CLADOCERA

Behavioural and physiological mechanisms behind extreme longevity in *Daphnia*

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Abstract The combined effect of external environment and energy allocation strategy of the organism on longevity can be exceptional. In a cold oligotrophic fishless habitat, individual *Daphnia* can live for over a year, several times the usual *Daphnia* lifespan. This extreme lifespan is in part a consequence of the overwintering strategy which includes storing resources and delaying reproduction until another spring. Yet, contrasting strategies may be applied by *Daphnia*, resulting in over twofold differences in lifespan within a single habitat. We identify physiological mechanisms mediating such differences in longevity in closely related *Daphnia* of two lineages coexisting within a high altitude lake, testing the predictions that long-lived animals stay in colder waters and have lower metabolic rates, irrespective of temperature. Vertical distribution of the animals was assessed during three summer stratification seasons, and metabolic activity was measured as oxygen consumption and RNA:DNA ratio. The results not only support our predictions but also reveal that habitat choice is dependent on reproductive status rather than genotype. The young individuals of the overwintering lineage may delay reproduction in part by staying in colder waters than the reproducing adults, which together with low intrinsic metabolic rates may underlie the longevity of *Daphnia* of this lineage.

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Introduction

In lakes and ponds of the temperate zone, life cycle of most planktonic cladoceran species is short and in the order of weeks (Threlkeld, 1987). This may be a result of selection by temporarily changing abiotic conditions: drying or overfreezing in shallow habitats, or oxygen deficits in deep lakes of high trophic level. This is also in part a consequence of fish predation pressure acting as a strong selective force under which probability of surviving to subsequent reproductive period is low, and thus *Daphnia* follow the *r* strategy, maximizing fitness by investing in early reproduction (Threlkeld, 1979; Boersma et al., 1998; Pijanowska et al., 2006; Dawidowicz et al., 2010). In deep

oligotrophic habitats with no oxygen deficits, where fish predation pressure is reduced or absent, released from the selective pressure on early reproduction, *Daphnia* can maximize fitness by applying different strategies, including those involving the delay of the onset of reproduction and lengthening the lifespan (Dawidowicz et al., 2012). This is possible at the concurrent permanence and stability of the habitat, which favour the evolution of longevity (Pianka, 1970; Grime, 1977).

Indeed, exceptionally long lifespan combined with a biennial life cycle has been found in *Daphnia* from high altitude or latitude fishless lakes (Gliwicz et al., 2001; Larsson & Wathne, 2006; Slusarczyk, 2009). Where it was found, this strategy of overwintering in the water column and delaying reproduction until the second breeding season coexisted with a contrasting strategy: that of winter diapause in the form of resting eggs, produced parthenogenetically. The situation in deep oligotrophic fishless Czarny Staw pod Rysami in the High Tatra Mountains is an example. The lake is inhabited by cladocerans of two distinct lineages belonging to one species: European *Daphnia pulicaria* clade within tenebrosa group of *Daphnia pulex* species complex (Dufresne et al., 2011; R. Vergilino, personal communication). At least one of the lineages reproduces exclusively by obligate parthenogenesis (Dufresne et al., 2011), which prevents interbreeding. In each of the High Tatra lakes inhabited by these two lineages, they either coexist or occur alone, but no more than a few genotypes (clones) are found together, and not more than two are found dominating, these differing in overwintering mode (Černý & Hebert, 2011). Individuals of these two lineages differ conspicuously in the colouring of the fat reserves, which gives them either a transparent or an orange appearance. Indeed, the occurrence of two *Daphnia* colour morphs was recorded in Czarny Staw as long as a century ago (Minkiewicz, 1911). The quality of the fat stores appears meaningful, as these two differ in the way they manage and expend the very limited resources, and as such—in their overwintering strategy and consequently, in longevity (Gliwicz et al., 2001; Slusarczyk, 2009).

Individuals of the 'transparent', exclusively asexual, lineage complete their life cycle in the water column within one growing season. They hatch from parthenogenetic resting eggs in spring and begin reproduction after a relatively short period of growth;

in late summer, they produce, or their offspring produce, parthenogenetic resting eggs and die soon. The population survives winter only as the diapausing eggs, and thus the individual post-hatching lifespan does not exceed 5–6 months in these annual animals (Slusarczyk, 2009). The exceptionally long-lived individuals of the 'orange' lineage do not produce resting eggs in Czarny Staw though genetic data do not exclude their occasional sexuality (Dufresne et al., 2011; M. Slusarczyk & J. Mergeay, unpublished data). They follow a capital breeding strategy with delayed reproduction in a biennial cycle. They do not reproduce during their first season, when they were born, but grow, acquire and store resources to survive the winter in the water column and begin reproduction the following spring. Their individual lifespan can reach 13–14 months. As a consequence, in summer, there are three easily distinguishable groups of animals present in the lake: (i) the young-of-the-year from the biennial, overwintering 'orange' lineage, (ii) the previous year born, reproducing individuals from this biennial lineage and (iii) individuals of the annual, ephippia-producing 'transparent' lineage (Slusarczyk, 2009).

These life histories have most probably diverged as a result of conflicting selective pressures acting on energy allocation into either soma or reproduction (Stearns, 1992). However, this may have been followed by and have additional grounds in more mechanistic differences in the use of energy. According to the classical rate-of-living theory, individual lifespan depends on the rate of energy expenditure—rate of metabolism, and on genetically determined amount of energy consumed in adult life—metabolic potential (Sohal et al., 2002). As metabolic potential is similar in closely related species (Speakman, 2005), it can be expected that the twofold differences in *Daphnia* lifespan of two closely related, coexisting lineages will be associated with different metabolic rates. Such differences can be grounded both genetically and environmentally.

Temperature is one of the most important environmental factors strongly influencing energetic demand. In ectotherms, lower temperature naturally leads to lowering metabolic rates and lengthening lifespan (Miquel et al., 1976; McArthur & Sohal, 1982; Leiser et al., 2011), which has also been observed in *Daphnia* (MacArthur & Baillie, 1929; Orcutt & Porter, 1984). In thermally stratified lakes, lower temperatures are

experienced by animals staying in deeper layers, thus a single lake presents planktonic animals with a gradient of thermally different habitats, and several studies have shown that populations may segregate between these. This includes large and small individuals of the same species having different migratory behaviours (Kessler, 2004; Kessler & Lampert, 2004; Reichwaldt & Abrusán, 2007; Reichwaldt, 2008), as a consequence of their having different optimal habitats (Reichwaldt & Abrusán, 2007).

Apart from differences implicated by habitat choice, the animals of the long- and short-lived lineage might differ in their metabolic activity irrespective of the environmental temperatures. A widely used method for assessing metabolic rate in laboratory conditions is the oxygen consumption measurement (Wieser et al., 1992; Houthoofd et al., 2002), whereas RNA:DNA ratio has been used in different organisms as an indicator of physiological condition and metabolic activity in the field (Wagner et al., 1998; Meesters et al., 2002; Cullen et al., 2003; Buckley & Szmant, 2004). Under laboratory conditions, the latter has also been shown as an indicator of growth (Zhou et al., 2001; Ibiam & Grant, 2005), dependent on the developmental stage of the animal (Gorokhova & Kyle, 2002; Ibiam & Grant, 2005).

The aim of this study was to identify mechanisms underlying the extreme differences in longevity in the closely related *Daphnia* of the two lineages coexisting within the lake. We tested the hypotheses that the observed differences in life histories, and in lifespan in particular, (i) are mediated by habitat choice in the vertical gradient of temperature, and (ii) that they are also a consequence of differences in basic physiology—metabolic rates. We thus predicted the short-lived *Daphnia* (i) to stay on average in shallower, warmer waters and (ii) to have higher metabolic rates measured as oxygen consumption and RNA:DNA ratio, than the long-lived *Daphnia*.

Methods

We assessed vertical distribution of *D. pulicaria* in Czarny Staw pod Rysami (1,581 m above sea level) in the High Tatra Mountains in Southern Poland. The animals were sampled at the time of summer stratification, in August of three different years (1999, 2003 and 2010), during the day and at night each year. Stratified live samples were taken in vertical hauls using a quantitative Apstein closing plankton net (150-µm mesh size) from discrete 5-m (surface layers) or 10-m (deeper layers) strata, covering the whole water column at the deepest spot of the lake. The net was equipped with a long collar and a separately operated closing line attached to the largest ring of the net to prevent loss or admixture of individuals from different strata (de Bernardi, 1984; Masson et al., 2004). Quantitative subsamples were analysed under dissecting microscope. *Daphnia* of three groups were counted: (i) short-lived individuals of the ephippia-producing lineage and the long-lived: (ii) young and (iii) adult individuals (born in the year of sampling or the previous year, respectively) of the overwintering lineage. The counts were used to calculate the density of the animals at different depths. Temperature and oxygen profiles were taken at each plankton sampling date and the mean temperature of each layer from which the animals were taken was estimated. In the first sampling year (1999), 2 l water samples were taken from depths between 5 and 65 m, each 10 m, and filtered through glass-fibre filter (30 µm) to measure particulate organic carbon content (Wetzel & Likens, 1991). In 2003, chlorophyll *a* concentration in water samples taken from mid-depth of each layer from which zooplankton samples were taken was determined fluorometrically (Turner Designs) (Welschmeyer, 1994).

Comparisons between the three groups (i.e. including the age class discrimination in the biennial, Table 1) were made for all 3 years on weighted mean depths (Basedow et al., 2010, Ringelberg, 2010) and weighted mean temperatures at which animals of the three groups resided at the times of sampling, both measures correcting for the different widths of the sampled strata. ANOVA was performed, followed by between group comparisons with post hoc Tukey's test at $\alpha = 0.05$.

As physiological assays were meant to be measures of intrinsic processes, independent of the temperature of the environment, the samples for nucleic acids ratio analysis and for oxygen consumption experiment were taken from Czarny Staw at a time point when temperature in the water column was close to uniform, at the beginning of the autumnal mixing. *Daphnia* for these assays were taken from a single layer, 15–25 m depth, in 2010, when temperature in the water column

Table 1 Summary of the results for the three groups of *D. pulicaria* from Czarny Staw

	Short lived	Long-lived young (1st year)	Long-lived adults (2nd year)
Lineage	Ephippia producing (annual, 'transparent')	Overwintering (biennial, 'orange')	
Stage	Reproductive	Juvenile	Reproductive
Size (mm)	1.6	1.7	3.0
Depth (m)	14.3 ± 2.8	24 ± 2.6	14.5 ± 2.3
Temp. (°C)	8.4 ± 0.6	6.2 ± 0.4	8.1 ± 0.7
OC (mgO_2 mg^{-1} day^{-1})	0.089 ± 0.009	0.074 ± 0.001	0.076 ± 0.004
RNA:DNA	33.8 ± 1.4	27.5 ± 1.4	30.2 ± 2.1

Size averaged size of *Daphnia* in August–September, given according to Slusarczyk (2009); *Depth* weighted mean depth; *Temp.* weighted mean temperature at which animals resided, averaged over all sampling times; *OC* oxygen consumption. Mean ± SE. Group effect was significant for all measured parameters, see details in text

ranged from 7°C at the surface to 4°C at the bottom. 24 individuals of each of the three studied groups were separated straight from the plankton samples, transferred to 1.5-ml tubes, flash frozen in liquid nitrogen on site and later stored at −85°C until RNA:DNA analysis. After having been sampled from the water column, *Daphnia* used in the oxygen consumption experiment were acclimated for 6 h and the experiment was performed at 12°C, surface temperature in Czarny Staw that summer. 10 individuals of each group were placed in 50-ml vials of aerated Czarny Staw water, all in six replicates (3 groups × 10 individuals × 6 vials = 180). Oxygen concentration was recorded with a ProOdo optic probe (YSI) while filling the vials, which were then capped under the water surface with care taken not to close any air bubbles inside, and the second oxygen recording was done after 14 h of exposure. Four individuals from each vial were dried and weighed.

RNA and DNA were extracted and analysed using the fluorochrome RiboGreen in combination with RNase treatment according to a modified protocol of Vrede et al. (2002). To each 1.5-ml tube containing one *Daphnia* individual, 200 μl of TE buffer was added and the animal was homogenized with an RNase-free plastic pellet pestle. Thereafter, the samples were vortexed on a microplate shaker (15 min, 4°C, 900 rpm), sonicated 3 × 10 s in ice water, vortexed again (5 min, 4°C, 900 rpm) and centrifuged (5 min, 4°C, 15700×g). Supernatants were transferred into new 1.5-ml tubes and diluted 1:3 with the TE buffer. Duplicate or triplicate 100 μl subsamples were transferred to a black 96-well microplate, 2,000 time diluted fluorochrome RiboGreen (Molecular Probes) reagent solution (100 μl) was added and the samples

were gently mixed for 5 min in darkness. Fluorescence was then measured in a spectrofluorometer using a microplate reader (Victor 3, Perkin Elmer) at 485-nm excitation wavelength and 535-nm emission wavelength. After the first fluorescence measurements, 26 μl RNase solution (1.8 Kunitz, ribonuclease A from bovine pancreas; Sigma) was added to all samples, microplate was incubated on the microplate shaker (20 min, room temperature, 300 rpm), and fluorescence was subsequently measured as above. To estimate the contribution of DNA in the samples, the fluorescence after RNA digestion was subtracted from the total fluorescence before RNase treatment, and subsequently RNA:DNA ratio was calculated. For details on buffers and solutions preparation, see Vrede et al. (2002). All *Daphnia* used for oxygen consumption and RNA:DNA assays were either juveniles or females bearing subitaneous eggs.

Results

The mean midsummer densities of *Daphnia* in the water column throughout the study period were 145 ± 35 ind. m^{-3} of the annuals and 483 ± 169 ind. m^{-3} of the biennials, and the relative abundance of the first to second year individuals of the latter lineage was about 10:1. The vertical distribution of the animals in the water column differed between groups.

There were significant effects of group, time of day and year on the depth at which *Daphnia* stayed in the lake (three-way ANOVA, group effect: $F_{2,17} = 41.24$, $P = 0.0003$, time of day effect: $F_{2,17} = 22.84$, $P = 0.0031$, year effect: $F_{2,17} = 41.50$, $P = 0.0282$; All

two-way and three-way interactions were not significant ($P > 0.069$)). Young individuals of the biennial, overwintering lineage stayed on average deeper than both the reproducing adults of the same lineage and than *Daphnia* of the annual, ephippia-forming lineage (Table 1; Fig. 1). The animals stayed deeper during the day than at night (20.0 ± 2.2 m vs. 15.2 ± 2.7 m (mean $\pm$ SE), respectively) and at different depth each year (16.7 ± 2.6 m in 1999, 23.5 ± 3.1 m in 2003, and 12.5 ± 1.8 m in 2010). The differences in vertical distributions were similarly meaningful when translated into temperatures of occurrence (temperature and food concentration profiles are presented in Fig. 2). There was a significant effect of group (three-way ANOVA, $F_{2,17} = 18.43$, $P = 0.0027$), time of day ($F_{2,17} = 25.12$, $P = 0.0024$) and year ($F_{2,17} = 6.91$, $P = 0.0277$) on the mean temperature at which the animals were found (Fig. 3). All two-way and three-way interactions were not significant ($P > 0.06$). Accordingly with the differences in depth, young

biennials stayed in colder waters than both the reproducing adults of the same lineage and than the annuals (Table 1; Fig. 3). The animals stayed in colder water during the day than at night (6.8 ± 0.4°C vs. 8.4 ± 0.6°C (mean $\pm$ SE), respectively) and in colder water in 2003 than in 2010 (7.0 ± 0.8°C vs. 8.4 ± 0.4°C, respectively). In addition, neither lineage nor size had an effect on mean depth or temperature of occurrence. Significant effect of stage (reproductive vs. juvenile) on mean depth was seen (one-way ANOVA, $F_{2,17} = 9.75$, $P = 0.0066$), juvenile individuals staying deeper than the reproductive ones (24.0 ± 2.6 vs. 14.4 ± 1.8 m).

Both assays returned parallel results on metabolic activity. Oxygen consumption per mass differed between the three groups of animals (one-way ANOVA, $F_{2,17} = 5.92$, $P = 0.0127$) and was higher in the annuals than in the overwintering *Daphnia* of both ages (Table 1; Fig. 4). RNA:DNA ratio also differed between these groups (one-way ANOVA, $F_{2,61} =$
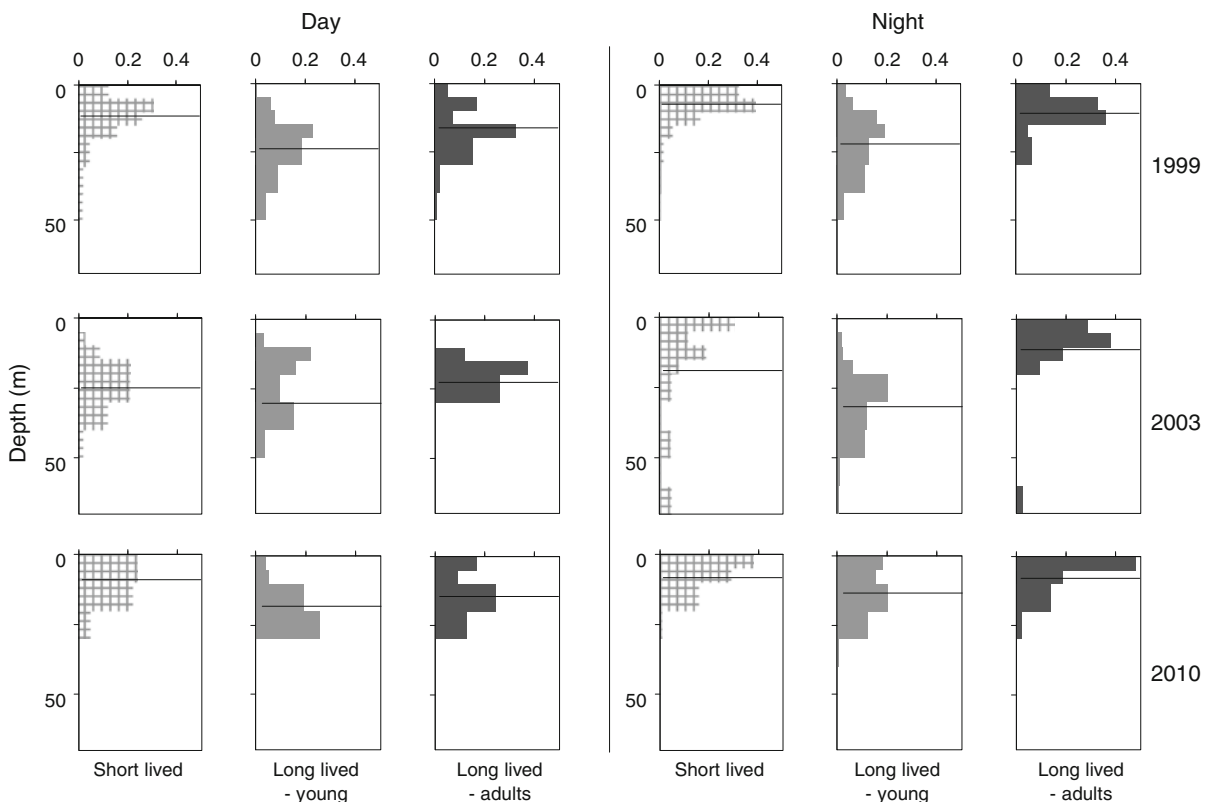

Fig. 1 Day and night vertical distribution of *D. pulicaria* presented as the share of the group population present in the water column of Czarny Staw. *Horizontal lines* mark the weighted mean depth

Fig. 2 Temperature (*left*) and food concentration (*right*) profiles in Czarny Staw at the times of sampling. In 1999, food concentration was measured as organic carbon; in 2003, as chlorophyll *a* content; for 2010, no data is available
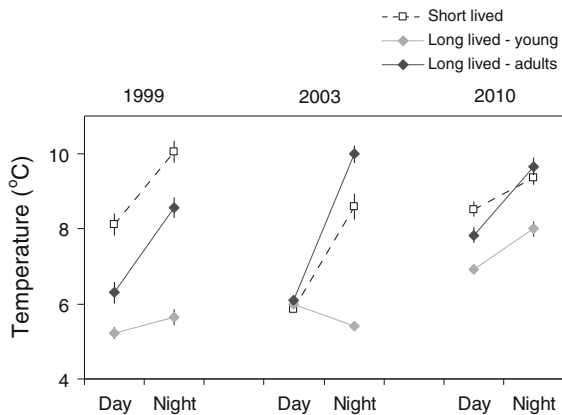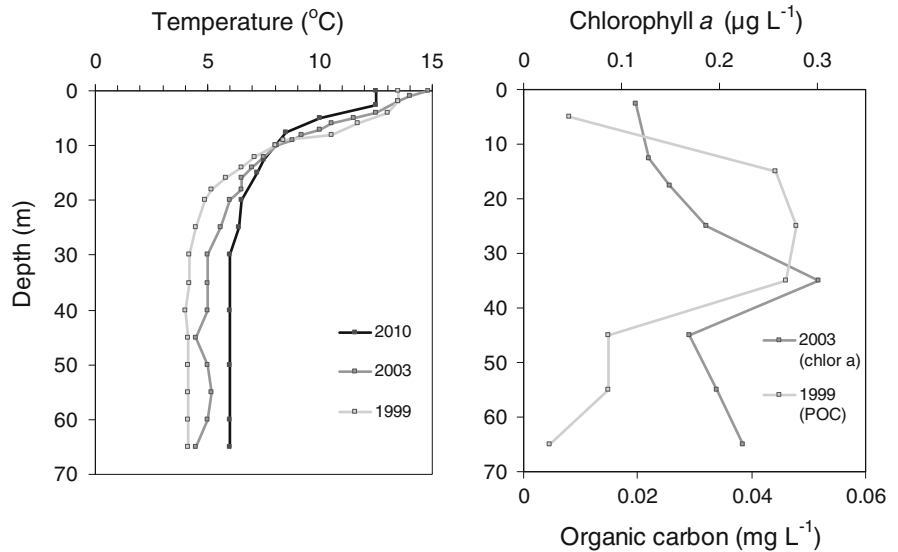

Fig. 3 Weighted mean temperature at which *D. pulicaria* stayed in Czarny Staw (data for 3 years, mean ± SE)
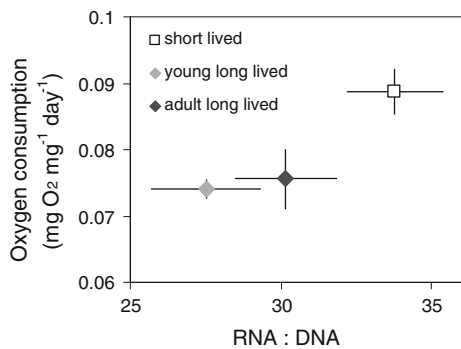

Fig. 4 Metabolic activity of *D. pulicaria* of the three studied groups as measured by RNA:DNA ratio and oxygen consumption (mean ± SE)

3.47, $P = 0.0376$), and was significantly higher in the ephippia producing than in the young overwintering *Daphnia* only (Table 1; Fig. 4).

Discussion

Temperature governs metabolism through its effect on the rate of biochemical reactions, and the rate of metabolism observed at the level of the organism increases exponentially with temperature (Gillooly et al., 2001). In high altitude or latitude oligotrophic lakes, *Daphnia* are faced with very low temperatures all season long. Low temperatures naturally extend cladoceran lifespan through lowering metabolic rates, and this lengthening is reinforced by very low concentrations of food. Such conditions, together with the length of the growing season strongly limited by long periods of ice cover, demand strategies of surviving winter other than those effective in lowland lakes. Two possible strategies enable planktonic species surviving in time constrained habitats, where the temperatures do not allow for more than two or three generations, and food conditions secure reproductive success only early in the season (Edmondson, 1955; Stross & Kangas, 1969; Larsson & Wathne, 2006). The first is the early reproduction and shortening the life cycle through obligatory parthenogenesis and production of resting eggs even by the first generation that hatches in spring. The second is

delaying reproduction while accumulating resources to survive winter in the water under the ice cover and reproduce early in the second growing season.

The aim of this study was to test the hypothesis that the observed differences in life histories, and in lifespan in particular, are mediated by habitat choice of *Daphnia* representing two coexisting strategies. As in a thermally stratified lake, faster growth and development are feasible in the layers closer to the surface, and slowed down in the deeper layers, we hypothesized that individuals of the short-lived annual lineage stay closer to the surface than the long living individuals of the overwintering biennial one—the former shortening, the latter lengthening developmental time.

The results obtained stay in accordance with this 'rate-of-living' hypothesis (Pearl, 1928). Analysis of the plankton samples collected from the cold lake in the summer showed that young individuals of the biennial lineage stayed on average deeper in the water column than the reproducing adults of the same lineage and than the shorter-lived individuals of the ephippia forming lineage. In consequence, the young biennials chose on average $\sim 2°C$ colder water than the animals of the other two groups—considerable difference, taking into account low and narrow range of temperatures in the lake. The animals migrated between the colder and warmer waters on diurnal basis. Such migratory behaviour, i.e. avoiding surface waters during the day, observed in the absence of fish is most probably caused by ultraviolet radiation, an important factor shaping zooplankton vertical distribution in high altitude or latitude ultraoligotrophic habitats (Tartarotti et al., 1999; Rhode et al., 2001; Williamson et al., 2001), preventing the animals from fully profiting from higher temperatures in the subsurface water during daylight hours. The between-year variability in depth and temperature distribution is interrelated, depth of occurrence being influenced by temperature profile and other resulting physical and biotic gradients in the lake. The pronounced differences in both depth and temperature distribution between 2003 and 2010 may be partly due to differences in light conditions on the dates of sampling, sunny versus overcast, respectively (data not presented; weather record not available for 1999), which strengthened the between day and night differences.

The fact that the first and second year individuals of the overwintering lineage stayed at different depths reveals that the depth selection behaviour of these animals is not only genotype but also age or stage dependent. Juveniles of the biennials appeared to delay the onset of reproduction by staying in colder waters than the reproductively active adults of both lineages. It seems also that the young individuals of the overwintering lineage, staying deepest (24 m on average), were more influenced by algal distribution in the water column than were the other two groups: the highest food concentrations were observed 25–35-m down. Indeed, food threshold concentrations are higher for small or young *Daphnia* (Gliwicz, 1990; Kreutzer, 1999). The observed distribution could be an example of a niche segregation resulting from inter- or intraspecific competition for food between small and large sized individuals, if the vertical distribution of the animals was size dependent. However, the equally small sized individuals of the ephippia-producing lineage were staying in most cases in food poor surface waters, together with the large adults of the overwintering lineage. Vertical distribution of the animals was neither size nor lineage, but stage, i.e. reproductive status, dependent.

The young *Daphnia* of the overwintering lineage may delay reproduction in part by staying in deeper, colder, although richer in food waters, but then as adults they move to higher temperatures to shorten the development time of the eggs they bear and to produce another clutch of offspring before the food resources are depleted. *Daphnia* of the ephippial lineage stay in warmer waters throughout the free-swimming part of their life cycle, which enables them to complete reproduction within one season. This suggests that the two lineages of *Daphnia* achieve such different life histories, both through differences in energy allocation and through manipulating their metabolism through behavioural thermoregulation.

Daphnia of the studied lineages differ in their early reproductive investments. Although the young-of-the-year of both lineages attain similar body size during the growing season, only individuals of the ephippia-producing lineage begin to reproduce that same season, which is within the first 2 months of their life (Slusarczyk, 2009). Individuals of the same age and size, but belonging to the other lineage, delay reproduction for almost a year, investing the scarce resources in accumulating energy reserves and in further growth instead. Allocating energy into soma means investing in the increased probability of survival and future reproduction (Kozłowski, 1993).

Allocating these limited resources into reproduction means the loss of this energy to maintaining the soma, storing resources or further growth. At the end of the growing season, individuals of the ephippia-producing lineage are smaller than the current year individuals, now the only individuals of the overwintering lineage left in the water column. The annuals are deprived of fat stores after the reproductive season, the reserves being put into ephippia production, and soon disappear from the water column (Slusarczyk, 2009).

We expected that the differences in lifespan are not only mediated by habitat choice but also by differences in basic physiology, the short-lived *Daphnia* having higher metabolic activity measured as oxygen consumption and RNA:DNA ratio than *Daphnia* of the long-lived lineage when assayed at the same temperature.

Indeed, the measurement of metabolic activity revealed lower oxygen consumption in both young and adult individuals of the long-lived overwintering lineage than those of the ephippia-producing lineage. This finding stays in accordance with the expectations linking longevity to the rate-of-living. Oxygen consumption assessed several hours after taking animals from the lake, after acclimation and the duration of the experiment yielded results similar to metabolic activity measured through the RNA:DNA ratio in animals taken straight from the lake. In the latter, though, a significant difference was seen only between the annuals and young biennials. That the annuals had the highest and the young biennials the lowest RNA:DNA ratio might confirm the usefulness of this measure as a metabolic activity indicator. However, it also indicates that inferring nutritional condition upon this measure (Wagner et al., 1998) might not be straightforward, as depending on current energy allocation pattern in the organism. Our results suggest the nucleic acid ratio could rather be used as an indicator of reproductive status in the field, as it was lower in juveniles than in egg-carrying females, clearly due to RNA:DNA ratio being the highest in developing embryos (Gorokhova & Kyle, 2002).

As oxygen consumption was lineage dependent and lower in the biennials than in annuals, the results of the present study suggest that lower intrinsic metabolic rates of the biennials, together with the staying deeper in the water column of their young, may be their strategy for longevity. The observed lowest transcriptional activity in the young biennials could then be a

consequence of that strategy and a direct result of delayed reproduction. Although single time point measurements preclude whole-season generalisations, the above results were obtained for the end of summer/early fall, when water mixing started. This is the time when the young individuals of the biennials finish accumulating resources before overwintering, and the last surviving second-year adults of this lineage disappear from the water column (Slusarczyk, 2009). At the same time, many of the annuals, more metabolically active according to our results, are still reproductively active and carry ephippium.

In summary, our data indicate that both behavioural and physiological mechanisms underlie the extreme differences in longevity in these closely related *Daphnia* and the exceptionally long lifespan of the overwintering ones. Further studies could verify these conclusions through the measurement of seasonal dynamics in metabolic activity in these animals, their sensitivity to changes in temperature and identifying possible additional effects of food availability and distribution. Such studies would also help in identifying the mechanisms underlying apparently stable, over 100 years in the case of Czarny Staw, coexistence of two closely related lineages within a habitat.

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