CATEGORY: Global change ecology - Original research

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

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

ABSTRACT

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

KEYWORDS

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Plankton collection and maintenance

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

Humic substances and fish kairomones

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

stress on the physiology of aquatic organisms (Meinelt et al. 2007; Euent et al. 2008; Steinberg et al. 2010). HF of a single batch was mixed into water with and without fish kairomones shortly before use in the olfactory trials. Five concentrations were used, resulting in DOC exposure scenarios for the copepods within an environmentally relevant range from very low to mesohumic (Table 1). For comparison, DOC concentrations across numerous lakes in Quebec 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 (Granéli et al. 1996).

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

Experimental design and procedures

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

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

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

We tested responses of the copepods under the following conditions:

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

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

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

Physiological stress indicator

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

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

Statistical analyses

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

To assess differences in the horizontal distribution of individuals in the choice flume, we calculated the mean distance of the 20 individuals from the center of the flume where the right and left arm meet and the animals were placed. Mean compartment distances from zero (i.e. -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 mean distance from the center. From the individual mean distances of 20 copepods, a mean value and its 95% confidence intervals (CIs) were calculated to compare the avoidance of

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

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

RESULTS

Olfactory trials without kairomones or HS

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

Effects of population identity, HS concentration, and exposure duration

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

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

Olfactory trials under acute stress

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

Olfactory trials after prolonged stress

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

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

Physiological stress

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

DISCUSSION

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

Physiological stress and altered behavior

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

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

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

Population-specific responses to humic stress

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

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

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

Potential zooplankton community effects of lake brownification

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

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

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

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

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

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

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	

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

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

FIGURE LEGENDS

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

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

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

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

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

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

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

Fig. 1

680 **Fig. 2**

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Fig. 5

Fig. 6