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ORIGINAL ARTICLE

Contrasting diurnal patterns in antioxidant capacities, but not in expression of stress protein genes among copepod populations from clear versus glacially fed alpine and subalpine lakes

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Short-term changes in environmental conditions largely influence planktonic organisms, but their responses will depend on the habitat characteristics. Here we studied diurnal patterns in antioxidative metabolites (lipophilic and hydrophilic antioxidant capacities) and in the expression of stress protein genes (heat shock proteins, *hsp*) of copepods to identify short-term stress responses in clear and turbid alpine lakes, as well as in less transparent subalpine ones. *Cyclops abyssorum tatricus* showed diurnal variation in antioxidant capacities with maxima around noon in clear, but not in glacially fed, turbid lakes. Low fluctuations of these metabolites were also observed in another copepod, *Acanthodiaptomus denticornis*. Although levels of *hsp* genes differed between populations living in clear or glacially fed lakes, there was no diurnal rhythmicity in gene expression. Our data show that when planktonic organisms may be at greatest risk of oxidative damage, such as during the daytime in high UV radiation environments, they activate antioxidant responses. Conversely, in less transparent lakes, the physiological response seems to be unnecessary. The

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difference in gene expression levels suggests an ecological, albeit not acute, role of these genes in copepods experiencing daily environmental fluctuations.

KEYWORDS: diurnal rhythmicity; heat shock proteins; zooplankton; high mountain lakes

INTRODUCTION

Lakes are characterized by environmental heterogeneity with large spatial and temporal variation in physico-chemical parameters and biological processes. In these ecosystems, organisms experience environmental changes on a daily basis, including fluctuating levels of solar radiation and large temperature differences if migrating through the water column. Compared to planktonic organisms from lowland lakes, organisms inhabiting high-elevation lakes face more challenging conditions. In addition to lower temperatures, zooplankton from clear alpine lakes is exposed to high solar radiation levels. UV radiation (UVR) not only increases with elevation (Blumthaler *et al*., 1997), but also typically can penetrate the entire water column still with substantial intensity (Morris *et al*., 1995; Laurion *et al*., 2000). Thus, alpine zooplankton experiences large daily fluctuations in this important ecological driver (Williamson *et al*., 2011). In response, these organisms evolved several adaptations including the avoidance of the upper water layers during the day (Kessler *et al*., 2008), even in systems devoid of predators (Fischer *et al*., 2015; Tartarotti *et al*., 2017), and the accumulation of high concentrations of photoprotective compounds such as mycosporine-like amino acids (MAAs) (Tartarotti *et al*., 2001, 2017; Persaud *et al*., 2007).

Zooplankton from glacier-fed lakes or less transparent subalpine lakes are more protected from solar UVR (Laurion *et al*., 2000; Rose *et al*., 2014; Tartarotti *et al*., 2017). They typically migrate into deeper water layers to avoid predation pressure (Lampert, 1989) and have low photoprotection levels (Tartarotti *et al*., 2001, 2017; Persaud *et al*., 2007). Higher carotenoid contents have also been reported in copepods from alpine than from subalpine lakes (Byron, 1982), and lower concentrations were observed in copepods from glacier-fed turbid lakes than from clear lakes in the Himalayas (Sommaruga, 2010). Ringelberg and Hallegraeff (1976) found diurnal variations in the carotenoid content of copepods from a mountain lake, which they related to changes in feeding activity and speculated on a protective function of these pigments. In fact, some carotenoids act against photooxidative stress and are part of the antioxidant system present in the organism (Krinsky, 1979). Generally, little is known about other antioxidant defenses in copepods. Differences in antioxidant enzyme activities (catalase; CAT) were observed between copepods and cladocerans (Souza *et al*., 2007) and rapid enzymatic responses were found in freshwater, as well as in marine copepods after UVR exposure (Hylander *et al*., 2012; Souza *et al*., 2012; Won *et al*., 2014; Kim *et al*., 2015). While photoprotective compounds such as MAAs are retained in copepods over longer time periods (days to weeks; Moeller *et al*., 2005), antioxidant enzyme activities such as glutathione *S*-transferase (GST) and superoxide dismutase (SOD) change within hours (Souza *et al*., 2012; Won *et al*., 2014). In zooplankton, studies reporting short-term changes in antioxidant activities under field conditions are scarce. Differences in antioxidant enzyme activities with significant diurnal variation in alpine, but not in arctic *Daphnia*, as observed by Borgeraas and Hessen (2002), suggest species- and population-specific adaptive responses to oxidative stress.

At a molecular level, diurnal up- or downregulation of gene expression may be essential for organisms living under constantly changing environmental conditions. In *Daphnia pulex*, diel rhythmicity in the expression of genes, including oxidative detoxification and sensory process genes, is known (Rund *et al*., 2016), and recently, strong diel cycling in clock genes was found in the marine copepod *Calanus finmarchicus* (Häfker *et al*., 2018). Transcriptional oscillations of heat shock protein (*hsp*) genes during the tidal cycle in the mussel *Mytilus californianus* (Gracey *et al*., 2008) and diurnal variability of HSP70 (protein level) in the chiton *Acanthopleura granulate* (Schill *et al*., 2002) emphasize the importance of stress proteins in aquatic organisms. HSPs are a central component of the cellular stress response; they act as molecular chaperones and play a critical role in the recovery of cells from stress by maintaining the integrity of the cellular protein pool (Feder and Hofmann, 1999; Sørensen *et al*., 2003). The expression of *hsp* genes may follow the daily fluctuations that planktonic organisms are subjected in their natural environment.

We hypothesized that the antioxidant capacities and *hsp* gene expression differ in copepods that inhabit either clear, highly UV-exposed or glacier-fed turbid alpine lakes, as well as less transparent subalpine lakes, and predict diurnal variation of both physiological and molecular measures at times of high irradiance (i.e. ice-free summer period) in the copepods from clear lakes. While the focus of most studies examining antioxidant activities in zooplankton has been on particular enzymes

Table I: Main characteristics of the study lakes including geographic location, elevation, lake area, maximum lake depth (z_{max}), mean specific (25°C) electrical conductivity (cond), mean pH and water optical properties (mean dissolved organic carbon content (DOC), vertical attenuation coefficient (k_d) at 320 and 380 nm and depth of 1% of surface irradiance for 320 nm ($Z_{1\%320}$) and 380 nm ($Z_{1\%380}$) UV)

Lake	GKS	FAS4	FAS3	PIB
Latitude/longitude	47°13′N, 11°00′E	47°04′N, 10°13′E	47°04′N, 10°13′E	47°11′N, 10°50′E
Elevation (m a.s.l.)	2 417	2 416	2 414	913
Lake area (km^2)	0.017	0.019	0.021	0.17
Z_{max} (m)	9.9	15.0	17.0	24.6
Cond (μS cm^{-1})	20.0	66.4	44.4	73.0
pH	7.1	7.2	7.3	7.2
DOC (mg L^{-1})	0.35	0.30	0.17	2.13
K_{d320} (m^{-1})	0.24	0.23	2.20	3.49
K_{d380} (m^{-1})	0.11	0.16	1.30	1.20
$Z_{1\%320}$ (m)	19.51	20.19	2.09	1.32
$Z_{1\%380}$ (m)	41.3	29.06	3.54	3.83

(Borgeraas and Hessen, 2002; Hylander *et al.*, 2012; Souza *et al.*, 2007, 2012; Won *et al.*, 2014), here, we report the overall antioxidant capacity providing insight into the *in vivo* balance between reactive oxygen species (ROS) and antioxidant compounds (Ghiselli *et al.*, 2000). Lipophilic antioxidants such as vitamin E and β-carotene act as antioxidants in their lipid soluble form within lipid environments (Limón-Pacheco and Gonsebatt, 2009) and may counter the detrimental effects of ROS (Kelman *et al.*, 2009), whereas water-soluble molecules (e.g. glutathione, ascorbic acid) are potent radical-scavenging agents in the aqueous phase of the cytoplasm (Niki, 1991). In addition, we assessed the expression patterns of *hsp60*, *hsp70* and *hsp90* genes, which are responsive to, among others, thermal, oxidative, chemical and UVR stress (Feder and Hofmann, 1999; Sørensen *et al.*, 2003; Richter *et al.*, 2010).

METHOD

Zooplankton sampling and sample processing

We collected the cyclopoid copepod *Cyclops abyssorum tatricus* Kozminski from the clear alpine lakes Gossenköllesee (GKS) and Faselfadsee 4 (FAS4), as well as from the turbid glacier-fed lake Faselfadsee 3 (FAS3). The calanoid copepod *Acanthodiaptomus denticornis* Wierzejski was collected from the subalpine Lake Piburger See (PIB) (Table I). Samples were taken by vertical net (50 μm mesh size) tows made at the center of the lakes. As UVR reaches the bottom in Lakes GKS and FAS4 and as the copepods are distributed close to it during the day (Table I; Tartarotti *et al.*, 1999, 2017), net tows were taken from ~1 m above the sediment to the surface. In Lakes FAS3 and PIB, tows were made from 10 m and 4 m depth to the surface, respectively. These sampling depths reflect the water column where light is still available and sufficient copepods can be caught in Lake FAS3 (Tartarotti *et al.*, 2017) and where 1% UV-A radiation at 380 nm is still present in Lake PIB (Table I). Copepod samples were taken every ~6 hours over 24–30 hours (at 13:00 = midday, 19:00, 24:00 = midnight, 06:00 and 13:00 = midday of the following day for all lakes; and 19:00 of the following day for Lakes GKS and PIB). The lakes were visited one (FAS3, 3–4 August 2015; FAS4, 12–13 August 2015; helicopter flights were necessary because of the remoteness of the sites) or two times (GKS, 19–20 July and 21–22 August 2010; PIB, 09–10 August and 25–26 August 2010) during the ice-free summer period. For all analyses, we selected copepodid CIII–CIV life stages, because these stages were present at all sites in sufficient numbers. Immediately after sampling, copepods (60 copepods per sample, 3–5 biological replicates) were flash frozen in liquid nitrogen for antioxidant capacity measurements. For copepod gene expression samples (Lakes FAS3 and FAS4; 60 copepods per sample, 5 biological replicates), the time from collection to flash freezing was less than 30 min (sorting <10 min per sample). Samples were stored at −80°C.

Antioxidant capacities

We measured antioxidant capacities as described previously (Tartarotti *et al.*, 2014). Briefly, the copepods were cleaved in a Speedmill (Analytik Jena) followed by centrifugation (11 600 *g*, 3 min) using a sodium hydrogen phosphate buffer (0.1 M, pH 6.5). The cooled supernatant was directly used to determine the antioxidant capacity of water-soluble antioxidants or was further processed for the extraction of lipid-soluble antioxidants following Bligh and Dyer (1959). We analyzed antioxidant capacities based on Popov and Lewin (1999) in

a PhotoChem device (Analytik Jena) via photo-chemiluminescence. Copepod protein content was measured according to Bradford (1976), and antioxidant capacities were expressed as nM trolox or ascorbic acid equivalents [mg protein]$^{-1}$ for lipophilic and hydrophilic antioxidants, respectively.

Extraction of RNA and cDNA synthesis

RNA extraction and cDNA synthesis were done as described in detail previously (Tartarotti *et al*., 2018). We used TRIzol reagent (Invitrogen) to extract total copepod RNA following the manufacturer's instructions. We homogenized the samples and added gDNA eliminator solution (Qiagen) to eliminate potential contamination with genomic DNA. The RNA extract was further purified (RNeasy Mini Kit, Qiagen; according the manufacturer's protocol). Total RNA was measured (NanoDrop ND1000, Thermo Fisher Scientific) to assess RNA quality (mean A260/280 ratio: 2.10 ± 0.04) and run on a 1.2% agarose gel to assess RNA integrity. We quantified the RNA concentration in triplicate with a plate reader (2030 Multilabel Plate Reader Victor X4, Perkin Elmer) and the Quant-iT RiboGreen Assay Kit (Life Technologies). Copepod RNA (450 ng) and random hexamer primers (#S0142, Thermo Fisher Scientific) were used for first-strand cDNA synthesis. cDNA was synthesized using MMLV H minus reverse transcriptase (#EP0452, Thermo Fisher Scientific) following the manufacturer's protocol with slight modifications by using less reverse transcriptase (100 U in 50 μL reaction volume). To test for potential genomic DNA contamination, we included "no reverse transcriptase" (NRT) controls. Before quantitative polymerase chain reaction (qPCR) analysis, cDNA was frozen at −20°C.

qPCR

We used oligonucleotide primers (hsp60 forward [f]: GGCTGGAGACGGTACCACAA, reverse [r]: ACCTGCCTTGGCAATTGC; efficiency [e]: 92%; hsp70 f: CAACCAGAAGCAGGGAAAGAAG, r: CCACCCCCGAGGTCAAA; e: 96%; and hsp90 f: AACATCAAGCTTGGTATCCATGAA, r: GAGGAGCCCGGCTAACTTCT; e: 96%) and qPCR conditions as described previously (Tartarotti *et al*., 2018). Briefly, qPCR reactions (triplicate technical repeats) were run in a QuantStudio3 real-time PCR detection system (Thermo Fisher Scientific), using a mixture comprising 1× PowerSybr Green PCR Master Mix (Thermo Fisher Scientific), forward and reverse qPCR-specific primer (500 nM each), non-acetylated bovine serum albumin (Sigma-Aldrich) and cDNA. NRT controls were included. The qPCR conditions were 50°C/2 min; 40 cycles of 95°C/15 s, 60°C/1 min. Data acquisition and analysis were done with the QuantStudio^{TM} Design and Analysis Software v1.4.1 (Thermo Fisher Scientific). Serial dilutions of gene-specific quantified *C. abyssorum tatricus* cDNA were made for the determination of real-time PCR efficiency. We calculated absolute copy numbers (absolute quantification method) by plotting the CT values versus the log10 of the initial copy numbers, quantified with the Quant-iT Picogreen dsDNA Assay Kit (Life Technologies) and the specific molecular weight of each amplicon. The copy numbers were normalized to 10 ng of total copepod RNA. After amplification, PCR products were subjected to melt-curve analysis.

Temperature, incident solar radiation and UV attenuation measurements

During sampling, water temperature was recorded and global solar radiation was measured automatically at 15-min intervals at Lakes GKS and PIB (star-pyranometer Type 8 102, Schenk). Due to potential variability in radiation on a daily basis, measurements were integrated over the whole wavelength band (300–3 000 nm). Global irradiance was obtained from the nearest weather station (linear distance 6.7 km; Station Galzig) located at 2079 m a.s.l. to reflect the solar radiation present at Lakes FAS3 and FAS4. We are aware that weather conditions can be localized in mountainous areas; however, we noted the cloud cover (or lack of) during the sampling days, which reflected well the meteorological data. For colored dissolved organic matter (CDOM) absorption measurements, lake water (~0.5 m depth) was filled in pre-combusted glass bottles, filtered through Whatman GF/F filters (pre-combusted) and absorbance was measured by spectrophotometry (Hitachi U-2000) using quartz glass cuvettes. The absorbance value at 690 nm was used to correct UV absorbance for the presence of particulate matter. Absorption coefficients were calculated as CDOM absorption: $CDOM = 2.303\ D/r$ where D is the corrected absorbance and r is the path length in meters. The K_d was estimated by the CDOM absorption measurements as described in Laurion *et al*. (2000). At Lakes FAS3 and FAS4, underwater irradiance-depth profiles were taken with a PUV-501B profiler radiometer. The K_d in the water column was determined from the slope of the linear regression of the natural logarithm of downwelling irradiance versus depth.

Data treatment

Analysis of variance (ANOVA) with all pairwise multiple comparison procedures (Holm–Sidak method), or

one-way ANOVA on ranks (Kruskal–Wallis test) when equal variance failed, was used to test for differences in antioxidant capacities and *hsp* gene expression across the sampling time points. We used Kruskal–Wallis one-way ANOVA on ranks to assess differences in antioxidant capacities among lakes and copepod populations. Differences in *hsp* gene expression between Lakes FAS3 and FAS4 were analyzed with *t*-tests. We ran all statistical analyses using the software Sigma Stat (Version 3.5). Antioxidant data and gene expression data are reported as mean + standard error (SE) and + standard deviation (SD), respectively.

RESULTS

In July and August, the period of high solar irradiance (Supplementary Fig. S1a and b) and higher temperatures, the lipophilic antioxidant capacities of *C. abyssorum tatricus* from the clear Lake GKS showed a statistically significant diurnal pattern, with highest levels found at midday (Fig. 1a and c; ANOVA July: $F_{5,17} = 4.293$, $P = 0.018$; August: $F_{5,17} = 11.365$, $P < 0.001$). In July, we observed the lowest antioxidant capacities at midnight and again in the evening hours of the second sampling day (Fig. 1a), while in August lowest levels occurred in the early morning and evening hours (Fig. 1c). Significant diurnal variability of the hydrophilic antioxidant capacities was also found in July, with highest levels observed at midday (Fig. 1b; ANOVA $F_{5,17} = 3.995$, $P = 0.023$). However, there was a lower and statistically non-significant variation in these metabolites in August (Fig. 1d; ANOVA on ranks, $H = 1.826$, df = 5, $P = 0.873$). For the lipophilic antioxidant capacities in the *C. abyssorum tatricus* population of Lake FAS4, we observed a similar diurnal pattern to that of the copepods from GKS, with highest levels found around noon (Fig. 1e; ANOVA $F_{4,13} = 3.676$, $P = 0.049$). In contrast to GKS, in this population, we observed the highest hydrophilic antioxidant capacities during the night (Fig. 1f). In the *C. abyssorum tatricus* population from the glacier-fed lake, Lake FAS3, the variability in the antioxidant capacities was not significant over the 24 h period, but there was a tendency to observe higher levels around midnight (Fig. 1g and h; ANOVA lipophilic antioxidant capacities, $F_{4,13} = 0.506$, $P = 0.733$; hydrophilic antioxidant capacities, $F_{4,14} = 1.340$, $P = 0.321$).

Similarly, in *A. denticornis* from the subalpine Lake PIB, there was some variability in the antioxidant capacities, but no diurnal pattern was observed (Fig. 2; ANOVA 09–10 August and 25–26 August, lipophilic antioxidant capacities, $F_{5,19} = 1.652$, $P = 0.211$ and $F_{5,18} = 1.963$, $P = 0.152$; hydrophilic antioxidant capacities, $F_{5,23} = 0.659$, $P = 0.659$ and $F_{5,22} = 0.918$, $P = 0.493$).

In Lakes GKS and PIB, higher, although not statistically significant, copepod antioxidant capacities were found during midsummer compared with late summer (Figs 1a–d and 2; ANOVA on ranks, Dunn's method, $P > 0.05$). *Cyclops abyssorum tatricus* populations from Lakes FAS3 and FAS4 had low lipophilic antioxidant capacities (Fig. 1e and g). The levels of these metabolites were significantly lower than the ones from the GKS population, when compared with both midsummer (July) and late summer (August) data (ANOVA on ranks; Dunn's method, $P < 0.05$). However, hydrophilic antioxidant capacities from FAS3 and FAS4 populations were similar (compared with July data; $P > 0.05$) or even higher (August data; $P < 0.05$) than the ones from the Lake GKS population (Fig. 1).

There was no diurnal pattern in the expression of *hsp* genes over the 24 h period, regardless of the origin of the copepod population (clear versus turbid lake) (Fig. 3). The *hsp60*, *hsp70* and *hsp90* gene expression showed low variation in FAS3 (Fig. 3b, d and f; *hsp60* ANOVA on ranks, $H = 7.528$, df = 4, $P = 0.110$; *hsp70* ANOVA, $F_{4,22} = 1.098$, $P = 0.388$; *hsp90* ANOVA, $F_{4,24} = 1.078$, $P = 0.394$), while there was some variability in the *hsp60* (ANOVA, $F_{4,24} = 5.374$, $P = 0.004$) and *hsp90* (ANOVA, $F_{4,24} = 3.854$, $P = 0.018$), but not *hsp70* (ANOVA, $F_{4,23} = 0.312$, $P = 0.866$) gene expression in FAS4 (Fig. 3a, c and e). When comparing the two sites, we found significantly higher levels of *hsp60* gene expression in the *C. abyssorum tatricus* population from turbid Lake FAS3 than clear FAS4 (*t*-test; df = 47, $t = 9.38$, $P < 0.001$). The *hsp70* gene expression was similarly low in both populations (*t*-test; df = 45, $t = -1.67$, $P = 0.103$), while the *hsp90* gene expression was significantly higher in the population from the clear than from the turbid lake (*t*-test; df = 48, $t = -12.14$, $P < 0.001$) (Fig. 3).

DISCUSSION

Planktonic organisms exposed to large daily environmental changes respond by either avoiding or minimizing stressors at the times they are most likely to experience stressful conditions. We observed diurnal rhythmicity in antioxidant metabolites of the copepod populations from clear alpine lakes, with peaks of lipophilic antioxidant capacities around noon (Fig. 1a, c and e). Hydrophilic antioxidant capacities, however, showed a more diverse response ranging from daytime maxima to minima (Fig. 1b, d and f). In a previous study on antioxidant enzyme activities, Borgeraas and Hessen (2002) observed maxima in CAT and SOD activities around noon in a

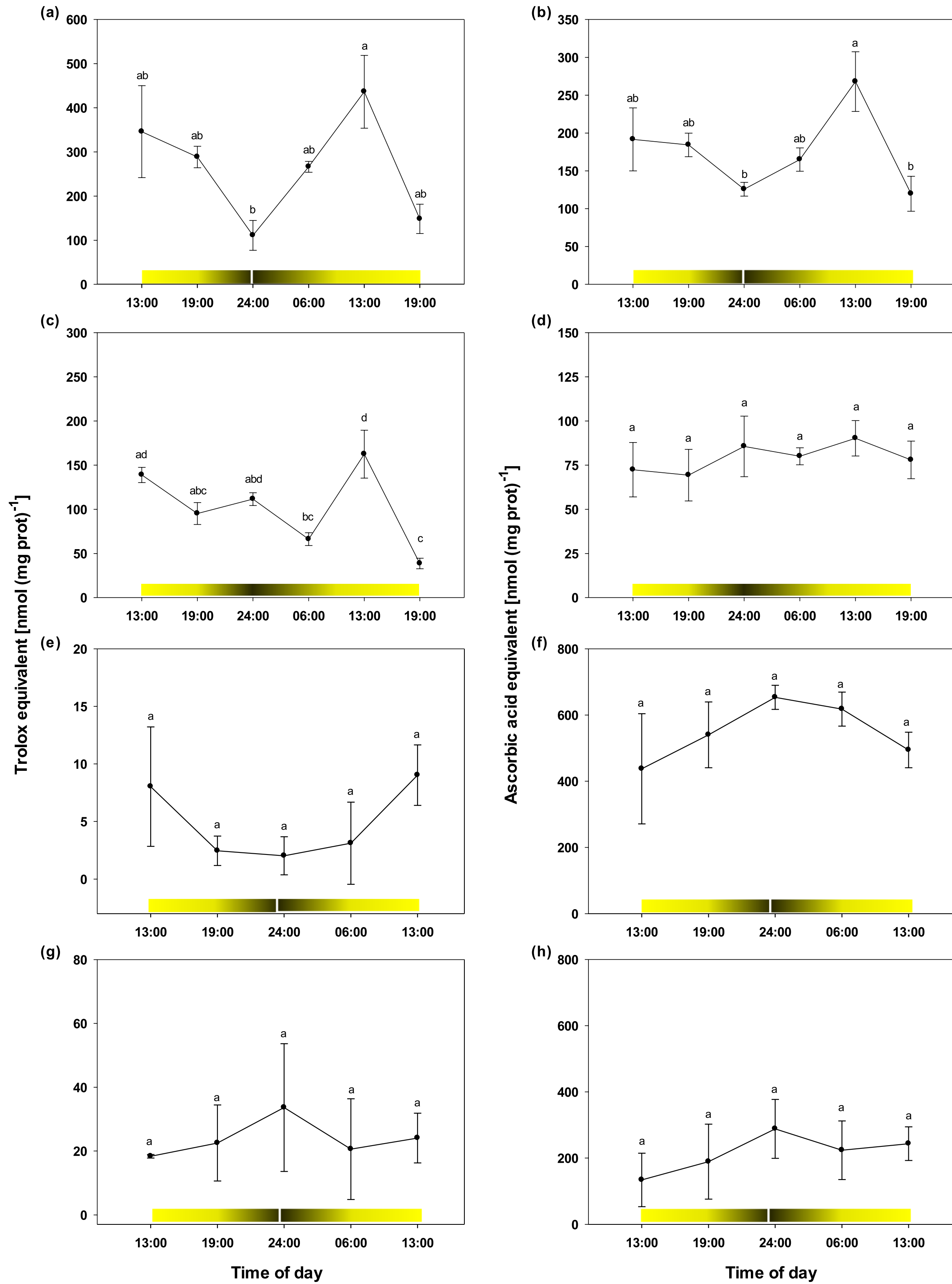

Fig. 1. Diurnal changes in lipophilic (left) and hydrophilic (right) antioxidant capacities of *C. abyssorum tatricus* ($n = 3$–4) from the clear alpine Lake GKS in July (**a** and **b**) and August (**c** and **d**), clear alpine Lake FAS4 (**e** and **f**) and glacially turbid Lake FAS3 (**g** and **h**). Data represent means + SE. Different letters above the data points indicate significant differences between sampling times after one-way ANOVA. Please note the difference in y-axis scale. Abbreviations for the lakes are defined in Table I.

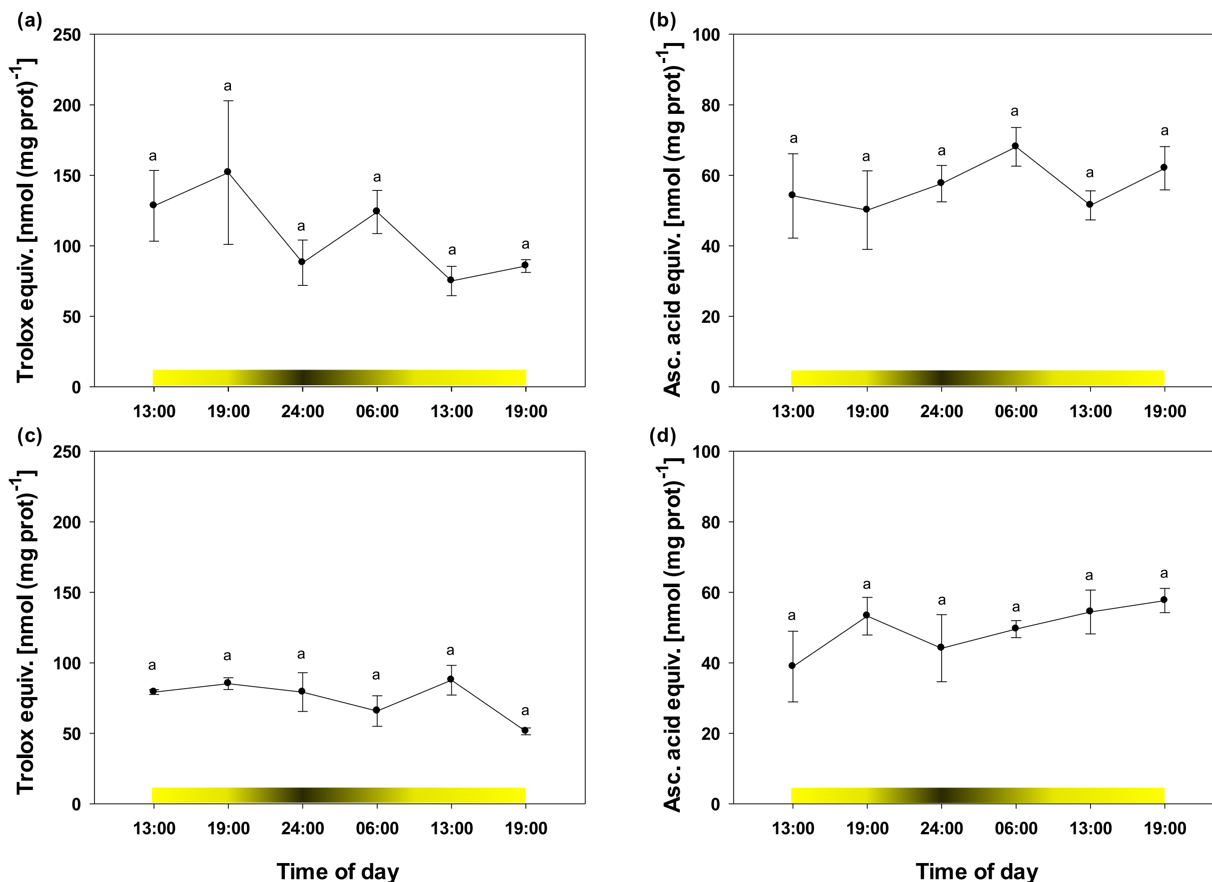

Fig. 2. Diurnal changes in lipophilic (left) and hydrophilic (right) antioxidant capacities of *A. denticornis* ($n = 3–5$) from the subalpine Lake PIB on 09–10 August (**a** and **b**) and 25–26 August (**c** and **d**). Data represent means + SE. Different letters above the data points indicate significant differences between sampling times after one-way ANOVA.

hyaline morph of alpine *Daphnia longispina*, while in a pigmented population of the same species, significant diurnal variation was found only in GST activity, with a minimum at midday. In addition, the same authors reported only small and insignificant diurnal fluctuations in antioxidant enzymes in another *Daphnia* species, the arctic melanic *D. tenebrosa*. Such low variations were also observed in the antioxidant activities of copepods from subalpine and less transparent lakes (Figs 1g and h and 2). These findings suggest strong species- and even population-specific variability in antioxidant activities, depending also on the environment in which an organism lives (e.g. clear versus turbid) and on its respective photoprotection level (e.g. unpigmented versus pigmented).

Several factors may influence and induce diurnal changes in antioxidant activities. In the Antarctic midge larvae *Belgica antarctica*, the total soluble antioxidant capacity did not vary following exposure to a variety of environmental stressors, including extreme temperatures (Lopez-Martinez *et al.*, 2008). However, in the same organisms, mRNA expression patterns of CAT and SOD change in response to incident sunlight. In our study systems, there was low variation (~1°C) in water temperature at a certain depth between day and night. The difference in temperature within the water column was 5–7°C (July and August) in GKS and 5–6°C in lakes FAS3 and FAS4. In the lake with the largest temperature differences, PIB (16°C in mid-August and 18°C at the end of August), *A. denticornis* antioxidant activities did not vary significantly (Fig. 2), although this species is known to vertically migrate (Taleb *et al.*, 1992) and to be present from the deepest strata to surface waters in Lake PIB (unpublished data). Moreover, there was a similar temperature range between clear and turbid alpine lakes; thus, temperature is unlikely to have caused diurnal variation in the antioxidant capacities of *C. abyssorum tatricus* from the clear lakes.

Solar irradiance levels follow a diel cycle and one of the main differences among our study lakes were the higher fluxes of solar irradiance at the alpine sites (Supplementary Fig. S1) and their higher UVR trans-

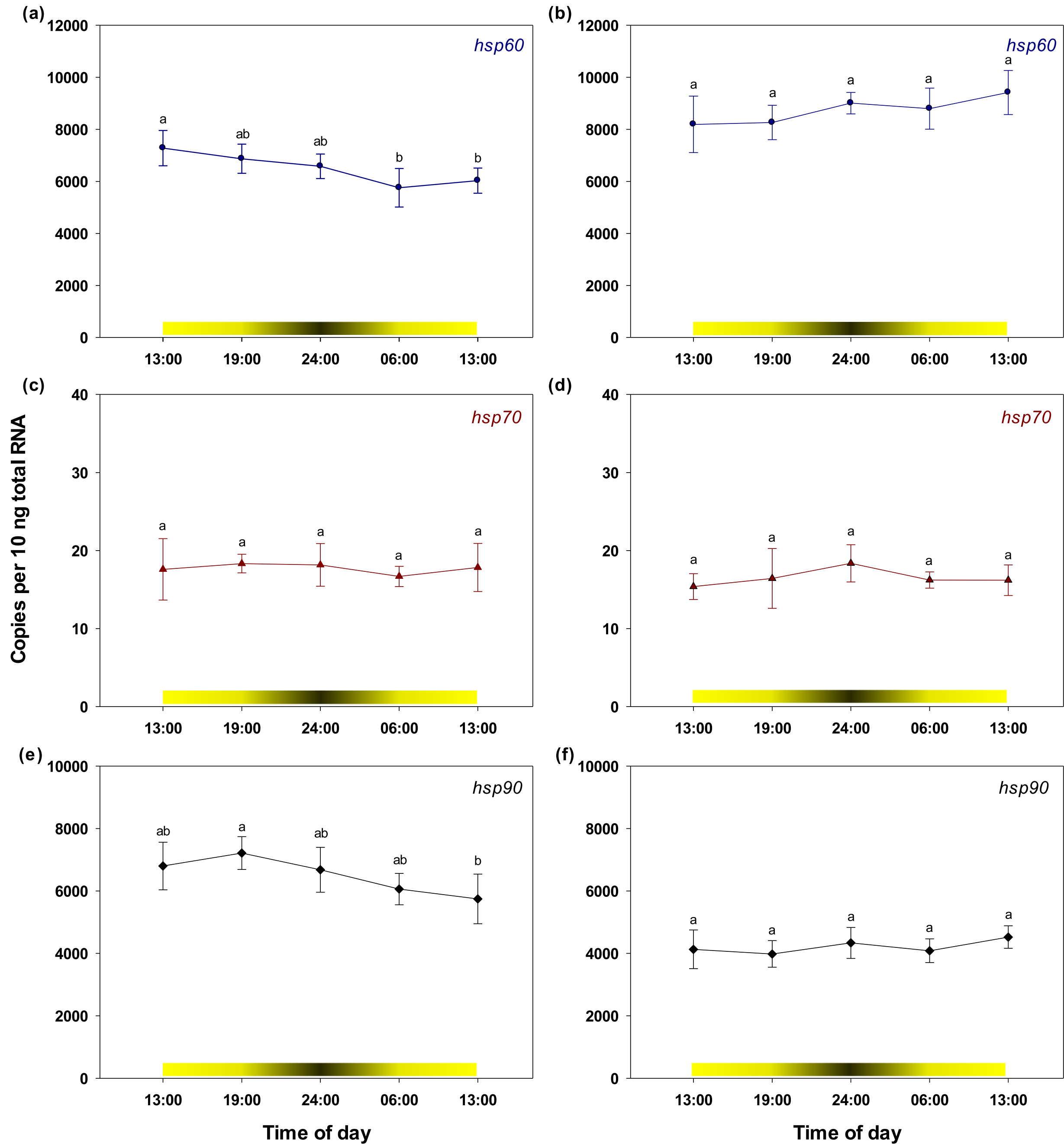

Fig. 3. Expression of heat shock protein genes (*hsp*) in *C. abyssorum tatricus* ($n = 5$) from the clear alpine Lake FAS4 (left) and glacially turbid Lake FAS3 (right) over the 24 h study period. Shown are mean expressions + SD. Different letters above the data points indicate significant differences between sampling times after one-way ANOVA. Abbreviations for the lakes are defined in Table I.

parency when compared with the glacially turbid or lowland ecosystems (Table I). Our data on the antioxidant capacities in the copepod population from Lake GKS also show how sensitive aquatic organisms respond to changes in daily irradiance. Both in July and August, the antioxidant capacities were lower at midday on the first than on the second sampling day (Fig. 1a-d). This difference probably reflects the partly overcast weather in the morning on the first and the cloudless morning with higher daily integrated solar radiation on the second sampling day (20.5 MJ m^{-2} day^{-1} on the first versus 28.1 MJ m^{-2} day^{-1} on the second sampling day in July,

18.4 versus 24.1 MJ m^{-2} day^{-1} in August; Supplementary Fig. S1a,b). The formation of ROS in sunlit surface waters will cause the need of high intracellular antioxidant levels, and this need will be higher in clear than in less transparent lakes and at times of high UVR exposure (i.e. noon), especially under sunny weather conditions.

Apart from UVR, also hypoxia can induce oxidative stress in animals inhabiting aquatic environments (Glippa *et al*., 2018). Some crustaceans such as mysid shrimps protect themselves from oxidative damage by "preparation for oxidative stress" (POS) (Hermes-Lima *et al*., 1998), resulting in tolerance to large variations in dissolved oxygen concentrations (Webster *et al*., 2015). While copepods may experience a gradient from oversaturation to oxygen-depletion in Lake PIB, the water column of alpine lakes is well oxygenated during summer, thus, this abiotic factor is unlikely to play a role in the diurnal rhythmicity of antioxidant capacities in *C. abyssorum tatricus* populations from clear high-elevation lakes. However, the concept of POS may hold for copepods from clear alpine lakes in response to high radiation levels.

While measuring lipophilic and hydrophilic antioxidant capacities has the advantage of covering all types of antioxidants present in an organism, it remains unknown how discrete antioxidants respond to stressful conditions. The antioxidant defense system of an organism is complex, and while certain antioxidants may increase and others decrease under stress conditions, our data show clear diurnal patterns in the overall antioxidant capacity (Fig. 1). However, as discussed by Costantini and Verhulst (2009), it is not clear whether an increase in antioxidative capacity of an organism really indicates a response to elevated oxidative stress or displays an accumulation of unused antioxidants due to missing stress. A detailed analysis of the whole organisms' oxidative stress status, enzyme activities and non-enzymatic metabolites would be necessary to accurately interpret the measured capacities.

Although the copepod populations from Lakes GKS and FAS4 have one of the highest levels of photoprotection (MAAs) reported for freshwater zooplankton (Tartarotti *et al*., 2001, 2017; Tartarotti and Sommaruga, 2006), they also activate alternative defense systems and increase their antioxidant capacities at the time of day when solar UVR exposure risk is highest. In contrast, our data on *A. denticornis* and *C. abyssorum tatricus* populations from turbid lakes, which have only low photoprotection (Tartarotti *et al*., 2001, 2017), suggest that these copepods do not display higher antioxidants levels during the day, probably because UVR is rapidly attenuated in these lakes (Table I). Interestingly, diurnal variations in carotenoids, as part of the antioxidant response, with highest levels during daytime are found in *A. denticornis* from a clear mountain lake (Ringelberg and Hallegraeff, 1976). Our data set for *A. denticornis* is relatively small (one sampling location and only antioxidative metabolites); however, the sampling of *A. denticornis* was replicated, showing—similarly to the *Cyclops* population from the turbid alpine lake—low fluctuations in antioxidant capacities during both sampling campaigns. Thus, these data support the idea that such physiological responses are not in play for copepods from less transparent habitats. Nevertheless, further studies are needed to determine whether the overall antioxidant capacity shows similar diurnal fluctuation in *A. denticornis* populations from more UV-transparent waters than our study system, Lake PIB.

The findings of the present study make also aware of how important the time of day in zooplankton collection is, and that the handling time, which was shown to be a potential stressor in marine copepods (Nilsson *et al*., 2018), should be kept as short as possible when measuring parameters such as antioxidant capacities. Moreover, our data indicate longer-term temporal variability in antioxidant capacities, with decreasing levels during the course of the summer (Figs 1a–d and 2). Seasonal changes in antioxidant capacities have been shown for alpine (Tartarotti *et al*., 2018) but not for subalpine copepods before. Compared with previous studies (Tartarotti *et al*., 2014, 2017), the lipophilic antioxidant capacities of the Lake FAS3 and FAS4 populations were low (Fig. 1e and g), while the levels of these metabolites in the GKS population (Fig. 1a and c) are high, even higher as recently reported (Tartarotti *et al*., 2018). However, hydrophilic antioxidant capacities (Fig. 1b, d, f and h) were in the same range as those found in former studies (Tartarotti *et al*., 2017, 2018).

Although diel rhythmicity in gene expression can be observed in crustaceans (copepods, Häfker *et al*., 2018; cladocerans, Rund *et al*., 2016) and diel patterns of HSPs have been shown in marine invertebrates (Schill *et al*., 2002; Gracey *et al*., 2008), we found no diurnal variation in the expression of *hsp* genes in *C. abyssorum tatricus* (Fig. 3). In a previous study, we observed *hsp70* in its constitutive and inducible form, whereas *hsp60* and *hsp90* were only constitutively expressed in this copepod species after stress exposure (UVR) (Tartarotti *et al*., 2018). This suggests that the energetically costly production of increasing gene expression (i.e. costs of transcription and translation; Somero, 2002) such as of *hsp70*, occurs only under acute stress. The data of the present study indicate that the copepods do not rely on upregulation of stress proteins on a daily basis. However, the gene expression levels differed between copepod populations. The higher levels of the mitochondrial *hsp60* gene in the copepods from the turbid lake (Fig. 3a and b) might

reflect an increased energy demand of these organisms to cope with stress caused by high particle loads. Glacial mineral particles are known to negatively affect zooplankters such as *Daphnia* (Koenings *et al*., 1990), who respond by up- and downregulation of specific cellular antioxidant enzymes (Laspoumaderes *et al*., 2017). Copepods are able to feed selectively (DeMott, 1986), thus potentially avoiding some of the problems that filter-feeders face in particle-rich environments. However, there are examples of species-specific effects of suspended sediments on vital rates (e.g. ingestion rate) and of higher energy demand as caused by changes in swimming behavior in the presence of non-ingestible particles in marine copepods (Hansen *et al*., 1991; Arendt *et al*., 2011).

In addition to small *hsps* such as *hsp20*, *hsp70* and *hsp90* seem to be part of the cellular protection against stressors like UVR in copepods (Kim *et al*., 2015). While the *hsp70* gene expression was generally low and showed similar levels between the Lake FAS3 and FAS4 populations (Fig. 3c and d), we found pronounced *hsp90* gene expression with higher expression in the copepods from the high UV environment (Fig. 3e and f). *Hsp90* is abundantly produced by cells under normal physiological conditions (Sørensen *et al*., 2003). Lejeusne *et al*. (2006) speculate on a possible involvement of HSP90 in the response of crustaceans to acute stress (e.g. heat shock) rather than chronic exposures; however, *C. abyssorum tatricus* did not upregulate the corresponding gene when exposed to acute UVR stress (Tartarotti *et al*., 2018). Nevertheless, pronounced *hsp90* gene expression may be typical for copepods from clear alpine lakes, as similarly high levels were observed in the *C. abyssorum tatricus* population from GKS during summer (Tartarotti *et al*., 2018). Interestingly, *hsp90* gene expression was even higher during the period with ice-cover (Tartarotti *et al*., 2018), suggesting that this gene plays a role in seasonal acclimatization (e.g. seasonal change in water temperature). It remains to be studied whether other stress-related genes such as small *hsps*, DNA damage response and repair genes or free radical detoxification genes (like CAT and SOD) and genes involved in the glutathione pathway are more responsive in copepods when exposed to a daily cycle of environmental changes.

Mikulski *et al*. (2017) showed that rapid temperature changes, such as those experienced by zooplankton when migrating through thermally stratified lakes, can modify hsp levels (HSP70) in *Daphnia magna*. However, our data on copepods do not suggest a strong temperature influence on *hsp* gene expression because only low fluctuations of *hsp* gene expression were observed (Fig. 3). This indicates that either the temperature gradient was not steep enough or that because *C. abyssorum tatricus* is a small (~1 mm) copepod species, it does not experience abrupt temperature changes by migrating relatively slowly through the water column.

CONCLUSION

We highlight that in UV-exposed aquatic ecosystems such as clear alpine lakes, copepods not only rely on high contents of photoprotective compounds (Tartarotti *et al*., 2001, 2017) and efficient DNA repair mechanisms (Tartarotti *et al*., 2014), but that they also activate antioxidant responses during the daytime, when they may be at greatest risk of oxidative damage. In contrast, copepods from more turbid environments (i.e. higher concentrations of CDOM or inorganic particles) show little variation in antioxidant capacities on a daily basis, suggesting that such (photo) protective responses are not essential for these organisms. The expression of *hsp* genes, a potentially energetically costly stress response, does not follow a diurnal cycle; however, expression levels differ between copepod populations from clear or glacially turbid lakes, indicating differences in the ecological importance of these genes.

SUPPLEMENTARY DATA

Supplementary data can be found at *Journal of Plankton Research* online.

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DATA ARCHIVING

We intend to archive our data at the Dryad Digital Repository.

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