *Humic substances and fish kairomones*

127 HuminFeed® (<http://www.humintech.com>; henceforth HF) was used as source of HS. HF
128 is an industrially processed leonardite containing 43% organic carbon, 82% humic substances,
129 18% low-molecular-weight compounds, and no polysaccharides (Meinelt et al. 2007). HF has
130 been previously used as a standard source of humic substances to investigate effects of humic

131 stress on the physiology of aquatic organisms (Meinelt et al. 2007; Euent et al. 2008; Steinberg
132 et al. 2010). HF of a single batch was mixed into water with and without fish kairomones shortly
133 before use in the olfactory trials. Five concentrations were used, resulting in DOC exposure
134 scenarios for the copepods within an environmentally relevant range from very low to
135 mesohumic (Table 1). For comparison, DOC concentrations across numerous lakes in Quebec
136 ranged from 3.0 to 15.5 mg L⁻¹ (Robidoux et al. 2015) and in Sweden from 3.9 to 19.4 mg L⁻¹
137 (Granéli et al. 1996).

138 Water containing fish kairomones was freshly prepared before use in the experiments. Four
139 individuals of zooplanktivorous perch (*Perca fluviatilis*, 71.3 ± 4.3 mm length) were immersed
140 for 4 h in a 40-L (0.1 fish per L) aerated tank kept in the dark to produce the kairomones.
141 Although average fish densities in Lake Stechlin are considerably lower (0.01-0.02 per m³;
142 Mehner et al. 2011), densities of shoaling fish can be locally very high and potentially close to
143 those used in our experiment. Perch were pre-fed with live zooplankton (>250 µm) from Lake
144 Stechlin. Water in the tank was fully oxygenated groundwater that had been passed over a sand
145 filter and an ion exchange resin to reduce its high concentrations of calcium and iron. Before
146 use in the experiments, the water was filtered (Whatman grade no. 4 filter paper, 25 µm average
147 pore size) and transferred to five 8-L tanks.

148

149 ***Experimental design and procedures***

150 A two-armed choice flume designed to conduct pairwise choice experiments was used to
151 determine the olfactory ability of copepods to discriminate between water previously containing
152 fish and untreated control water in both the presence and absence of HS (Fig. 1). Two tanks
153 containing water either with or without fish cues were connected to the choice flume with tubing
154 to create a constant gravity-driven flow of ~5 mL min⁻¹. Individual copepods were released at
155 the junction of the horizontal arms where flow from the two different water sources meets. The
156 released copepods were free to move 15 cm into either of the two arms (4 cm diameter)

157 connected to the tanks (Fig. 1). The horizontal alignment of the flume prevented the copepods
158 from vertical migrations.

159 Each trial consisted of a 60-s acclimatization period followed by a test period of 120 s,
160 where the position of the copepod on the right or left side of the flume was recorded at 5-s
161 intervals. The flume was completely emptied after the test period and thoroughly rinsed with
162 tap water. Water sources were switched from one side of the flume to the other after every
163 fourth trial to control for potential side preferences not related to the water source. A total of 20
164 individual copepods were tested per treatment combination. Two copepods that did not swim
165 in either direction against the water flow during the acclimatization period were removed from
166 the study. We recorded the time (s) the individual copepods spent (1) in each of the two arms
167 of the choice flume (i.e. with or without kairomones) and (2) in each of three 5-cm
168 compartments on the left and right side of the flume (Fig. 1). Additionally, we recorded the
169 number of visited compartments during the test period as a measure of locomotor activity. All
170 observations were made under indirect, dim visible light with no light source pointing directly
171 on the flume. There was no evident effect of light source on behavior since the copepods showed
172 no preference for either side of the choice flume.

173 We tested responses of the copepods under the following conditions:

- 174 (i) Water on either side of the flume contained neither fish kairomones nor HS to assess
175 the horizontal distribution of copepods in the choice flume when both predator cues
176 and HS are absent (control).
- 177 (ii) Water contained fish kairomones on one side of the flume and control water without
178 kairomones in the other to assess responses of freshly collected copepods to acute
179 stress by exposure to HS at different concentrations (acute HS stress).
- 180 (iii) Water contained fish kairomones on one side of the flume and control water without
181 kairomones on the other to assess responses to HS exposure at different

182 concentrations after maintaining the copepods at the respective test concentration
183 for 5 days (prolonged HS stress).

184 To assess the effects of prolonged exposure, 50 individuals of each species were kept at
185 the five different HS concentrations (Table 1) for 5 days in 2-L glass beakers containing 1.5 L
186 of lake water that had been passed over a 90- μ m mesh. Two glass beakers were used per
187 treatment, one for the olfactory experiment and the other to assess copepod physiological status.
188 Water with a given HS concentration was prepared in a single batch and distributed among all
189 beakers to avoid any potential differences in HS concentration between species. The copepods
190 were fed at the beginning and after 3 days with the *Cryptomonas* culture (cell density: 6.5 ± 1.9
191 $\times 10^3$ mL⁻¹, ~ 0.35 mg C L⁻¹). Twenty individuals of one beaker were haphazardly chosen for
192 each treatment of the olfactory trials described above.

193

194 ***Physiological stress indicator***

195 The effect of HS on the physiology of the two copepod species was assessed by
196 determining the oxidative balance between antioxidant capacity and oxidative damage at the
197 cellular level as an indicator of oxidative stress. Oxidative stress occurs when the cellular
198 balance between the production of reactive oxygen species and antioxidant defenses shifts
199 towards the former (Sies 1991) and can adversely affect life-history traits and fitness
200 (Monaghan et al. 2009). After 5 days of exposure to HS (see above), all copepods were
201 collected, allocated to 3-5 subsamples per HS concentration, each comprising 4 *H.*
202 *appendiculata* or 9 *E. gracilis*, which were placed in Eppendorf tubes, flash-frozen in liquid
203 nitrogen, and stored at -80 °C. The frozen samples were later homogenized in 250 μ L cold
204 (4°C) potassium phosphate buffer (PPB; 0.1 M, pH 7.2) by bead-beating for 2 min at 20 Hz
205 with stainless steel milling balls (4 mm diameter) in a Mixer Mill MM 400 (Retsch®, Haan,
206 Germany). The homogenate was centrifuged at 10,000 g for 10 min at 4 °C and the supernatant
207 was used for analyses. Intracellular soluble antioxidant capacity was determined using the

208 oxygen radical absorption capacity (ORAC) assay (Prior et al. 2003, modified for copepods by
209 Gorokhova et al. 2013), and oxidative damage was measured using the thiobarbituric acid
210 reactive substances (TBARS) assay (Oakes and Van Der Kraak 2003). Total protein content
211 was measured according to Bradford (1976) using bovine serum albumin as standard. The raw
212 data of the ORAC and TBARS assays were first normalized to the quantity of proteins and the
213 ratio between ORAC and TBARS was used as an indicator of the oxidative balance.

214

215 *Statistical analyses*

216 All statistical analyses and tests of their underlying assumptions (normality of the residuals,
217 homogeneity of variance) were performed in R version 3.2.3 (R Core Team 2012). The
218 significance level was set at $P < 0.05$ unless specified otherwise. To investigate the effect of
219 HS on the ability of copepods to avoid predation threat, we analyzed the time that the copepods
220 spent in the arm of the flume containing water with kairomones (square root-transformed to
221 meet assumptions of normal distribution and homogeneous variances) and the number of visited
222 compartments (as a proxy of locomotor activity) using general linear models with copepod
223 identity and duration of exposure to humic stress as factorial and HS concentration as
224 continuous explanatory variables. The response variable ‘time copepods spent in the flume arm
225 containing kairomone water’ accounts for the avoidance response of the copepods to fish
226 kairomones and thus includes the kairomone effect.

227 To assess differences in the horizontal distribution of individuals in the choice flume, we
228 calculated the mean distance of the 20 individuals from the center of the flume where the right
229 and left arm meet and the animals were placed. Mean compartment distances from zero (i.e. -
230 12.5, -7.5, -2.5, 2.5, 7.5, and 12.5 cm, see Fig. 1) were used in the calculation of an individual’s
231 mean distance from the center. From the individual mean distances of 20 copepods, a mean
232 value and its 95% confidence intervals (CIs) were calculated to compare the avoidance of

233 copepods to water containing fish kairomones across the different treatment combinations.
234 Significance was based on non-overlapping 95% CIs.

235 To assess the effects of prolonged HS stress on the oxidative balance of copepods, we
236 compared the treatments with HS addition to the control treatment without HS. Dunnett's post
237 hoc tests with P-values adjusted according to Holm were conducted to compare responses to
238 HS addition with the controls (0.1 mg DOC L⁻¹). Statistical tests to assess oxidative stress were
239 based on the log response ratio of the oxidative balance (i.e. ORAC:TBARS ratio). Taking the
240 logarithm ensured that any increases or decreases of the ratio were given the same weight.

241

242 **RESULTS**

243 *Olfactory trials without kairomones or HS*

244 In the absence of fish kairomones and HS, the average time individuals of both copepod
245 species spent on either side of the choice flume during the 120-s trials was not significantly
246 different from the expected 60 s (Fig. S1; one sample t-test, *E. gracilis*: $t = 1.05$, $df = 19$, $P =$
247 0.31 , *H. appendiculata*: $t = 0.73$, $df = 19$, $P = 0.47$). This lack of preference for the left or right
248 side of the flume was also reflected in the horizontal distribution of the copepods in the choice
249 flume in that the mean distance from the centre of the flume was near zero (Fig. 2a, b). The
250 distribution of copepods across the flume was more variable for *E. gracilis* than for *H.*
251 *appendiculata*, as shown by larger CIs, indicating that *E. gracilis* moved further away from the
252 starting point (i.e. the center of the choice flume) than *H. appendiculata*. In addition, the larger
253 number of compartments visited by *E. gracilis* revealed a higher locomotor activity than shown
254 by *H. appendiculata* (Fig. S2; two sample t-test, $t = 2.93$, $df = 38$, $P = 0.006$).

255

256 *Effects of population identity, HS concentration, and exposure duration*

257 The two copepods were strongly affected by HS concentration and exposure duration (Fig.
258 3; Table 2, high variance explained by these main effects). In general, both species spent

259 increasingly more time in compartments containing fish kairomones when the HS concentration
260 increased (Fig. 3; Table 2, HS concentration main effect), and this increase differed between
261 the two tested populations (Fig. 3; Table 2, significant interaction of HS concentration and
262 population identity). Prolonged exposure reduced the ability of both copepods to avoid water
263 containing fish kairomones, but the strength of this effect depended on the population tested
264 (Fig. 3; Table 2, main effect of exposure time, significant interaction of population identity and
265 exposure time).

266

267 *Olfactory trials under acute stress*

268 The time individuals of both copepod species spent in compartments containing fish
269 kairomones was very short in the absence of HS (Fig. 2c, d). Instead, the copepods stayed
270 almost exclusively in the arm of the flume supplied with water not previously containing fish
271 (Figs. 3a, b, S3), indicating a distinct ability of copepods to perceive predator cues and avoid
272 potential predation threat. This response pattern was maintained, although gradually weakened,
273 with the concentration of added HS increasing up to 7.2 mg C L⁻¹ (Fig. 3a, b). At 19.3 mg C L⁻¹
274 ¹, however, predator threat evasion appeared to be partly diminished for *E. gracilis* (Fig. 3a)
275 and completely impaired for *H. appendiculata* (Fig. 3b). This pattern is also reflected in the
276 mean distance of the copepods from the center of the choice flume (Fig. S4), which increased
277 with increasing HS concentration for *H. appendiculata* (Fig. 4b) and above 3.6 mg C L⁻¹ also
278 for *E. gracilis* (Fig. 4a). The locomotor activity of *E. gracilis* decreased with increasing HS
279 concentration (Fig. 5a), whereas no consistent change was observed for *H. appendiculata* along
280 the HS gradient (Fig. 5b).

281

282 *Olfactory trials after prolonged stress*

283 The ability of *E. gracilis* to sense predatory cues was greatly reduced by prolonged HS
284 exposure (5 days), as indicated by individuals spending more time in compartments with fish

285 kairomones already at 1.8 mg C L⁻¹ (Figs. 3c, 4a, S5) compared to acute exposure, where the
286 copepods showed a significant response only at 7.2 mg C L⁻¹ (Figs. 3a, 4a). For *H.*
287 *appendiculata*, the mean distance from the flume center continuously increased with increasing
288 HS concentration in the trials testing for both acute and prolonged stress (Fig. 4b). A significant
289 difference in predator cue recognition between the two exposure times was only apparent at 7.2
290 mg C L⁻¹ (Figs. 3b, d, 4b). Predator avoidance by *E. gracilis* under prolonged exposure was
291 completely suppressed at 3.6 mg C L⁻¹ (Fig. 4a), and that of *H. appendiculata* only at 19.3 mg
292 C L⁻¹ (Figs. 4a, S5). Copepod locomotor activity was unaffected by exposure duration (Fig. 5;
293 Table 2); whereas the activity of *E. gracilis* decreased with increasing HS concentration, there
294 was no consistent change along the HS gradient for *H. appendiculata*.

295

296 ***Physiological stress***

297 HS also affected the oxidative balance of both copepod species. The pattern observed along
298 the HS gradient is best described as a biphasic response known as hormesis, which is
299 characterized by stimulation at low and inhibition at high concentrations (Fig. 6). Specifically,
300 the oxidative balance was higher than that in the controls at the lowest HS concentration (5 mg
301 L⁻¹) but lower at 3.6 and 7.2 mg C L⁻¹ (Fig. 6). The magnitude of the increase and decrease was
302 similar (i.e. about a doubling and halving of the oxidative balance, respectively) and there were
303 no notable species-specific differences along the HS gradient. At the highest HS concentration,
304 the oxidative balance approached the level of the controls (Fig. 6), suggesting that other
305 physiological pathways than those involving antioxidant defenses might counteract stress
306 caused by HS at very high concentrations. Prolonged exposure to HS also had a direct negative
307 effect on copepod survival, as mortality increased with increasing HS concentration after 5 days
308 of exposure (Table 3; linear regressions, *E. gracilis*: $R^2 = 0.51$, $P = 0.02$; *H. appendiculata*: R^2
309 $= 0.43$, $P = 0.04$).

310

311 **DISCUSSION**

312 The observed responses of the two copepod populations to the presence of fish kairomones
313 revealed that both *E. gracilis* and *H. appendiculata* are clearly capable of detecting olfactory
314 cues and avoiding predator threat. This result is in line with previous experimental studies on
315 other copepod species that observed avoidance behavior by kairomone-induced vertical
316 migration (Neill 1990, 1992; Cohen and Forward 2005; Jamieson 2005; Minto et al. 2010;
317 Gutierrez et al. 2011). More important, our results also show that HS can impair the ability of
318 copepods to evade predator threat by interfering with the recognition of chemical cues released
319 by fish or by inducing stress that alters copepod behavior. The implication is that by preventing
320 predator evasion, increases in HS concentrations in surface waters can reduce the fitness of prey
321 species. This, together with the finding that females of a freshwater fish (*Xiphophorus*
322 *birchmanni*) lose their preference for conspecific male cues upon HS exposure (Fisher et al.
323 2006), reinforces the conclusion that chemical signal transduction both within and between
324 species, ranging from cue perception to behavioral responses, can be disrupted as a result of
325 surface water brownification.

326

327 ***Physiological stress and altered behavior***

328 Direct physiological effects of HS on freshwater invertebrates have been attributed to the
329 induction of oxidative stress, production of chemical defense proteins, and variations in
330 detoxification enzyme activities (Steinberg et al. 2006; Timofeyev et al. 2006; Steinberg et al.
331 2010). Our assessment of oxidative balance indicates that HS caused oxidative stress also in the
332 two populations of copepods we investigated. The biphasic pattern observed along our
333 experimental HS gradient suggests a hormetic effect for both copepods, with stimulation at low
334 and a negative response at elevated HS concentrations. This pattern is consistent with previous
335 results showing HS-mediated hormesis of antioxidant enzymes in other crustaceans, *Gammarus*
336 *lacustris* and *Daphnia galeata* (Meems et al. 2004; Timofeyev et al. 2006). According to

337 hormesis theory (e.g. Calabrese 2005), the stimulation at low HS concentration may be
338 beneficial for organisms by training their defense systems, whereas HS becomes toxic at
339 elevated concentrations. That our copepods showed greater oxidative stress at high HS
340 concentrations is also in line with the greater mortality observed in these conditions after 5 days
341 of exposure, indicating direct toxicity of HS on both copepods. This toxic effect could have
342 influenced the results we obtained after prolonged exposure, at least for *E. gracilis*, whose
343 avoidance ability was more strongly reduced than after acute exposure. However, potentially
344 reduced fitness was unlikely to affect our results on acute stress, because the acute stress trials
345 were limited to 120 s and used freshly caught copepods that had not previously experienced
346 stress by HS. Thus, our results on acute stress are unlikely to be notably influenced by toxic
347 effects on copepod performance, although copepod behavior could have been affected.
348 Prolonged exposure to HS, however, could exacerbate the negative effect on avoidance ability
349 by directly increasing mortality or by physiologically weakening the copepods as a result of
350 oxidative stress.

351 It currently remains unknown which mechanism was responsible for the observed effects.
352 HS could alter the chemical structure of fish kairomones such that these molecules become
353 undetectable to the chemoreceptors of the copepods. However, since HS bind to hydrophobic
354 chemicals such as steroid pheromones (Mesquita et al. 2003), rather than to hydrophilic ones
355 such as fish kairomones (von Elert and Loose 1996), this scenario is rather unlikely.
356 Alternatively, HS could block or damage the chemoreceptors, as found in a goldfish where HS
357 reduced the response of the olfactory epithelium and olfactory bulb to sexual pheromones
358 (Hubbard et al. 2002). Both of these mechanisms could cause the observed impairment of
359 information flow. In addition, HS could alter copepod behavior by imposing stress. Thus, future
360 studies need to distinguish between the modification by HS of kairomone chemical structure,
361 impairment of copepod chemosensory ability as a cause of infodisruption, and HS-induced
362 stress that elicits other behavioral effects on copepods.

363

364 *Population-specific responses to humic stress*

365 A notable outcome of our experiments is that the two tested copepod populations showed
366 differences in their abilities to respond to fish kairomones. Under conditions of acute exposure,
367 the avoidance ability of *H. appendiculata* was reduced at low HS concentrations compared to
368 that of *E. gracilis*, suggesting a higher sensitivity to HS of *H. appendiculata*. After prolonged
369 exposure, the two species showed opposite patterns, but this result needs to be interpreted with
370 caution because of potentially concomitant direct toxic effects of HS after 5 days of exposure.
371 Furthermore, the threshold-like response of *E. gracilis* to increasing HS concentrations was
372 dependent on exposure time, whereas *H. appendiculata* showed a similar and steady reduction
373 in its ability to avoid water containing fish kairomones at both exposure times. This suggests
374 that surviving *H. appendiculata* is better at evading predator threat after prolonged HS stress
375 than *E. gracilis*, in spite of a higher mortality at elevated HS concentrations than *E. gracilis*. It
376 is important to note, however, that our experiment was not designed to assess the response of
377 both species in general, because populations from different lakes could deviate in their
378 sensitivities to both HS and predator kairomones.

379 The alteration of predator avoidance under HS stress that we observed could increase the
380 susceptibility of copepods to predation in the future as brownification of surface waters
381 continues, because this effect may ultimately reduce the distance between copepods and
382 planktivorous fish. The differences we observed between the two copepod populations further
383 suggest that *H. appendiculata* populations could be more prone to predation than *E. gracilis*
384 under conditions of acute humic stress, whereas *E. gracilis* population could be more affected
385 when exposed to prolonged humic stress. Conversely, the direct negative effect of HS on
386 copepod survival observed here may favor *E. gracilis* over *H. appendiculata* populations,
387 because the latter showed a higher mortality after prolonged exposure. Selection is expected to
388 act more strongly on populations less tolerant to humic stress, as a result of both direct

389 physiological effects and changes in predator avoidance, potentially leading to changes in
390 community composition. For instance, since *E. gracilis* is much smaller than *H. appendiculata*,
391 it is harder for fish to visually detect this prey species, even though the ovigerous (egg sac-
392 carrying) females of *E. gracilis* we used are more susceptible to fish predation than males or
393 females without egg sacs (Winfield and Townsend 1983; Svensson 1992).

394

395 ***Potential zooplankton community effects of lake brownification***

396 Increasing evidence indicates that chemical cues of predators induce diel vertical migration
397 (DVM) in copepods as a defense strategy against predation (Neill 1990, 1992; Cohen and
398 Forward 2005; Jamieson 2005; Heuschele and Selander 2014). This includes *E. gracilis*, where
399 the migration amplitude depends on season and other factors (Ringelberg et al. 1991). *E.*
400 *gracilis* can use fish kairomones as a warning signal triggering DVM (Jamieson 2005) but, as
401 our data show, may lose this ability under elevated HS levels resulting from surface water
402 brownification. Very little is known about the DVM behavior of *H. appendiculata*, probably
403 because this species is rarely dominant in lake zooplankton communities (Walseng et al. 2006).
404 However, *H. appendiculata* more often than *E. gracilis* appears to occur in lakes characterized
405 by lower HS concentrations (Berzins and Bertilsson 1990), suggesting that *E. gracilis* may cope
406 better with increased lake brownification expected in the future.

407 The HS-mediated reduction in the ability to evade predator threat could increase the
408 susceptibility of copepods to predation in lakes. Alternatively, however, brownification could
409 protect zooplankton by impairing the visual detection ability of fish, although experimental
410 studies on fish predation in relation to brownification have shown mixed results. Brownification
411 has decreased predation efficiency of some visually hunting planktivorous fishes (Estlander et
412 al. 2010, 2012), but no effects have been observed in other cases (Horppila et al. 2011; Jonsson
413 et al. 2012). For instance, perch (*Perca fluviatilis*) consumed significantly less phantom midge
414 larvae (*Chaoborus flavicans*) in highly humic than in clear water (Estlander et al. 2012),

415 whereas a significant effect of reduced water clarity on roach (*Rutilus rutilus*) feeding on
416 copepods has not been observed (Jonsson et al. 2012). Further, the average body size of
417 zooplankton in lakes with fish increases with increasing cDOM concentrations (Symons and
418 Shurin 2016), suggesting that brownification weakens top-down control of large zooplankton
419 species, which is consistent with the hypothesis that fish predation is less intense at high HS
420 levels. Thus, the consequences on the zooplankton community resulting from species-specific
421 differences in both zooplankton and fish responses to HS could be complex and entail cascading
422 effects on lake food webs and ecosystem functioning.

423

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433

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617

618 **Table 1** Summary of water-chemical and physical characteristics along the experimental humic
619 substance gradient prepared using HuminFeed[®] (HF). Dissolved organic carbon (DOC) and
620 water color increased along the HF gradient

621

HF (mg L ⁻¹)	DOC (mg L ⁻¹)	Color intensity (absorption at 436 nm)	pH
0	0.1	<0.01	7.1
5	1.8	0.03	7.2
10	3.6	0.06	7.2
20	7.2	0.13	7.1
50	19.3	0.32	7.2

622

623

624 **Table 2** ANOVA results of general linear models testing for the effects of copepod population
625 (P), duration of exposure to humic stress (= exposure time; E) and humic substance
626 concentration (HS; continuous) on copepod avoidance behavior assessed as the time spent by
627 copepods in the arm of the choice flume containing kairomones (square root transformed data)
628 and on the number of visited compartments (untransformed data). Significant effects are
629 indicated by asterisks: * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$

630

Source of variation	Time spent in the choice-flume arm containing kairomones				Number of visited compartments			
	df	Sum of squares	Variance (%)	F-value	df	Sum of squares	Variance (%)	F-value
Copepod population (P)	1	0.37	<0.1	0.1	1	17.4	2.3	11.3***
Exposure time (E)	1	327.4	9.6	68.1***	1	0.1	<0.1	0.1
HS concentration (HS)	1	1115.5	32.7	231.9***	1	78.3	10.2	50.6***
P × E	1	64.5	1.9	13.4***	1	7.5	1.0	4.9*
P × HS	1	22.7	0.7	4.7*	1	54.0	7.1	35.0***
E × HS	1	1.3	<0.1	0.3	1	1.0	0.1	0.7
P × E × HS	1	0.11	<0.1	0.0	1	3.7	0.5	2.4
Residuals	390	1875.8	55.0		390	602.8	78.8	

631

632

633 **Table 3** Mortality of *E. gracilis* and *H. appendiculata* after 5 days of exposure to humic
 634 substance (HS; HuminFeed) at different concentrations
 635

Population	Glass 1			Glass 2	
	HS (mg C L ⁻¹)	No. dead individuals	Mortality (%)	No. dead individuals	Mortality (%)
<i>E. gracilis</i>	0.1	2	4	1	2
	1.8	1	2	1	2
	3.6	8	16	5	10
	7.2	7	14	7	14
	19.3	8	16	7	14
<i>H. appendiculata</i>	0.1	3	6	5	10
	1.8	8	16	11	22
	3.6	12	24	10	20
	7.2	15	30	12	24
	19.3	12	24	14	28

636

637

638 **FIGURE LEGENDS**

639

640 **Fig. 1** Schematic of the experimental choice flume. Individual copepods were released at the
641 center of the horizontally aligned experimental arena (location zero), where they could freely
642 choose to move 15 cm in opposite directions of the flume. Both arms of the flume (4 cm
643 diameter) were divided into three compartments (left side: -15 to -10 cm, -10 to -5 cm, -5 to 0
644 cm; right side: 0 to 5 cm, 5 to 10 cm, 10 to 15 cm). Mean compartment distances from the centre
645 of the flume are indicated in bold. The two arms were connected to tanks supplying water that
646 either contained or did not contain fish kairomones. Dashed arrows symbolize direction of the
647 water flow ($\sim 5 \text{ mL min}^{-1}$)

648

649 **Fig. 2** Responses of *Eudiaptomus gracilis* (a, c) and *Heterocope appendiculata* (b, d) in acute
650 stress trials without (a, b) and with (c, d) fish kairomones expressed as time spent in different
651 compartments of the choice flume. The black vertical lines indicate the center of the flume and
652 the thick solid lines indicate the mean distance of 20 individual copepods from the center with
653 the dashed lines denoting 95% confidence limits

654

655 **Fig. 3** Responses of (a,c) *Eudiaptomus gracilis* and (b,d) *Heterocope appendiculata* to
656 increasing humic substance (HS) concentrations in (a,b) acute and (c,d) prolonged exposure
657 trials, expressed as time spent in compartments without (white boxes) and with (gray boxes)
658 fish kairomones. N = 20 copepods in each trial

659

660 **Fig. 4** Responses of (a) *Eudiaptomus gracilis* and (b) *Heterocope appendiculata* to increasing
661 humic substance (HS) concentrations in acute and prolonged exposure trials, expressed as the
662 mean distance of 20 individual copepods from the flume center. Negative values indicate
663 avoidance of fish kairomone containing water. Differences between HS levels within a given

664 exposure treatment are indicated by different letters, based on non-overlapping 95% confidence
665 intervals (error bars)

666

667 **Fig. 5** Number of choice-flume compartments visited as an estimate of locomotor activity of
668 (a) *Eudiatomus gracilis* and (b) *Heterocope appendiculata* at increasing humic substance (HS)
669 concentrations during acute and prolonged exposure trials. N = 20 copepods in each trial

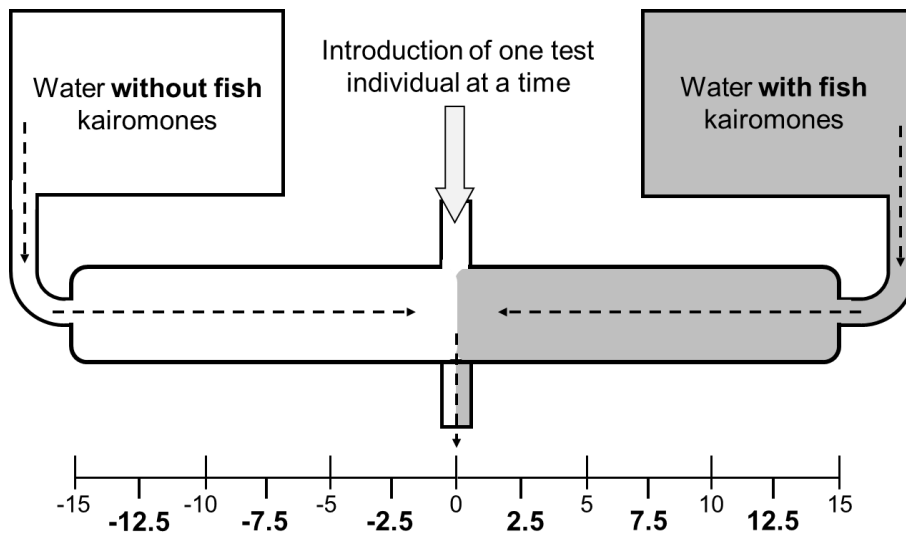
670

671 **Fig. 6** Normalized oxidative balance (ORAC:TBARS ratio) as an indicator of oxidative stress
672 of *Eudiatomus gracilis* and *Heterocope appendiculata* after prolonged exposure to increasing
673 humic substance (HS) concentrations. Error bars indicate standard deviations. Asterisks
674 indicate significant differences from the controls at the $P < 0.05$ (*) and $P < 0.01$ (**) level

675

676 **Fig. 1**

677

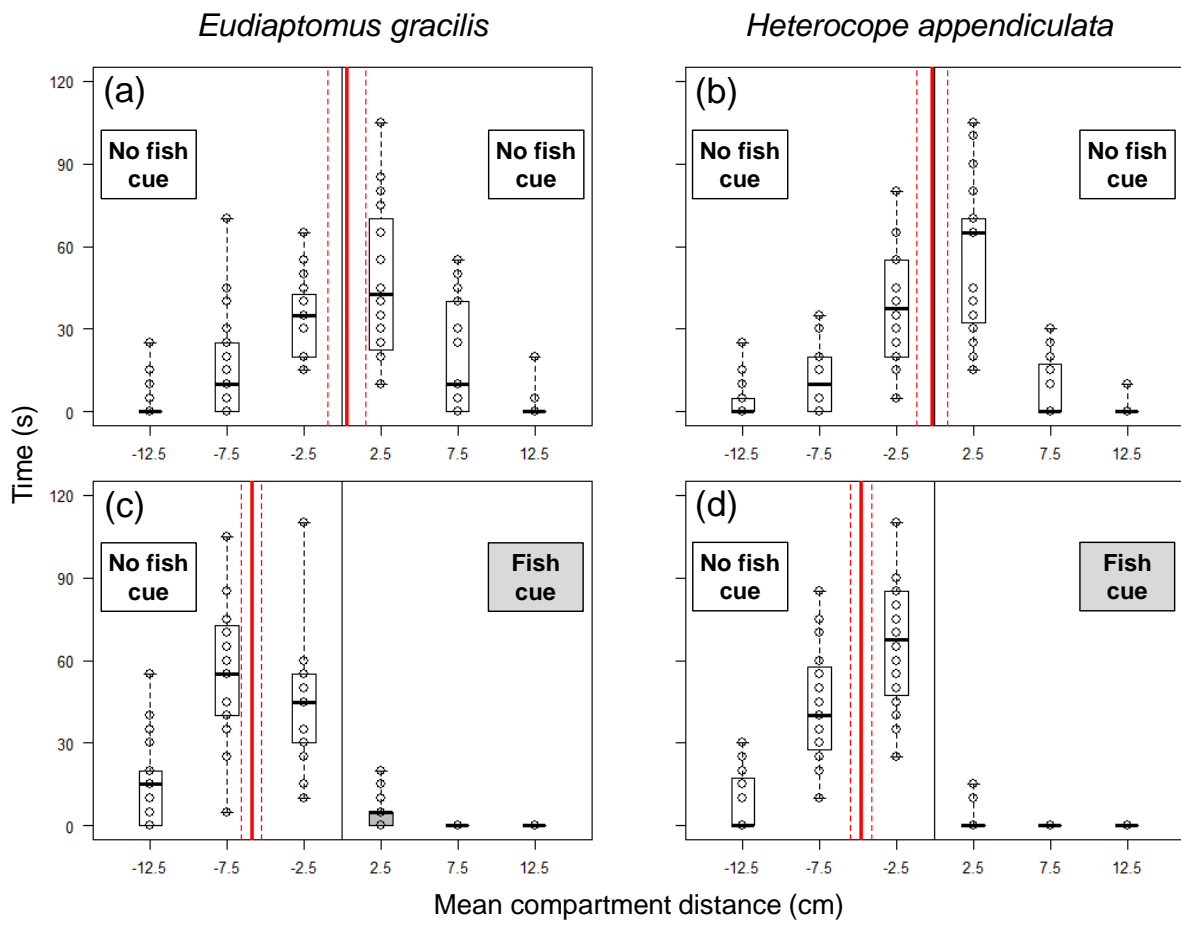


678

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680 **Fig. 2**

681

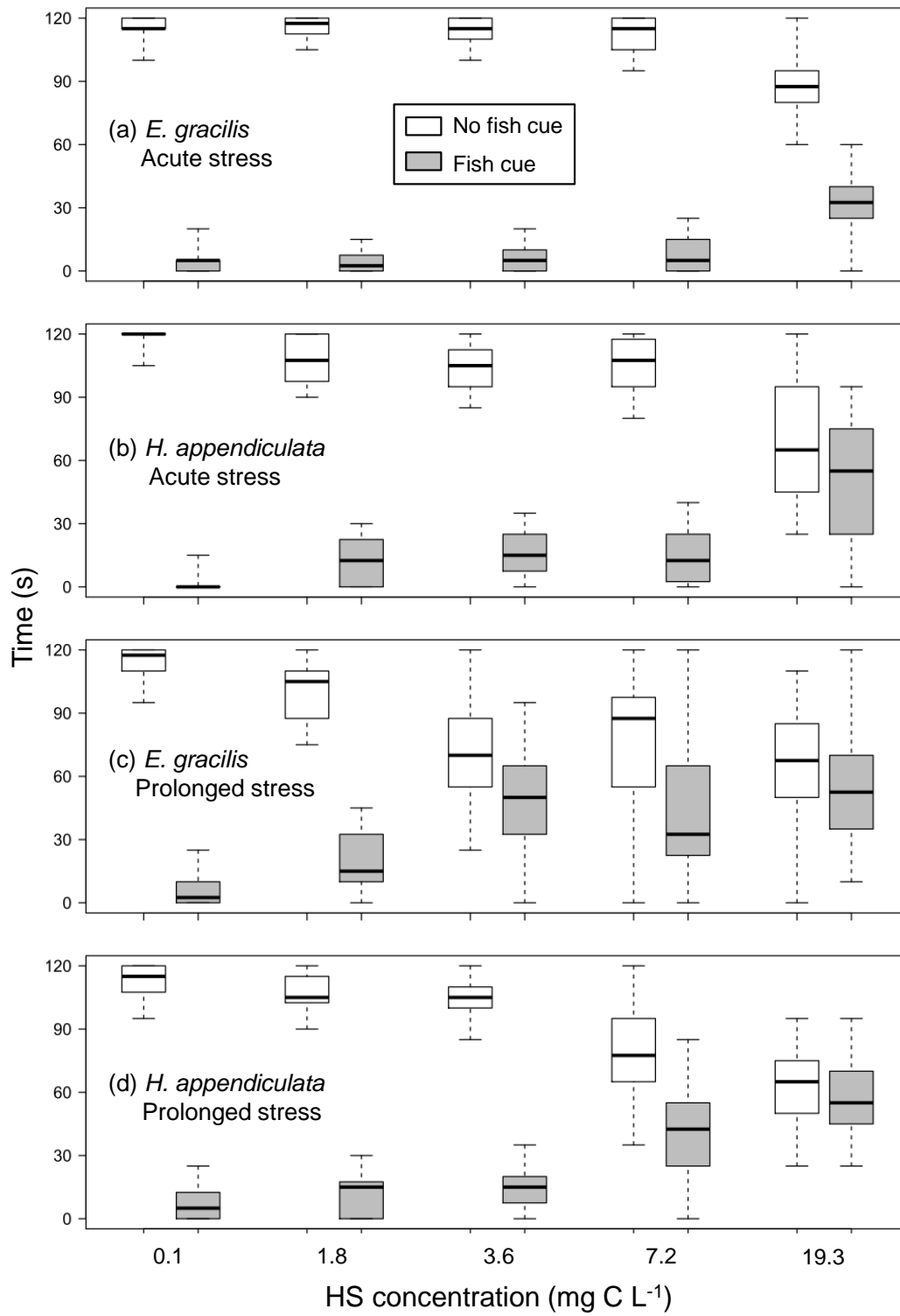


682

683

684 **Fig. 3**

685

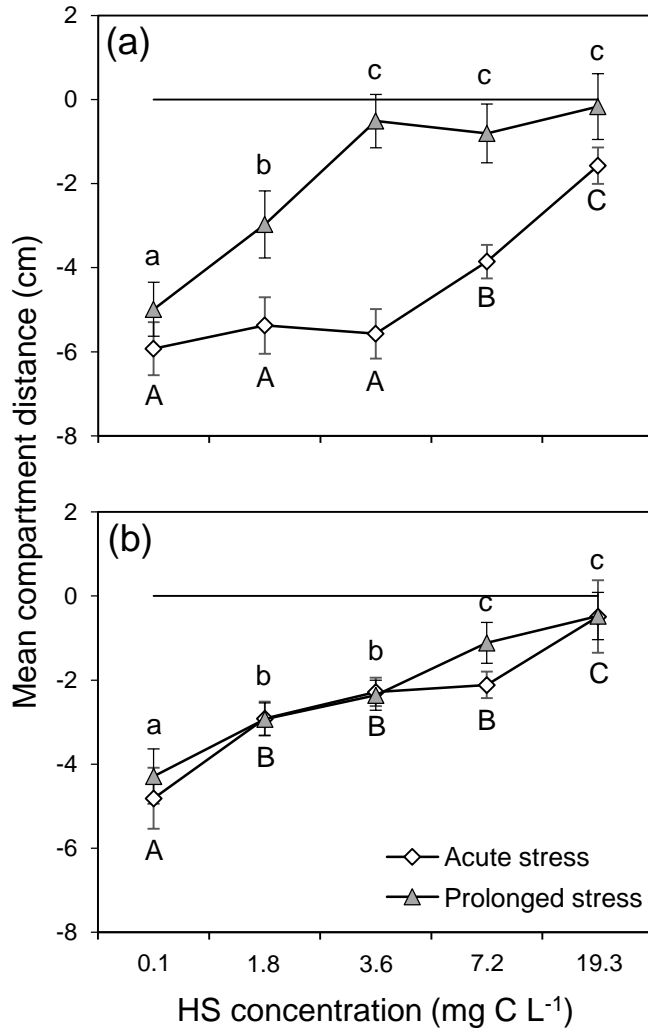


686

687

688 Fig. 4

689

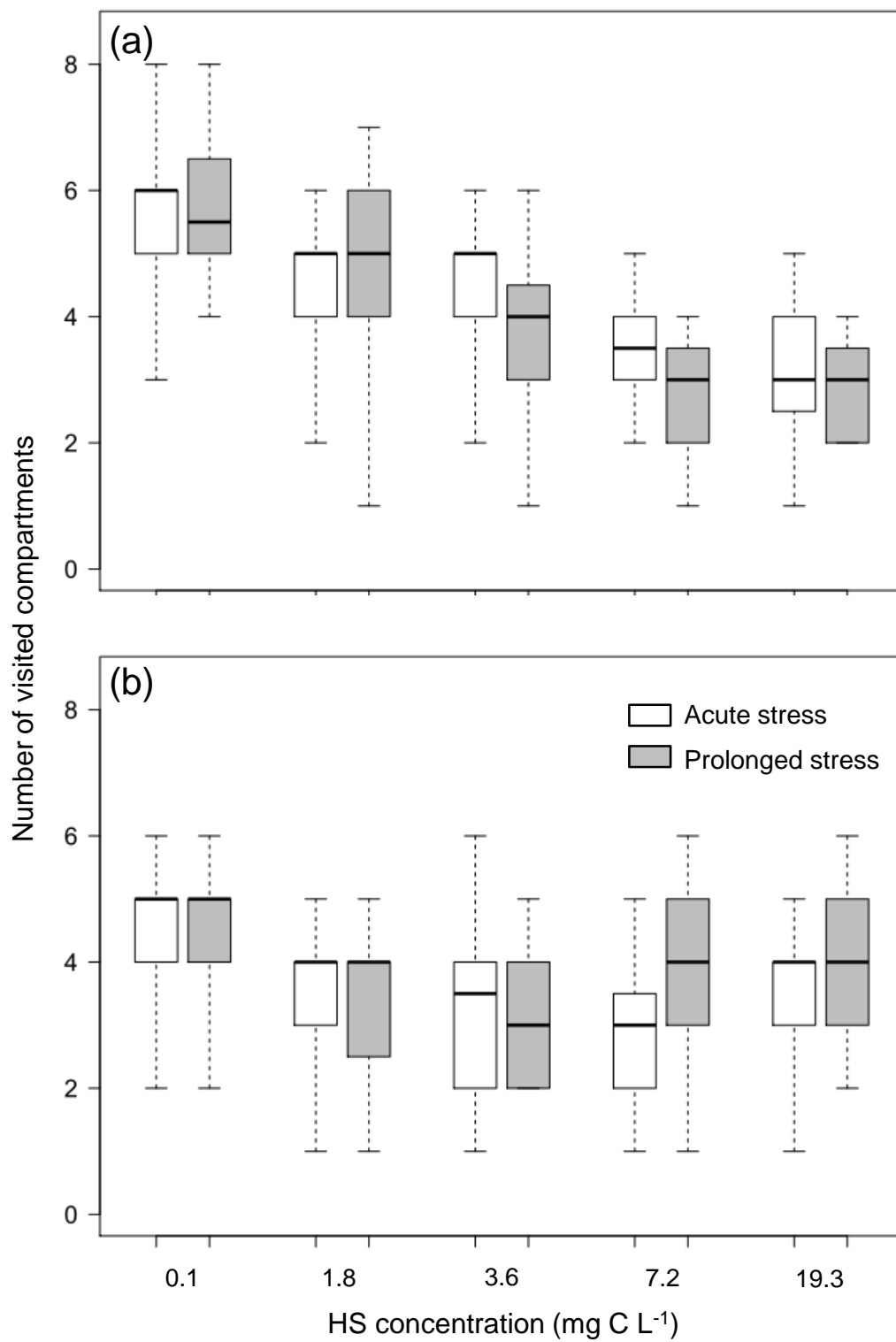


690

691

692 **Fig. 5**

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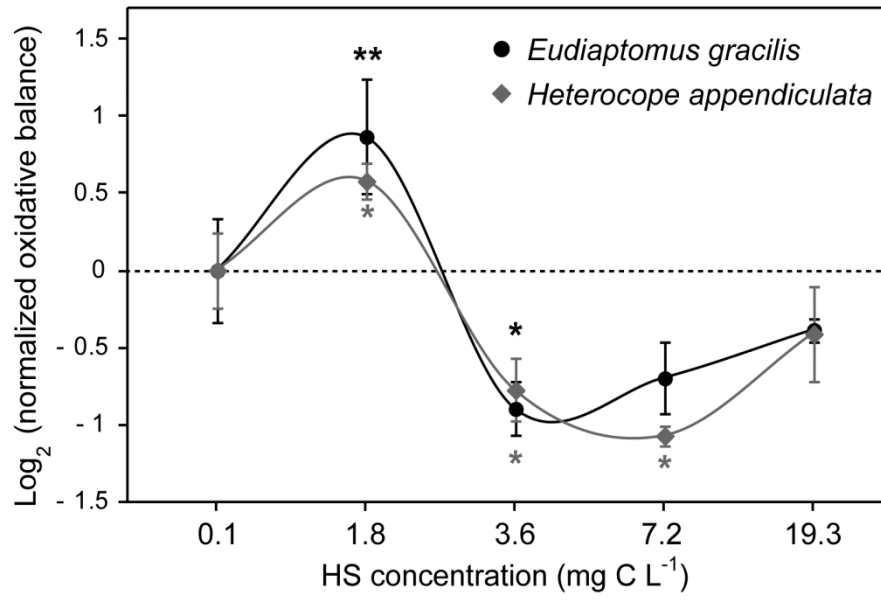


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696 **Fig. 6**

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