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Changes in food characteristics reveal indirect effects of lake browning on zooplankton performance

Laetitia Minguez,[1,2*,†] **Erik Sperfeld**,[1,3*,†] **Stella A. Berger**,[1,4] **Jens C. Nejstgaard**,[1,4] **Mark O. Gessner**[1,4,5]

[1]Department of Experimental Limnology, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Stechlin, Germany
[2]Université de Lorraine, CNRS, LIEC, Metz, France
[3]Animal Ecology, Zoological Institute and Museum, University of Greifswald, Germany
[4]Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), Berlin, Germany
[5]Department of Ecology, Berlin Institute of Technology (TU Berlin), Berlin, Germany

Abstract

Browning caused by colored dissolved organic matter is predicted to have large effects on aquatic ecosystems. However, there is limited experimental evidence about direct and indirect effects of browning on zooplankton in complex field settings. We used a combination of an ecosystem-scale enclosure experiment and laboratory incubations to test how prolonged browning affects physiological and life-history traits of the water flea *Daphnia longispina*, a key species in lake food webs, and whether any such effects are reversible. Daphnids and water were collected from enclosures in a deep clear-water lake, where the natural plankton community had been exposed for 10 weeks to browning or to control conditions in clear water. Daphnid abundance was much lower in the brown than in the clear enclosure. Surprisingly, however, daphnids continuously kept in brown enclosure water in the laboratory showed increased metabolic performance and survival, and also produced more offspring than daphnids kept in clear enclosure water. This outcome was related to more and higher-quality seston in brown compared to clear water. Moreover, daphnids transferred from clear to brown water or vice versa adjusted their nucleic acid and protein contents, as indicators of physiological state, to similar levels as individuals previously exposed to the respective recipient environment, indicating immediate and reversible browning effects on metabolic performance. These results demonstrate the importance of conducting experiments in settings that capture both indirect effects (i.e., emerging from species interactions in communities) and direct effects on individuals for assessing impacts of browning and other environmental changes on lakes.

Many aquatic ecosystems across the northern hemisphere are receiving increasing amounts of dissolved organic carbon derived from the terrestrial environment (tDOC), a phenomenon that has been referred to as browning or brownification (Solomon et al. 2015; Williamson et al. 2015; Creed et al. 2018). Humic substances constitute the main fraction of tDOC in aquatic ecosystems and cause a typical tea-stained water color due to their strong chromophoric properties (Evans et al. 2006; Monteith et al. 2007; Williamson et al. 2015; Kelly et al. 2016; Wolf and Heuschele 2018). Reported DOC concentrations range from < 1 mg L^{-1} in the most transparent waters to 100 mg L^{-1} or occasionally more in polyhumic waters (Steinberg et al. 2006; Creed et al. 2018). Several mechanisms have been suggested to explain the increase in tDOC inputs into aquatic ecosystems. These include recovery from acidification (Evans et al. 2006; Monteith et al. 2007), intensified precipitation and altered temperature patterns induced by climate change (Freeman et al. 2001), and changes in catchment hydrology (Evans et al. 2005) and land use (Kritzberg 2017). Even though the relative importance of potential drivers of browning are still debated, current trends suggest that tDOC concentrations continue to increase in inland waters. Information on how aquatic ecosystems and their organisms respond to these changes is of great importance for the future of freshwaters. Nevertheless, tDOC concentrations can also decrease when climate change reduces precipitation, leading to less leaching of soils and reduced

*Correspondence: laetitia.minguez@univ-lorraine.fr (L.M.); eriksperfeld@googlemail.com (E.S.)

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[†]These authors contributed equally to the study.

runoff (Fee et al. 1996; Schindler et al. 1996). Thus, the question arises not only to what extent aquatic organisms are affected by increasing browning, but also how they respond to recovery from browning.

Daphnia is a focal zooplankton genus to investigate ecological effects of DOC in freshwaters, because they play a pivotal role in the food webs of most lakes and ponds, both as efficient grazers of phytoplankton and as important prey for planktivorous fish and predatory invertebrates (Miner et al. 2012). Given *Daphnia*'s abundance in lakes and their high body phosphorus content and low N:P ratio (Urabe 1993), their central position in lake food webs entails not only a key role in carbon dynamics but also a major influence on nutrient cycling, most notably through phytoplankton grazing and phosphorus immobilization in the biomass of daphnids (Elser and Urabe 1999; Sterner and Elser 2002). In addition, as a generalist filter-feeder, *Daphnia* affects microbial communities by consuming protozoans and large bacteria (Langenheder and Jürgens 2001).

Direct effects of tDOC on aquatic consumers such as *Daphnia* can be positive or negative. On the one hand, tDOC reduces detrimental effects of UV radiation by attenuating solar radiation in the water column (Williamson et al. 1994; Wolf and Heuschele 2018). On the other hand, reactive oxygen species (ROS) that develop from tDOC exposed to UV radiation can cause severe cellular damage, most notably lipid peroxidation and DNA damage, prompting increased antioxidant enzyme activities (Souza et al. 2007; Wolf et al. 2017). Moreover, *Daphnia* and other aquatic consumers can take up tDOC when ingesting food, and once assimilated, tDOC can readily alter biochemical and physiological processes in organisms (Steinberg et al. 2003, 2006). Responses of zooplankton reported in the literature include DNA methylation and the induction of stress proteins (Steinberg et al. 2006; Menzel et al. 2011). tDOC can also induce internal oxidative stress related to an imbalance between the production of ROS and the activities of antioxidant enzymes (Steinberg et al. 2006; Menzel et al. 2011; Saebelfeld et al. 2017). Besides these demonstrated effects at the molecular and biochemical level, life-history traits such as life span and reproductive rate can increase or decrease depending on the investigated species (Bouchnak and Steinberg 2010; Steinberg et al. 2010*a*,*b*; Robidoux et al. 2015).

Indirect effects of browning by tDOC can arise from changes in resource supply in terms of both food quantity and quality. In particular, browning reduces primary production by attenuating solar radiation (Carpenter et al. 1998; Karlsson et al. 2009, 2015), thus reducing phytoplankton growth and biomass. However, reduced water transparency also decreases the light:nutrient supply ratio and hence algal carbon:phosphorus (C:P) ratios, thereby increasing the food quality of algal biomass for zooplankton (Sterner et al. 1997; Diehl et al. 2005; Striebel et al. 2008). Furthermore, terrestrial organic carbon can serve itself as a resource for zooplankton (e.g., Pace et al. 2004; Cole et al. 2011; Kelly et al. 2014) when available as particles (Cole et al. 2006) or when tDOC is taken up by bacteria and transferred through the microbial loop (Salonen and Hammar 1986; Cole et al. 2006; Tang et al. 2019). Thus, tDOC can support zooplankton populations even when phytoplankton is limiting (McMeans et al. 2015; Taipale et al. 2016), providing a subsidy for zooplankton unless the negative effect of reduced light availability on primary production overrules the positive effect caused by the allochthonous resource supply (Kelly et al. 2014; Cooke et al. 2015; Tanentzap et al. 2017). Results on tDOC effects obtained in previous studies are partially inconsistent, suggesting that allochthonous resource use can indeed increase if autochthonous resources decline, but that increasing allochthony (i.e., allochthonous carbon use in food webs) does not necessarily increase overall zooplankton production (Kelly et al. 2014; Karlsson et al. 2015; Tanentzap et al. 2017).

Clearly, the net effects on *Daphnia* exposed to tDOC depend on the balance of multiple positive and negative influences. Consequently, experiments taking both direct and indirect effects into account are required for meaningful predictions of tDOC or browning effects on zooplankton.

Here, we aimed at assessing the net outcomes of browning on the performance of *Daphnia* populations. In laboratory trials with daphnids and water from lake enclosures containing brown or clear water, we determined whether the metabolic performance and reproductive output are reduced when daphnids are (1) kept in brown water or are (2) transferred from clear to brown water in comparison to (3) daphnids kept continuously in clear water. We expected a negative effect of browning on *Daphnia* abundance, metabolism and life history, when using a humic substance that stains water without providing labile carbon or nutrients that often accompany tDOC inputs to lakes. We also investigated whether (4) daphnids are able to recover when being transferred back from brown to clear water. Finally, to elucidate mechanisms underlying the observed responses, we measured life-history traits and indicators of metabolic performance in the different browning scenarios and related them to various seston characteristics.

Material and methods

Experimental setup

The study consisted of two parts (Fig. 1). First, *Daphnia* populations were exposed to either brown or clear water for 10 weeks in two large field enclosures (Fig. 1a). Then daphnids collected from both enclosures were kept on clear or brown water in the laboratory for up to 3 weeks before measuring life-history traits and the elemental and biochemical tissue composition (Fig. 1b). The laboratory incubations involved a reciprocal transplant approach to investigate how fast indicators of metabolic performance and life-history traits change when daphnids are transferred from brown to clear water and vice versa (Fig. 1b).

Water and plankton were collected in two enclosures of a large experimental facility (www.lake-lab.de) located in Lake Stechlin (northeastern Germany, 53°8′35″N, 13°1′41″E). Lake Stechlin is

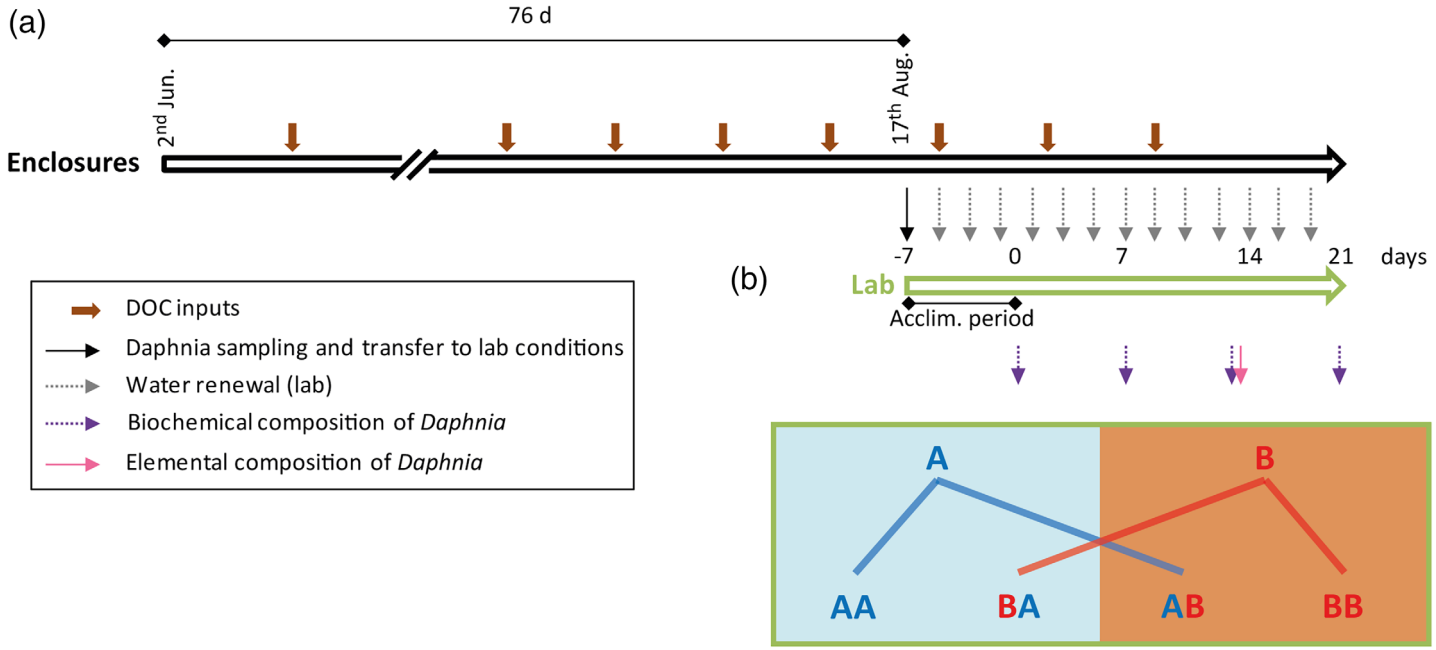

Fig. 1. Experimental design. (**a**) Time line of the enclosure experiment with tDOC inputs received as HuminFeed (HF) and (**b**) sampling scheme of *D. longispina* collected from a large enclosure receiving HF (B) or a control enclosure with clear water (A) and subsequently incubated in the laboratory for 3 weeks in water from either the original enclosure (AA and BB) or the other enclosure (AB and BA).

a deep clear-water lake with a mean depth of 22.8 m, a maximum depth of 69.5 m, and an average Secchi depth of 7.8 m measured between 21 July 2015 and 08 August 2015. The enclosures were 9 m in diameter and ca. 20 m deep, thus enclosing water volumes of ~ 1250 m^3 each. One of them served as a clear-water control. The other received HuminFeed® (henceforth HF; HuminTech GmbH, Grevenbroich, Germany) as a humic substance that simulates lake browning. HF exerts effects primarily by decreasing water transparency, because the carbon is very recalcitrant and the C:N and C:P ratios are very high. HF is a naturally occurring leonardite available as industrially processed, water-soluble granulated sodium salt of humic acids that contains 43% organic carbon, 82% humic substances, 18% low-molecular weight compounds, and no polysaccharides (Meinelt et al. 2007). It has been previously used in aquatic mesocosm experiments to simulate browning resulting from tDOC inputs (e.g., Urrutia-Cordero et al. 2016; Wilken et al. 2018) and in laboratory experiments to assess physiological responses of aquatic organisms (e.g., Meinelt et al. 2007; Steinberg et al. 2010*a*,*b*; Saebelfeld et al. 2017).

As the enclosures are permanent, the water of both enclosures was completely renewed on 02 June 2015, before the start of the experiment, by simultaneously pumping water from defined depths (epi- and hypolimnion) in and out of the enclosures in exchange with the surrounding lake water. The HF was added 1 week later (10 mg L^{-1}) and then again, from week 8 onward, at weekly intervals (4.5 mg L^{-1} to keep the water color at a constant level, Fig. 1a). A 500-μm plankton net was used to collect individual *Daphnia* after 10 weeks, on 17 August 2015. The daphnids were immediately brought to the laboratory on the lake shore, where gravid females were identified with the naked eye. Wide-mouth pipettes were used to transfer them individually to glass jars containing water from the respective enclosure after passing the water through a 90-μm mesh. The daphnids were kept under dim light at ~ 17°C (i.e., within the temperature range experienced in situ) and transferred every other day to freshly collected enclosure water passed through a 90-μm mesh. At their respective harvesting time for chemical analyses (see below), all *Daphnia* were identified using a microscope and found to belong invariably to the *Daphnia longispina* complex.

Laboratory incubations were started on 24 August 2016 after 1 week of acclimation (day t0 in Fig. 1a). Daphnids were kept either individually in glass jars containing 75 mL of water for 2 weeks or in pairs in 150 mL of water for up to 3 weeks. The subset of daphnids kept individually in 75 mL was used to measure molting and elemental tissue composition. Since the biomass of two individuals was needed for nucleic acid and protein analyses, a second subset with pairs of daphnids was kept in larger jars containing 150 mL. Surface water of the enclosures (20 cm depth) was sampled every second day and passed through a 90-μm mesh before transferring the daphnids to newly collected water. Part of the daphnids from the clear water enclosure was randomly selected for continued exposure to the ambient clear-water (A) conditions in the laboratory (treatment AA), whereas the other half from the A-enclosure was transferred to brown

(B) water (treatment AB) (Fig. 1b). Likewise, the daphnids originating from the enclosure with brown water were split to keep half of them in brown water (treatment BB) and transferring the other half to clear water from enclosure A (treatment BA). The level of replication was 8 for the 75-mL jars and 20–24 for the 150-mL jars.

In addition, we established treatments in which individual daphnids were kept in jars with 75 mL of particle-free (GF-75 glass fiber filter, 47 mm diameter, 0.3 μm average pore size; Advantec, Tokyo, Japan) enclosure water for 3 weeks and fed with *Cryptomonas* sp. at saturating concentrations ($3.5 \pm 1.0 \times 10^4$ cells mL^{-1}, ~ 1.8 ± 0.5 mg C L^{-1}). These additional treatments served as positive controls where daphnids were offered high-quality food (*Cryptomonas* sp.) in sufficient amounts to allow for maximum rates of growth and reproduction (e.g., Hiltunen et al. 2017, Supporting Information Fig. S1, Table S1). *Cryptomonas* sp. was obtained from an aerated semicontinuous culture grown in modified WC (Wright Cryptophyte) medium with vitamins (Guillard 1975) under low-light conditions to ensure high nutrient concentrations in the algae. The same treatments as above were used, designated AA*, AB*, BA*, and BB* ($n = 4$), with the asterisk indicating that the daphnids were fed *Cryptomonas* sp. instead of seston from the enclosures. The number of released offspring and dead individuals was counted at each transfer (i.e., every other day) to determine survival rate, individual clutch size, and the number of offspring per female. Individual clutch size was averaged per week and the number of offspring per female was summed up to obtain the cumulative number of offspring per week.

Chemical analyses

The water color of filtered (GF-75 filters) enclosure water was determined every week by reading absorbance on a spectrophotometer at 436 nm using a 1 cm cuvette (Nanocolor 500 D, Macherey-Nagel GmbH & Co. KG, Düren, Germany), and converting absorbance values to platinum (Pt) units according to calibration curves performed at NIVA and IGB (color 436 × 2402 = color in mg Pt L^{-1}). DOC was measured on a Shimadzu TOC 5000 (Tokyo, Japan) total carbon analyzer.

Elemental analyses of seston were performed on particles collected by first passing 1 L of enclosure water over a 90-μm mesh and then filtering the water through GF-75 filters. The filters were dried for at least 1 d at 55°C before subsamples were taken to determine particulate nitrogen and phosphorus by sequential alkaline and acid peroxodisulfate digestion (Ebina et al. 1983) and subsequent flow-injection analysis (FOSS FIAstar™, Höganäs, Sweden). Additional subsamples of the filters were used to determine total particulate carbon with a carbon analyzer (ELTRA SC 800, ELTRA GmbH, Haan, Germany). C:N, C:P, and N:P ratios were expressed in molar units. Daphnids were sampled at the beginning of the experiment (day t0) and after 14 d (individuals of the 75 mL jars) to determine body C, N, and P contents. Individual daphnids were transferred to preweighed aluminum microdishes, dried for at least 48 h at 60°C, and weighed to the nearest 1 μg. Single or several pooled daphnids, depending on size of the individuals, were used to analyze body N and P as described above for seston. To calculate C:N and C:P ratios of the daphnids, C content was assumed to be 44% of dry mass (Hessen 1990).

After 1 week of acclimation in the laboratory (day t0), five 150-mL jars containing two individual daphnids each were randomly selected to determine the initial nucleic acid and protein contents of *Daphnia* exposed to clear and brown water, respectively. Nucleic acid and protein contents were used as indicators to assess the metabolic performance of invertebrates as biological endpoints of physiological and nutritional state. The RNA content is a proxy for protein synthesis capacity, whereas DNA content informs on the body size and cell number of individuals (Furuhagen et al. 2014). *Daphnia* were transferred to nuclease-free Eppendorf tubes (five replicates per treatment), taking care to minimize the volume of water introduced into the tubes. The daphnids were immediately frozen in liquid nitrogen and stored at −80°C. A new set of five 150-mL jars per treatment was randomly selected every 7 d and processed in the same way.

Nucleic acids were analyzed following the methods of Vrede et al. (2002) and Gorokhova and Kyle (2002) with minor modifications. Microplate fluorometric high-range assays were used with the Quant-iT™ RiboGreen® RNA and Quant-iT™ PicoGreen® dsDNA kits (Molecular Probes, Eugene, OR, U.S.A.; cat. #R11490 and #P7589). RNA and DNA were extracted from individual *Daphnia* in 1X TE buffer containing Triton X-100 (0.1% final concentration). A volume of 200 μL buffer was added to each Eppendorf tube containing one or two individuals. Samples were homogenized by bead-beating using stainless-steel milling balls (4 mm diameter, nuclease-free) for 2 min at 20 Hz in a Mixer Mill (Retsch® MM 400, Haan, Germany). Another 300 μL of TE buffer without Triton X-100 was added to dilute and minimize the effect of Triton on signal intensity of the fluorescent dyes RiboGreen and PicoGreen. The homogenate was centrifuged at $10{,}000 \times g$ for 10 min at 4°C and the supernatant was collected for analyses. Since RiboGreen stains both RNA and DNA, a DNase digestion step was included in subsamples used to estimate RNA, as described in the assay kit protocol. RNase-free DNase (Promega GmbH, Mannheim, Germany, cat. #M6101) was used for this purpose. RNA concentrations were calculated based on a RiboGreen standard curve established with known amounts of 16S and 23S ribosomal RNA from *Escherichia coli* (component C of the RiboGreen kit). DNA contents were measured with PicoGreen, using lambda DNA as standard (component C of the PicoGreen kit). Total proteins were measured following the Bradford (1976) assay with bovine serum albumin as standard (Sigma-Aldrich GmbH, Munich, Germany, cat. #A2153). All measurements were performed using a Synergy 2™ multimode microplate reader (BioTek® Instruments, Winooski, VT, U.S.A.) in fluorescence or absorbance mode, depending on the specific assay. All samples, standards, and blanks were analyzed in duplicate, and averages were used in statistical analyses.

Seston size distribution

To estimate the abundance and mass of seston, 100 mL of enclosure water passed through a 90-μm mesh was preserved in

Lugol's solution. Three analytical replicates of the preserved samples were analyzed in a FlowCam VS (Fluid Imaging Technologies, Scarborough, ME, U.S.A.) equipped with a 10× objective and a 100-μm flow cell for particles ranging from 3 to 90 μm. The FlowCam was run in auto-image mode. All image collages were postanalyzed to separate seston particles into different size fractions: 3–20 μm (well-edible particles), 20–90 μm (particles that may interfere with the feeding apparatus of daphnids), and 3–90 μm (all particles). Abundances and particle sizes of these seston components were converted to carbon units of biomass by using the equations provided by Menden-Deuer and Lessard (2000). Additionally, cryptomonad abundance was determined based on the acquired and postanalyzed (i.e., sorted FlowCam) images and served as an indicator of high food quality.

Statistical analyses

Two-way ANOVAs were used to investigate the effects of treatment (i.e., different browning scenarios) and week (i.e., exposure duration) on the biochemical composition of *D. longispina*. Linear mixed effects (LME) models were applied to reproduction responses using the "lme4" package in R with treatment, week, and jar volume as fixed effects and jar ID as random effect to account for potential dependencies of measurements taken from the same jar in consecutive weeks. Tukey HSD post hoc tests were used to assess differences between treatments within each week. All explanatory variables were used as factors in the models to enable assessments of interactions (e.g., treatment by week). Survival curves were analyzed using the "survival" package (Therneau 2012) in R. Survival data of individuals kept in 75 and 150 mL jars were combined as there was no effect of jar size on survival (Supporting Information Fig. S2). Likelihood ratio tests within Cox regressions were applied to the survival data to test for differences among treatments; log-rank tests were used to test for significant differences between survival curves of two different treatments (Bewick et al. 2004). All analyses were run in the software R (version 3.2.0).

Results

Properties of enclosure water

The water of the enclosure without HF addition (A) was not noticeably colored (2.4 ± 2.0 Pt L^{-1}, mean $\pm$ 1 SD), but the weekly addition of 4.5 mg HF L^{-1} increased water color to up to 43.2 ± 4.4 Pt L^{-1} during the time of laboratory incubations. Despite this significant difference in water color (*t*-test, $t = 17.0$, $p < 0.001$), DOC concentrations in the enclosure receiving HF (7.1 ± 1.9 mg L^{-1}) were slightly, but not significantly higher than in the control enclosure (5.6 ± 0.8 mg L^{-1}, *t*-test, $t = 1.41$, $p = 0.21$), indicating that HF stains water strongly while having relatively minor effects on DOC concentration.

The carbon concentration of seston in enclosure B receiving HF averaged 520 μg L^{-1} (range 410–640 μg L^{-1}) over the course of the experiment, and was thus almost twice as high as in enclosure A without HF addition (average of 280 μg L^{-1}, range 220–350 μg L^{-1}; Table 1, Supporting Information Fig. S4). This indicates that food quantity was higher in enclosure B. FlowCam particle counts also showed that seston abundance and biomass in enclosures was significantly affected by the addition of HF, similar to the data on carbon concentrations (see above). The average abundance of small and thus well edible seston particles (3–20 μm) was 10,364 particles mL^{-1} in enclosure B receiving HF and 5887 particles mL^{-1} in the control enclosure A (Table 1, Supporting Information Fig. S5).

Table 1. Seston characteristics of enclosure water (mean $\pm$ 1 SD) without (A) and with (B) HF addition. Carbon (C), phosphorus (P), and nitrogen (N) concentrations and their ratios showed some fluctuations but no notable trends over time (Supporting Information Fig. S4). FlowCam data on particle abundance (no. mL^{-1}) and biomass (μg C L^{-1}) in different size fractions and cryptomonads are averages over 3 weeks. Abundance and biomass data showed similar variability over time (Supporting Information Figs. S5, S6). $N = 9$ or 10 for elemental data and $N = 5$ for FlowCam data. *p* values refer to *t*-tests: † for $p < 0.1$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

	Enclosure		
Seston characteristic	**Control (A)**	**With humic substances (B)**	***p* value**
C (μg L^{-1})	279.4 ± 41.1	519.3 ± 78.9	< 0.001***
N (μg L^{-1})	28.7 ± 2.1	41.0 ± 4.9	< 0.001***
P (μg L^{-1})	5.1 ± 0.7	5.8 ± 1.0	0.075†
C:N (mol mol^{-1})	11.4 ± 1.4	14.9 ± 2.4	< 0.001***
C:P (mol mol^{-1})	143.3 ± 30.4	233.9 ± 44.5	< 0.001***
N:P (mol mol^{-1})	12.5 ± 1.5	15.8 ± 2.4	0.002**
Abundance (3–20 μm)	5887 ± 3459	10,364 ± 2789	0.054†
Abundance (20–90 μm)	43 ± 17	186 ± 84	0.006**
Abundance of cryptomonads	67 ± 47	224 ± 404	0.414
Biomass (3–20 μm)	168.7 ± 108.5	327.4 ± 25.8	0.013*
Biomass (20–90 μm)	55.2 ± 18.2	277.2 ± 141.0	0.008**
Biomass (3–90 μm)	223.9 ± 120.7	604.6 ± 129.6	0.001**
Biomass of cryptomonads	3.9 ± 1.7	9.7 ± 14.8	0.411

Larger particles (20–90 μm) were even more than four times more abundant in water from enclosure B (186 particles mL^{-1} compared to 43 particles mL^{-1} in the control enclosure A; Table 1, Supporting Information Fig. S5). Thus, the mass of small and large seston was higher in enclosure water receiving HF (Table 1, Supporting Information Fig. S6).

The average abundance and biomass of cryptomonad algae, which were used as an indicator of high food quality, also tended to be higher in brown water, although this difference was not statistically significant (Table 1, Supporting Information Figs. S5, S6). In contrast, the average C:P and C:N ratios were higher in seston from the enclosure receiving HF compared to the seston from the clear-water enclosure (Table 1, Supporting Information Fig. S4), suggesting that food quality in terms of macroelement supply was lower in the enclosure with brown water.

Chemical composition of *Daphnia*

The N and P contents of *Daphnia* did not differ between treatments after 14 d (Table 2). Mean N concentrations ranged from 6.4% to 7.4% of dry mass, corresponding to mean molar C:N ratios of 7.1–8.3 (Table 2). Mean P concentrations of *Daphnia* tissue ranged from 1.4% to 1.7% of dry mass, corresponding to mean molar C:P ratios of 70–85 (Table 2).

At the beginning of the laboratory trial (t0), *Daphnia* originating from enclosure B receiving HF had 3–4 times higher nucleic acid and protein contents than those from the control enclosure A (Supporting Information Fig. S7). For DNA and RNA contents, a strong treatment effect was observed throughout the experiment (Table 3), which was mainly explained by higher DNA and RNA contents of daphnids kept in brown water (AB and BB) compared to daphnids kept in clear water (AA and BA) (Fig. 2a,b). Protein contents in weeks 2 and 3 showed similar patterns as DNA and RNA contents, but differed after the first week in that daphnids from enclosure B and kept in clear water (BA) had higher protein contents than those from enclosure A and kept in clear water (AA) (Fig. 2c; Table 3). In contrast, daphnids exposed to brown water in the laboratory (BB and AB) had intermediate protein contents (Fig. 2c).

Daphnia survival and reproduction

The survival probability of daphnids differed among treatments (Fig. 3a; likelihood ratio test in Cox regression = 32.3, df = 3, $p < 0.001$; 58 death events out of 212). It was highest when daphnids were kept in brown water in the laboratory, regardless of their origin (treatments AB and BB; Fig. 3a; log rank test: $\chi^2 = 0.2$, $p = 0.67$), and lowest in clear water when daphnids originated from the enclosure receiving HF (BA). The survival probability of daphnids from the control enclosure kept in clear water (AA) was intermediate (Fig. 3a), significantly lower or tending to be lower than the survival probability in brown water (log rank test: $\chi^2 = 4.4$, $p = 0.037$ and $\chi^2 = 3.0$, $p = 0.085$ for the BB and AB treatment, respectively), but significantly higher than that of daphnids from

Table 2. Phosphorus (P) and nitrogen (N) concentrations and ratios of nutrients to carbon (C) in *D. longispina* at the start of the experiment (t0) and after 14 d. *Daphnia* originating from the control enclosure (A) were kept in water of this enclosure (AA) or in water of the enclosure receiving HF (AB). Similarly, *Daphnia* originating from the enclosure receiving HF (B) were kept in the same water (BB) or in water of the control enclosure (BA). All values are means ± 1 SD. There were no significant differences between individuals at the start (ANOVA, $F_{1,2} < 2.3$, $p > 0.25$) nor among treatments (ANOVA, $F_{3,10} < 0.8$, $p > 0.55$).

Treatment	*n*	N (% dry mass)	P (% dry mass)	C:N (mol mol^{-1})	C:P (mol mol^{-1})	N:P (mol mol^{-1})
A (t0)	2	6.4 ± 0.8	1.5 ± 0.2	8.0 ± 1.1	77.6 ± 10.7	9.6 ± 0.1
B (t0)	2	7.4 ± 0.4	1.7 ± 0.04	6.9 ± 0.4	67.8 ± 1.5	9.8 ± 0.8
AA (14 d)	3	6.4 ± 0.2	1.4 ± 0.2	8.1 ± 0.2	82.8 ± 11.9	10.3 ± 1.2
AB (14 d)	4	6.5 ± 0.8	1.5 ± 0.2	7.9 ± 1.0	76.0 ± 7.7	9.6 ± 0.6
BA (14 d)	4	6.9 ± 1.2	1.5 ± 0.2	7.6 ± 1.4	77.8 ± 10.9	10.4 ± 1.6
BB (14 d)	3	7.2 ± 0.7	1.6 ± 0.1	7.2 ± 0.7	73.4 ± 3.2	10.3 ± 1.4

Table 3. Results of two-way ANOVAs to test for effects of treatments and week on RNA, DNA, and protein contents. * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

		DNA				RNA				Protein			
Source of variation	df	Sum Sq.	Var. (%)	*F*		Sum Sq.	Var. (%)	*F*		Sum Sq.	Var. (%)	*F*	
Treatment	3	1948.8	44.2	22.2	**	26,062	40.0	13.9	***	160.5	34.0	12.2	***
Week	2	310.0	7.0	5.3	***	4598	7.1	3.7	*	0.4	0.1	0.04	
Treatment × week	6	748.6	17.0	4.3	**	4387	6.7	1.2		100.6	21.3	3.8	**
Residuals	48	1405.9	31.9			30,056	46.7			210.5	44.6		

the enclosure receiving HF and kept in clear water (log rank test: $\chi^2 = 9.6$, $p = 0.002$ for the BA treatment).

Mortality in the laboratory trial was highest during the first 2 weeks when daphnids from the brown enclosure were kept in clear water (BA) and in the last week for daphnids that never experienced brown water (AA) (Fig. 3b). Daphnids kept in brown water (BB and AB) showed invariably low mortality (< 20%), whereas mortality of individuals kept in clear water (AA and BA) rose up to 50–60% after 3 weeks (Fig. 3a,b).

The average individual clutch size and the weekly cumulative number of offspring per female were similarly affected by

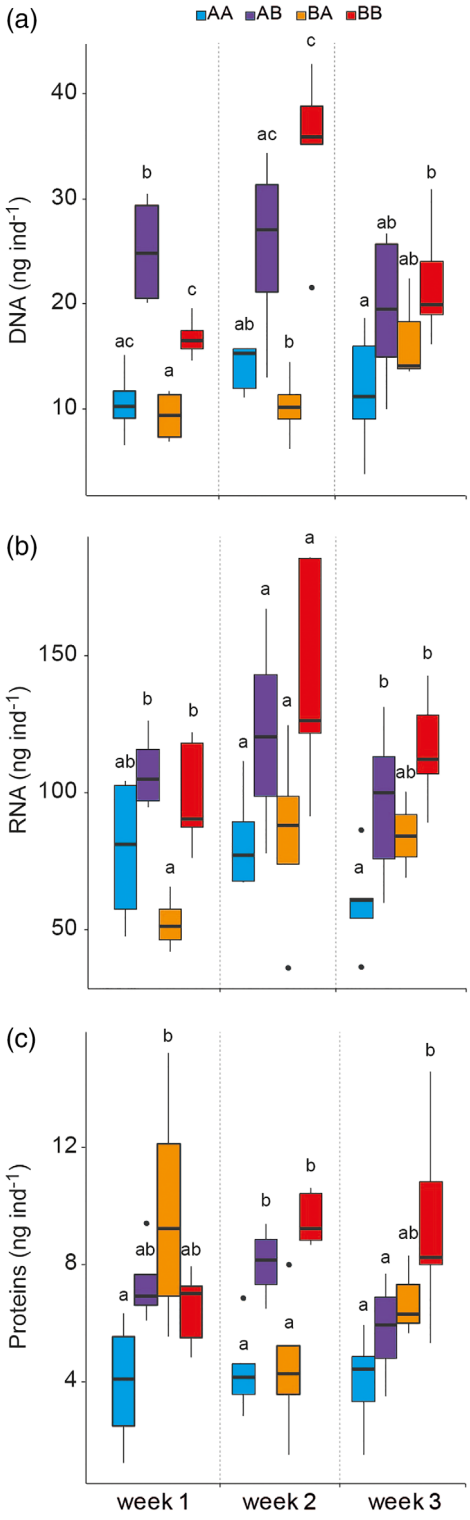

Fig. 2. (**a**) DNA, (**b**) RNA, and (**c**) protein contents of *D. longispina* after 1–3 weeks in laboratory conditions. *Daphnia* originating from the control enclosure (A) were kept either in water from this enclosure (AA) or in brown water from the enclosure receiving HF (AB); *Daphnia* originating from the enclosure receiving HF (B) were kept in water from the same (BB) or the control (BA) enclosure. Box plots show median values, quartiles (hinges), whiskers (1.5 × interquartile range), and outliers (dots). Different letters indicate significant differences ($p < 0.05$) between treatments within weeks according to Tukey's HSD post hoc test.

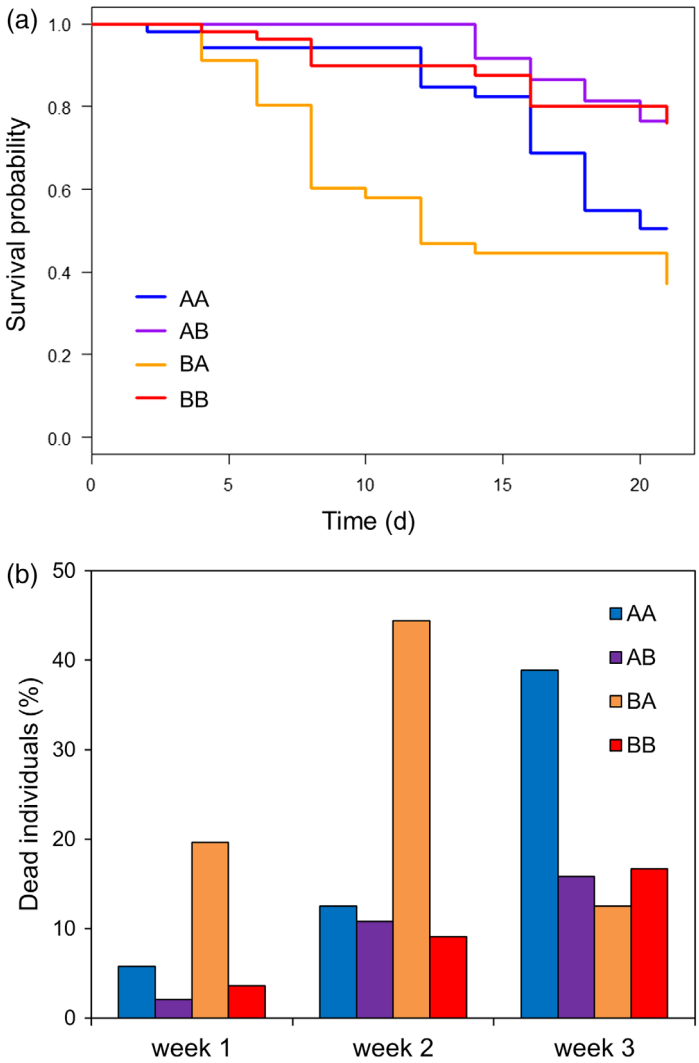

Fig. 3. (**a**) Survival probability and (**b**) mortality per week for *D. longispina*. Treatment codes AA, AB, BA, and BB as in Fig. 2.

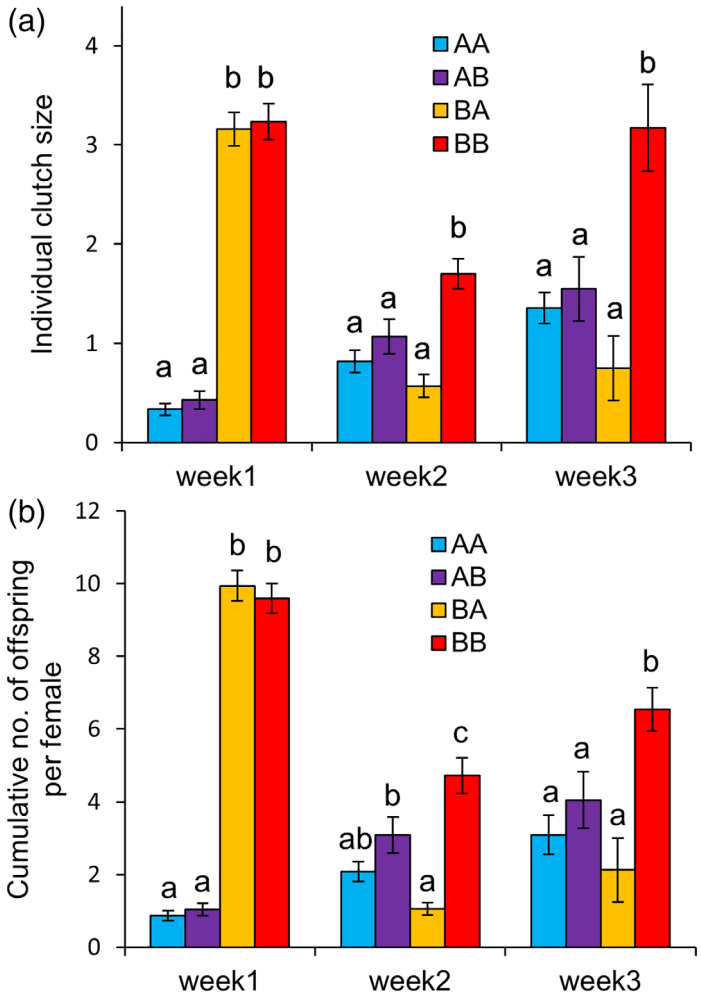

Fig. 4. (**a**) Individual clutch size and (**b**) cumulative number of offspring per female of *D. longispina* (means ± 1 SE). Treatment codes AA, AB, BA, and BB as in Fig. 2. Clutch size per female has been averaged per week. Cumulative offspring number per female refers to the sum of offspring per week. Letters indicate significant differences between treatments within weeks according to Tukey's HSD post hoc test.

treatments through time (Fig. 4; Table 4). Daphnids originating from the enclosure receiving HF (BA and BB) showed a high reproductive output in the first week, which then declined slightly for daphnids that stayed in brown water (BB), but strongly declined for the daphnids transferred to clear water (BA) (Fig. 4). In contrast, daphnids originating from enclosure A (AA and AB) had a low reproductive output during the first week, which then increased slightly in the following 2 weeks (Fig. 4). Molting frequencies of daphnids were similar across treatments during the first 10 d of the laboratory trials (three molts on average; Supporting Information Table S1).

Discussion

Increasing inputs of tDOC in freshwaters can reduce food availability for zooplankton by reducing algal growth due to reduced light penetration caused by water browning (Carpenter et al. 1998; Karlsson et al. 2009). In addition, tDOC can induce direct physiological oxidative stress in zooplankton, thereby impairing their metabolic performance and fitness (reviewed by Steinberg et al. 2006; Saebelfeld et al. 2017). However, tDOC can also serve as a resource for zooplankton by stimulating the microbial food web and by trophic upgrading of tDOC-metabolizing bacteria through heterotrophic microbes (Cole et al. 2006; Cooke et al. 2015; Tang et al. 2019). In the present study, the overall effect of browning on *Daphnia* was negative, as daphnids were less abundant in the brown enclosure where HF addition reduced light availability for phytoplankton growth (760 ± 585 compared to 13,895 ± 8635 daphnids m^{-3} in the control enclosure, mean ± 1 SD from seven sampling days, 28 July 2015 to 08 September 2015, *t*-test assuming unequal variances, $t = 4.0$, $p = 0.007$; Sperfeld et al. unpubl. data). Surprisingly, however, the daphnids collected from the enclosures and kept in brown enclosure water in the laboratory trials showed better physiological states, reproduction, and survival than daphnids kept in clear water. This counterintuitive finding is likely due to improved food quantity and quality for *Daphnia* in the brown-water

Table 4. ANOVA results for fixed effects of LME models fitted to the individual clutch size and cumulative number of offspring per female *D. longispina* with treatment, jar volume (75 or 150 mL), and week as fixed effects, and jar-ID as random effect (not shown). * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. Note that the effects including jar volume (marked in italics), although sometimes statistically significant, were very small compared to the effects of treatment and week (marked in bold).

		Individual clutch size				Cumulative no. of offspring			
	df (num)	df (denom)	Sum Sq.	*F*		df (denom)	Sum Sq.	*F*	
Treatment	**3**	**143.3**	**77.2**	**45.2**	***	**137.4**	**474.0**	**51.2**	***
Volume	*1*	*142.8*	*1.2*	*2.1*		*135.9*	*15.7*	*5.1*	*
Week	**2**	**163.1**	**36.5**	**32.0**	***	**156.8**	**298.2**	**48.3**	***
Treatment × volume	*3*	*142.1*	*0.84*	*0.49*		*135.2*	*14.5*	*1.6*	
Treatment × week	**6**	**159.8**	**99.0**	**29.0**	***	**153.4**	**862.7**	**46.6**	***
Volume × week	*1*	*148.7*	*4.8*	*8.4*	**	*142.6*	*2.0*	*0.64*	
Treatment × volume × week	*3*	*147.9*	*2.6*	*1.5*		*141.8*	*6.0*	*0.64*	

enclosure during our laboratory trial, although a positive physiological response to brown-water conditions could be another explanation. The impacts of browning on life-history traits and the physiological state of *Daphnia* were probably a result of indirect effects mediated by differences in daphnid densities. Thus, our study demonstrates the importance of experiments reflecting ecologically complex settings that capture such indirect effects resulting from species interactions in communities and potential plastic responses of organisms.

During the time of the laboratory trials, all indicators of seston quantity showed that food was much more abundant in brown than in clear water. The particle concentrations in water from the brown enclosure exceeded or were close to the threshold of 500 μg C L^{-1} below which *Daphnia* growth is food-limited (Lampert 1987), whereas daphnids kept in clear water experienced food levels well below that threshold (Supporting Information Figs. S4, S6). Enhanced food availability is hence likely to play a critical role in explaining the higher reproductive output of daphnids originating from the enclosure receiving HF (BA and BB) during the first week in the laboratory, an outcome that is also reflected in a low daphnid mortality in the brown water (AB and BB). The higher food quantity observed in brown water contrasts with the expectation that light-limited algal growth leads to lower phytoplankton abundance (Carpenter et al. 1998; Karlsson et al. 2009). However, this apparent contradiction can be reconciled by acknowledging that daphnids were much less abundant in the enclosure receiving HF compared to the control enclosure (following an initially strong decline in abundance), which alleviated the grazing pressure on phytoplankton in the brown enclosure, resulting in enhanced food supply, and finally supporting increased fecundity and survival for the remaining individual daphnids.

The elemental composition of seston suggests higher food quality in the control enclosure (i.e., lower seston C:P and C:N ratios than in the enclosure receiving HF). This result is also in contrast to expectations, because a low ratio of light to nutrient supply in the brown enclosure should have lowered seston C:N and C:P ratios (Sterner et al. 1997; Striebel et al. 2008). Likewise, high light to nutrient supply ratios in the clear enclosure should have caused high C:nutrient ratios of seston, due to the accumulation of carbon relative to nutrients in algal tissue because of a surplus of light available for photosynthetic carbon fixation (Sterner et al. 1997; Striebel et al. 2008). In addition, low C:nutrient ratios of seston in the clear enclosure could have been due to a high grazing pressure exerted by abundant grazers and the associated stimulation of nutrient recycling (Elser and Urabe 1999). In the brown enclosure, amorphous particles formed as a result of HF flocculation or attachment to particles (personal observation), which likely increased the average C:nutrient ratio of seston, as the organic carbon content of HF is high and nutrient contents are low. Nevertheless, the seston C:P ratio remained below 300 in both enclosures, a value that corresponds to the threshold elemental ratio above which growth of daphnids is limited when food is abundant (Urabe and Watanabe 1992; Lukas et al. 2011); when food quantity is limiting the critical ratio is even higher (Urabe and Watanabe 1992; Anderson and Hessen 2005). Thus, because seston C:P ratios were invariably below (A) or near (B) the threshold of 300 (Supporting Information Fig. S4), differences in food quality through P limitation were unlikely to have a strong negative effect on daphnid survival and reproduction, despite the difference in seston C:P ratios between brown and clear enclosures. These results imply that food quantity played a more important role than the elemental stoichiometry of the food.

A high C:P ratio of consumer body tissue (rather than of seston) is another indicator of P limitation (DeMott and Pape 2005; Lukas et al. 2011). However, we did not observe significant differences in C:P ratios between daphnids feeding on seston from the clear and brown enclosure, which further supports the conclusion that food quality defined by elemental ratios was unlikely to affect *Daphnia* performance. Instead, other aspects of food quality such as the occurrence of nutritious food particles could have played a role. The greater abundance of cryptomonads in the enclosure receiving HF (B) suggests indeed that some high-quality food was available in browning conditions. Cryptomonads are easily ingestible and digestible microalgae that contain large amounts of polyunsaturated fatty acids, which are important for *Daphnia* growth and especially reproduction (Ravet et al. 2003; Sperfeld and Wacker 2012). Cryptomonads are mixotrophs that can ingest bacteria, a mode of resource acquisition that is predicted to increase under reduced light availability (Wilken et al. 2018). Both bacterial abundances and rates of phytoplankton bacterivory have been shown to increase under browning scenarios (Wilken et al. 2018), thus increasing the importance of heterotrophic bacteria and mixotrophic microalgae as food for zooplankton (Cole et al. 2006; Hiltunen et al. 2017; Tang et al. 2019).

Even though food was more abundant and likely of better quality in the brown than in the control enclosure during the laboratory trials, daphnids were still limited by food quantity, quality, or both, since reproduction was lower than that of individuals kept in filtered (particle-free) enclosure water and supplied with high abundance of *Cryptomonas* cells to provide optimal food conditions (Fig. 4 and Supporting Information Fig. S1). This indicates that daphnids feeding on seston from the brown enclosure, still experienced suboptimal conditions, corroborating other studies that have shown tDOC supply to support *Daphnia*, but not to the extent that optimal growth is achieved (McMeans et al. 2015; Hiltunen et al. 2017). Nevertheless, tDOC addition still improved fecundity of daphnids compared to controls where no tDOC was added in our experiment.

Daphnids originating from the brown enclosure (B) in our study, and feeding on seston from that enclosure in the laboratory (BB), had invariably higher DNA, RNA, and protein contents than individuals originating and fed on seston from the control enclosure (AA). The higher metabolic performance and better physiological state of daphnids exposed to browning in the field and laboratory (BB) also corresponds to the measured higher reproductive output. Reproduction of daphnids is synchronized

with molting, and molting hormones stimulate the synthesis of RNA and DNA, resulting in cell multiplication and protein synthesis (Gorokhova and Kyle 2002). Furthermore, the biochemical composition of daphnids is influenced by dietary N and P (Hessen et al. 2007), suggesting that the significantly higher N content of seston from the enclosure receiving HF may have allowed individuals kept in brown water in the laboratory (BB and AB treatments) to build up more nucleic acids and proteins.

A distinct feature of our experimental design was the use of daphnids from a population that experienced browning over multiple generations in field conditions, whereas daphnids used in other studies were typically raised under optimal growth conditions in the laboratory before being used in experiments. Therefore, transgenerational, maternal, and acclimation effects could have influenced the positive responses of *Daphnia* to browning (i.e., increased metabolic performance and fitness) in the present study. This conclusion, however, must be qualified, in light of our intriguing finding that the observed physiological responses can be quickly reversed, as suggested by similarly high nucleic acid and protein contents of daphnids kept in brown water in the laboratory, regardless of their origin (i.e., in both the AB and BB treatments), and similarly low contents in daphnids kept in water from the control enclosure (i.e., in both the BA and AA treatments). Clearly, this pattern points to an overriding importance of current environmental conditions rather than to any kind of legacy effects.

Some of the plastic responses were very quick, since daphnids transferred from the control enclosure to brown water in the laboratory adjusted their nucleic acid and protein contents already within the first week, whereas the positive effects on fecundity and survival (i.e., increased fitness) were delayed. Similarly, daphnids from the brown enclosure that were transferred to clear water from the control enclosure adjusted their nucleic acid contents within the first week. Protein contents and fecundity remained significantly higher during the first week, but both declined by the end of the second week. Daphnids of this treatment (BA) also showed higher mortality during the first 2 weeks, suggesting that food quantity and quality after transfer to clear-water conditions was insufficient to sustain an elevated metabolism. These consistent responses suggest a limited buffer capacity of *Daphnia* to maintain elevated levels of nucleic acids and proteins, as well as high reproduction and survival rates, when the supply of (high-quality) food declined. At the same time, our results provide evidence for a remarkable ability of *Daphnia* to benefit quickly from improved food conditions. These responses are consistent with the dynamic energy budget theory, according to which allocation of resources to maintenance has priority over growth and reproduction when food supply is limiting (Sokolova et al. 2012).

Extrapolations of our results to real-world settings need to take into account that the industrially processed HF we used to simulate browning is a model substrate that does not reflect the exact properties of natural matter sources of tDOC. HF has chemical and physical characteristics that broadly resemble those of natural organic matter leached from watersheds (Meinelt et al. 2007). In particular, HF strongly affects water color while providing little extra nutrients. However, the carbon of HF is more recalcitrant than most natural DOC of terrestrial origin, and tDOC inputs to lakes during rain events, whether as surface run-off or groundwater, are often accompanied by nutrient inputs (Solomon et al. 2015), which may stimulate both primary and bacterial production. Consequently, our experiment could not reflect the full range of factors associated with lake browning in many situations, but would accurately mimic settings particularly where the main effect of increasing tDOC concentrations is water staining and a corresponding diminution of light availability.

Another important aspect to consider is that lake browning within the next decades will be accompanied by other drivers of environmental change, which could lead to synergistic or antagonistic effects. There is evidence, for instance, that even when browning alone has rather small effects on zooplankton, it can show important synergistic effects in combination with warming (Hansson et al. 2012; Nicolle et al. 2012). These and other studies point to the importance of considering multiple factors of environmental change in enclosure studies to predict complex responses of aquatic communities and ecosystems under global change.

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Author Contribution Statement

M.O.G., S.A.B., and J.C.N. designed, and S.A.B. and J.C.N. set up and coordinated the LakeLab experiment. L.M., E.S., J.C.N., and M.O.G. conceived and designed the laboratory study. L.M. and E.S. ran the laboratory trials and analyzed most data. S.A.B. performed phytoplankton and seston size class analyses by FlowCam. L.M. and E.S. wrote the manuscript with contributions by S.A.B., J.C.N., and M.O.G.

Conflict of Interest

None declared.

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