


Countergradient variation concealed adaptive responses to temperature increase in *Daphnia* from heated lakes

Marcin Krzysztof Dziuba ^{1,2*}, Lechosław Kuczyński ³, Łukasz Wejnerowski ¹, Sławek Cerbin ¹,
Justyna Wolinska ^{2,4}

¹Department of Hydrobiology, Faculty of Biology, Adam Mickiewicz University, Poznań

²Department of Ecosystem Research, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany

³Population Ecology Lab, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland

⁴Department of Biology, Chemistry, Pharmacy, Institute of Biology, Freie Universität Berlin, Berlin, Germany

Abstract

To test the general assumption that global warming will induce body size reduction in aquatic organisms, we used a system of lakes continually heated for six decades by warm water discharge from power plants. Their temperature elevation of 3–4°C corresponds with climate change forecasts for the end of the 21st century. We compared body size and reproduction of *Daphnia longispina* complex communities inhabiting heated and non-heated (control) lakes nearby. No difference in body size was found, but *Daphnia* communities from heated lakes had a wider thermal breadth for reproduction. The two lake groups varied in the taxonomic composition of *Daphnia* communities. Thus, to disentangle inter- and intraspecific sources of variation, and to examine evolution vs. phenotypic plasticity of investigated traits, we performed two life history experiments: (1) a between-species experiment compared *D. galeata* inhabiting heated lakes with *D. longispina* typical of nearby control lakes, under three temperature regimes; (2) a within-species experiment compared *D. galeata* from heated lakes with conspecifics from high latitude (cold control) and low latitude (warm control) lakes, under two temperature regimes. The experiments revealed countergradient variation: environmental constraints on body size in situ concealed evolution of larger potential body size in *Daphnia* from heated lakes. In turn, evolution of increased body size plasticity resulted in an efficient resource allocation trade-off: more effective reproduction at high temperature, at the cost of size reduction. We suggest that large size is adaptive during active overwintering, while plastic size reduction is a coping strategy for high temperatures.

*Correspondence: marcinkdziuba@gmail.com; marcin.dziuba@amu.edu.pl
[Leibniz-Institut für Gewässerökologie und Binnenfischerei]

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Additional Supporting Information may be found in the online version of this article.

Author Contributions Statement: M.K.D. and S.C. conceived the overall research project. M.K.D., L.W. and S.C. collected the field samples and M.K.D. processed them. M.K.D. and S.C. designed the between-species experiment and M.K.D. conducted it with the support of L.W. M.K.D. and J.W. designed and conducted the within-species experiment. L.K. designed statistical analyses and analyzed field data, L.K. and M.K.D. conducted statistical analyses of experimental data. M.K.D. conducted the body size contributors partitioning calculations. M.K.D. and J.W. wrote the manuscript with input from all co-authors. L.K. wrote specifically the parts concerning statistical analyses of field samples. All authors contributed critically to the drafts and gave final approval for publication.

Earth's climate is gradually warming and the global average temperature is expected to undergo an increase of 3–4°C by the year 2100 (IPCC 2019). Adverse effects of temperature elevation on biodiversity have already been observed, such as biological invasion, habitat shrinkage, and even species extinction (Thomas et al. 2004; Parmesan 2006). There is some evidence demonstrating organisms' adaptation to recent climate change, including shifts in species ranges and phenology, or plastic and genetic changes of phenotypes (Parmesan 2006; Bellard et al. 2012). However, these adaptations are sometimes too slow (Radchuk et al. 2019) and/or can result in trade-offs in resource allocation. In order to maintain high reproduction rates under temperature elevation, it is expected that ectothermic organisms will reduce somatic growth (temperature-size rule; Atkinson 1994; Kozłowski et al. 2004). Body size reduction is believed to be a universal response of organisms to climate warming (Gardner et al. 2011; Horne et al. 2015).

It is challenging to predict accurately the fate of organisms under forecast climate warming. The temperature increase already experienced (about 0.78°C since 1900) is only a foretaste

of what is expected by the end of the 21st century (IPCC 2019). Projecting the fate of organisms based on contemporary responses might therefore not be fully adequate. Experimental evolution allows the detection of evolutionary adaptation to anticipated levels of temperature elevation (e.g., Van Doorslaer et al. 2010; Geerts et al. 2015), but in some cases the restricted experimental time scales may be insufficient for evolutionary processes to occur. In general, the efficiency of experiments in detecting evolution depends on the model organism and the corresponding duration of the experiment (i.e., the practical trade-off between number of generations and experiment length) as well as on the standing level of genetic variation. Comparison of populations from different climate zones (e.g., Havens et al. 2015) is free of these limitations, but tends to juxtapose ecosystems that might be of distant geological origin and/or incomparable abiotic character. Such an approach, as well as comparison of organisms living at different times (paleoecology and resurrection ecology research methods; e.g., Orsini et al. 2013, Weider et al. 2018), could be prone to the impact of variables other than temperature (e.g., changes in predation or trophic state). Moreover, resurrection studies cannot go beyond the currently observed level of warming ($< 1^{\circ}\text{C}$), while climate models predict much stronger temperature increases in the coming decades. The drawbacks of the commonly used research methods listed above force us to seek novel approaches that would test future levels of temperature elevation while excluding biogeographical noise. At the same time they should involve extended periods of warming, allowing for adaptive organismal responses. A promising example of such an approach is comparison of urban (warmer) vs. rural (colder) ecosystems—it includes relevant temperature differences and does not juxtapose geographically distant ecosystems (e.g., Brans et al. 2017). Unfortunately, this approach can only be applied to a limited number of ecosystem types, which occur both in urban and rural areas. Here, we present a new approach that avoids the problems mentioned above: studying organismal adaptations to elevated temperatures in artificially heated (vs. non-heated) natural ecosystems.

We investigated thermal adaptation of the water flea *Daphnia* from lakes that had been exposed to artificially elevated temperatures for six decades in their natural environment (five lakes), in comparison to organisms that lived in nearby control sites (four lakes) at ambient temperature. The heated lakes have experienced a long-term temperature increase due to warm water discharge from local power plants' cooling systems. The temperature elevation is in line with forecast changes by the year 2100 (i.e., $3\text{--}4^{\circ}\text{C}$, Fig. S1). Both heated and control lakes are inhabited by the hybridizing *Daphnia longispina* complex, though the exact genetic/taxonomic structure differs between the lakes due to discrepancies in thermal regime (Dziuba et al. 2020). *Daphnia* play a vital function in freshwater ecosystems,

being a keystone herbivore and main fish food (Lampert 2006), therefore ascertaining how they will react to increased temperatures is important when projecting the fate of aquatic ecosystems under global warming. In particular, body size reduction—a common response of aquatic ectotherms to temperature increase (Daufresne et al. 2009; Horne et al. 2015)—might inflict negative consequences on entire ecosystems. Smaller *Daphnia* are known to produce less offspring (Bartosiewicz et al. 2015) and graze less efficiently (Gliwicz 1990), so reduction in body size could potentially lead to reduced overall pressure on primary producers. Smaller size and decreased reproduction of *Daphnia* could also result in deterioration of food resources for fish.

We tested the hypothesis that long-term temperature increase leads to reduction of average body size within the *Daphnia* community (interspecific level—community shift) as well as to selection of smaller individuals within species (intra-specific level—evolution) (Daufresne et al. 2009). We also determined if (and how) this long-term warming affects the organisms' reproductive output across a range of temperatures. We compared body size and reproduction between *Daphnia* originating from heated and control environments, using both in situ and laboratory surveys. In situ, we compared the *D. longispina* complex communities (composed of three hybridizing species: *D. galeata*, *D. longispina*, and *D. cucullata*) inhabiting either heated lakes or non-heated control lakes nearby. Reduced body size in *Daphnia* can be caused directly by temperature increase (due to the physiological advantage of size reduction, Kozłowski et al. 2004), or indirectly by temperature-mediated increase in planktivorous fish predation (e.g., Brucet et al. 2010; He et al. 2018). Fish predation has a particularly strong impact on body size in zooplankton communities (Brooks and Dodson 1965). In order to disentangle the direct effect of temperature from other confounding factors (e.g., predation, food regime, demographic effects; all of which were not controlled in the field study), we conducted two laboratory experiments. These experiments focused either on interspecific or intraspecific differences (Fig. 1), while otherwise investigating the same traits. Specifically, in the between-species experiment we compared *D. galeata* from heated lakes with *D. longispina* from nearby control lakes (*D. galeata* was present exclusively in heated lakes whereas *D. longispina* occurred mainly in control lakes; Dziuba et al. 2020). In the within-species experiment, we compared *D. galeata* from heated lakes with conspecifics from other control sites (representing different latitudes and therefore varying in thermal conditions). Both experiments were conducted under several thermal regimes, in order to unravel the effects of adaptive phenotypic plasticity (triggered by rapid changes in temperature) and evolutionary adaptation (induced by thermal conditions of environmental origin, Fig. 1). Overall, studying *Daphnia* from this system of heated lakes, both in-situ and in the laboratory, allowed us to integrate community ecology and

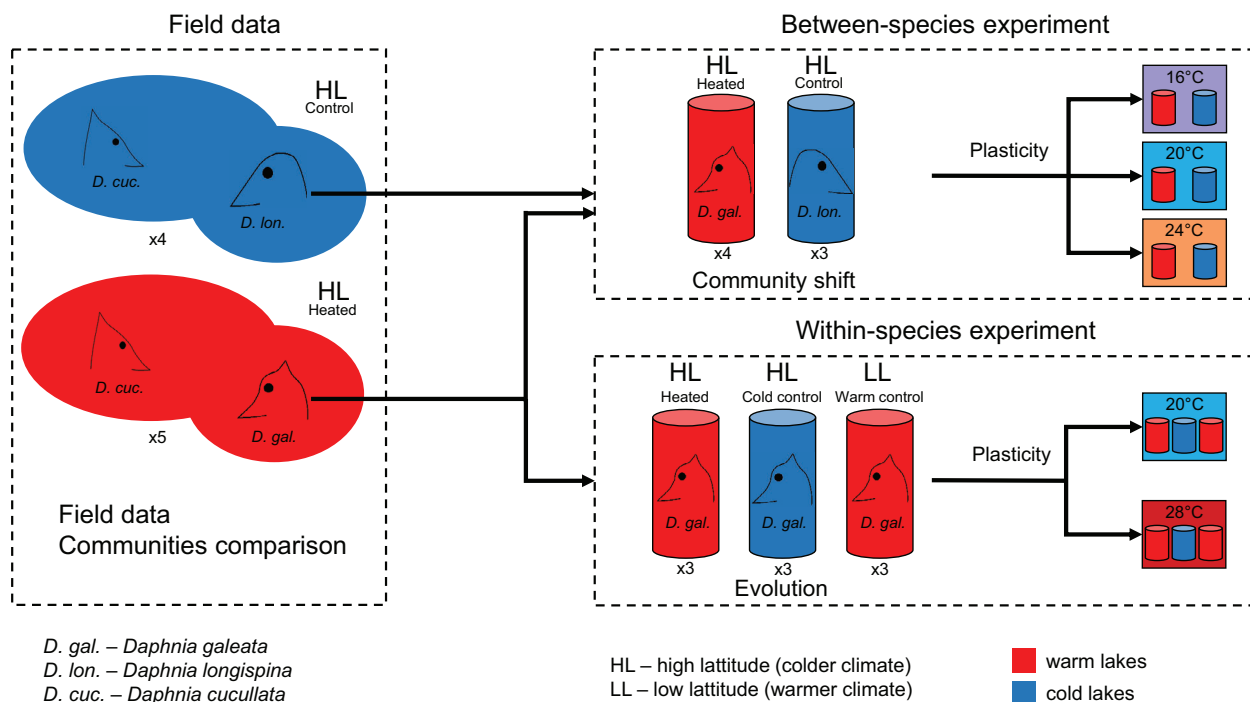


Fig 1. Graphical abstract of study design, and relationship between particular elements of the study. The field study (left panel) was conducted in order to compare entire *Daphnia* communities from heated and nearby non-heated control lakes. We further isolated *Daphnia* clones from heated and control lakes (*D. galeata* and *D. longispina*, respectively) in order to investigate the same traits experimentally, after excluding confounding environmental factors. The between-species experiment was designed to detect interspecific differences driving the split of communities from heated and nearby control lakes. Meanwhile, the within-species experiment was based on intraspecific comparison of *D. galeata* from heated lakes with conspecifics from two types of non-heated control: One at a similar latitude to the heated lakes (cold control: Germany) and one at a lower latitude than the heated lakes (warm control: Italy). Both experiments were conducted under several thermal regimes in order to evaluate the rate of thermal phenotypic plasticity, and disentangle it from evolutionary adaptation. Numbers below the lakes or experimental vials indicate the number of lakes investigated or from which clones were collected, respectively.

evolutionary ecology-based patterns in the search for adaptations to climate warming.

Materials and methods

Study sites and field sampling

Daphnia communities were investigated in five heated lakes (Licheńskie, Gosławskie, Pątnowskie, Mikozyńskie, and Łeśińskie) and four non-heated control lakes (Skulskie, Gopło, Budziszławskie, and Skulska Wieś) (Fig. 1, red and blue lakes, respectively). All are located in central Poland near the city of Konin and are in close proximity to each other – the greatest distance between lakes is ca. 30 km. The heated lakes have long experienced discharge of warm water from two lignite combusting power plants: Pątnów and Konin, operating since 1958 and 1970, respectively. A detailed description of the system can be found elsewhere (e.g., Socha and Hutorowicz 2009; Stawecki et al. 2013). Geographical positions and some physical characteristics of all lakes are provided in Table S1. The average summer temperature profiles of heated and control lakes illustrate the increase in temperature of ca. 3–4°C in the former (Fig. S1). Due to temperature increase, the frequency and

duration of ice-cover in heated lakes is reduced in comparison to control lakes (e.g., Socha and Hutorowicz 2009, personal observation). *Daphnia* were collected monthly between March 2014 and September 2015, by vertical towing of a 100- μ m plankton net through the deepest part of each lake (in the case of Lake Gopło, samples were collected from the deepest accessible point; some parts of this lake are protected and thus use is restricted). From each sample 50–100 (when possible) adult female individuals were measured from the tip of the head to the base of the tail spine, and the eggs in the brood chamber were counted. Physico-chemical parameters of the water column (i.e., temperature, conductivity, pH, total concentration of dissolved salts, oxygen concentration, and oxygen saturation) were monitored throughout the water column with 1-m resolution, and Secchi disc visibility was measured. There was no pattern of lake segregation into groups corresponding with heating mode (i.e., heated vs. control) with respect to any of these parameters except temperature (Dziuba et al. unpublished). All examined lakes are eutrophic (although control lake Budziszławskie is less turbid than the others), and have similar catchment use and the same microclimate. We lack data which would allow a comparison of fish and invertebrate

predators' communities between heated and control lakes. However, it is known that fish communities in heated lakes are dominated by planktivorous species (Tereshchenko et al. 2007). Heated lakes were intensively stocked with grass carp, silver carp, and big head carp (Ejsmont-Karabin and Węgleńska 1988; Hillbricht-Ilkowska and Zdanowski 1988b). This, together with elimination of some predatory fish species (e.g., pike, perch; Hillbricht-Ilkowska and Zdanowski 1988b) and temperature elevation-induced increases in foraging activity of fish on plankton (Ejsmont-Karabin and Węgleńska 1988; Hillbricht-Ilkowska and Zdanowski 1988a), likely resulted in increased predatory pressure on zooplankton in heated lakes (Ejsmont-Karabin and Węgleńska 1988; Hillbricht-Ilkowska and Zdanowski 1988a). Acknowledging that predation can strongly modify the body size of *Daphnia* communities in situ (Hart and Bychek 2011), we point out that the way to disentangle direct effects of temperature from other confounding factors (e.g., predation) is through comparison of body size in the field and under experimental conditions. In such a comparison, size measurements in the field serve as a proxy of the strength of selection (by extrinsic determinants) on body size of *Daphnia* in heated and control lakes, while life-history experiments reveal intrinsic body size determinants (evolution and phenotypic plasticity).

Life-history experiments

Between-species experiment—five *D. galeata* clones isolated from heated lakes (Licheńskie—1, Gosławskie—2, Pałnowskie—1, and Initial Cooling Pond—1 clone) were compared to five *D. longispina* clones isolated from nearby non-heated control lakes (Skulskie—2, Gopło—2, and Budziszawskie—1 clone, Fig. 1, Table S1). These clones were selected randomly from a larger collection of clones. *Daphnia galeata* was present exclusively in heated lakes whereas *D. longispina* occurred mainly in control lakes (Dziuba et al. 2020). Both species belong to the *D. longispina* species complex, frequently produce hybrids and backcrosses, and gene flow between them can be quite extensive (Giefler 1997; Petrusek et al. 2008; Yin et al. 2014). These species (and their hybrids) are morphologically very similar and difficult to distinguish (Dlouhá et al. 2010). Moreover, reports on differences in body size between *D. galeata* and *D. longispina* are inconclusive. Many articles show similar body size for the two species (e.g., Stich and Lampert 1984; Kirdyasheva and Kotov 2019), although others assert that *D. galeata* is slightly larger than *D. longispina* (Weider 1993; Pijanowska et al. 2007; Brzezinski 2015) or that *D. longispina* is larger than *D. galeata* (Gliwicz 1990; Burns 1995; Lass et al. 2001). *Daphnia* clones isolated from these lakes were maintained under stable conditions (20°C, fed ad libitum with green algae *Tetrademus obliquus* [Turpin] Wynne, three times per week). Detailed information concerning the clones used in this study is provided in Table S1; taxonomic assignment was conducted by genotyping at multiple microsatellite loci (using markers

described in Brede et al. 2006, and previously genotyped “reference clones”). The experiment was conducted at 16°C, 20°C, and 24°C (the latter two representing the summer temperature of the epilimnion in control and heated lakes, respectively), and involved two lake types × 3 temperatures × 5 clones = 30 experimental units (note, clones were used here as replicates). To avoid thermal shock, *Daphnia* were acclimated to the experimental temperatures for three generations. The experiment was initiated with the third clutch of newborns of third-generation mothers, separately for each temperature (but within a 3-day span). Each newborn (not older than 18 h) was transferred individually into 75 mL of medium mixed with *T. obliquus* in a dose of 1 mg C L⁻¹ in glass tubes. Tubes were placed in water baths and temperatures adjusted to ± 0.25°C accuracy by an aquatic heater (Aqua Medic Titanium Heater 100 W) submerged in the bath and connected to a temperature controller (Thermostab TS 500). Medium with algal suspension was fully refreshed every second day. Experimental *Daphnia* were inspected every 12 h throughout the entire lifespan; all offspring were counted and removed, while mothers were measured after each reproduction.

Within-species experiment—six *D. galeata* clones isolated from heated lakes (Licheńskie, Gosławskie, and Pałnowskie; two clones per lake) were compared to the same number of *D. galeata* clones isolated from two types of non-heated control lakes: (1) at a similar latitude to the heated lakes, north of the Alps in Germany (cold control: Müggelsee, Fasaneriesee, and Heimstettener See; two clones per lake) and (2) at a lower latitude than the heated lakes, south of the Alps in Italy (warm control: Lago di Endine, Lago d’Isèo, and Lago di Varese; two clones per lake; Fig. 1, Table S1). These clones were randomly selected from the available clonal collections. This experiment was performed at 20°C and 28°C (the latter representing a thermal extreme, noted on the hottest summer days in control lakes), and involved 3 regions × 3 lakes × 2 clones × 2 temperatures × 5 replicates = 180 experimental units. Pre- and experimental procedures were the same as those in the between-species experiment, except that newborns were not older than 12 h when transferred, experimental tubes held 50 mL volume, half of medium with algal suspension was refreshed every second day, clonal IDs and origins were blinded, and measurements were conducted until the third reproduction or death of each animal. Newborns produced by experimental *Daphnia* were preserved in ethanol after collection and their length was measured after the experiment.

Statistical analyses

Field data

Generalized Additive Mixed Models (GAMM) were used to test how body size and fecundity are related to water temperature. Smoothers for temperature were fitted separately for the heated and control lakes. Since the field study spanned two consecutive seasons, a year identifier was also included as a fixed predictor. Moreover, to account for any additional,

unexplained variance due to variability between lakes or sampling occasions, both lake and sample identifiers were included in the models as random intercepts. In the case of body size, the Gaussian distribution for errors and the identity link function were used. In the case of fecundity, to account for overdispersion, a compound Poisson-Gamma (Tweedie) distribution was used for the response (no. of eggs), with the log-link function. Full models were checked for the possibility of simplification by conducting backward model elimination within the information-theoretic framework (Burnham and Anderson 2003). Candidate model sets were generated (Table S2) by removing fixed effects in turn, though always retaining temperature and random structure in the model. Then, candidate models were compared based on the Akaike information criterion (AIC), with the model having the lowest AIC value being considered the best (Tables S3, S4). Models were compared based on Δ AIC values, calculated as the difference between the AIC of a particular model and the best model in the candidate set. Moreover, Akaike weights were calculated, representing a normalized estimate of the relative likelihood of each model being best in the set. As an overall measure of model fit, the percentage of explained deviance (D^2) was used (Weisberg 2005). To test specific hypotheses, several contrasts were derived from the final (i.e., the best according to the AIC criterion) GAM models expressing differences between control and heated lakes:

Difference between thermal optima: The optimum was defined as the temperature for which the maximum value for the fitted response (i.e., highest number of eggs) was recorded. The contrast was defined as the difference between optima for heated and control lakes (this contrast was calculated for fecundity only, since body size did not show unimodal response).

Difference between thermal breadths: The thermal breadth was defined as the range of temperatures for which the fecundity (no. of eggs) predicted from the model exceeds one.

Difference in response across the range of recorded temperatures: For each epilimnetic temperature (over the range 1–26°C with 0.1°C resolution), a difference between the fitted response for the heated and the control lakes was calculated, forming a smooth representation of that difference. This difference was calculated for both body size and fecundity (reported as reproductive advantage for the fecundity).

Inferences about the above contrasts were drawn by simulating from a posterior distribution of GAMM model parameters (Wood 2017). The procedure consisted of the following: (1) drawing a random sample from the estimated sampling distribution of a fitted model using the vector of coefficients corresponding to the basic functions of the GAMM fit and their variance–covariance matrix (using a prediction matrix obtained by setting the type parameter in the *predict.gam* R function to *lpmatrix*), (2) calculating predicted response curves for heated and control lakes using coefficients from the random draw, (3) calculating contrasts from these predictions,

(4) repeating this procedure 10,000 times to obtain the distribution of contrasts, and (5) calculating 95% empirical confidence intervals of contrasts (by finding 0.025 and 0.975 quantiles).

Life-history experiments

Between-species experiment. In order to test if *D. galeata* isolated from heated lakes are smaller than *D. longispina* from nearby control lakes, and if these species vary in plastic size modulation in response to experimental temperature, a linear mixed effects model was used, with origin (heated vs. control lakes), treatment temperature, and clutch (reproduction event) as fixed effects, and clonal ID as a random factor. Clutch was used as a proxy for time because *Daphnia* molt and grow after each reproduction. Contrasts between groups were calculated using estimated marginal means implemented in the *emmeans* R package (Lenth 2019). To identify differences in reproduction between species, number of offspring at reproduction event (i.e., clutch size) was analyzed using a general additive model with origin, temperature, and their interaction as parametric fixed effects, smoothers for age fitted separately for each origin-temperature combination, and individual identifiers as random intercepts. This model was fitted using a Tweedie distribution for the number of offspring with the log-link function. As with the body size model, contrasts for parametric effects were assessed using *emmeans*.

Within-species experiment. To ascertain if the altered thermal regime in heated lakes induced genetically-determined intraspecific difference in body size (evolution) and in strength of body size modulation (phenotypic plasticity), body size of *D. galeata* from three sites (varying in thermal regime) was compared. Data were analyzed in a similar way to the between-species experiment, with the origin factor now having three levels (heated, cold control, warm control). Since the within-species experiment was much shorter, with fewer reproduction events, the cumulative number of newborns (i.e., produced throughout the entire experiment) was analyzed. Due to a ca. 20% background mortality in this experiment (similar in all treatments), a zero inflated model was implemented. To compare reproductive efficiency of *D. galeata* originating from ecosystems with different thermal regimes, number of newborns was analyzed against origin and treatment temperature as fixed effects and clonal ID as a random effect. No significant impact of the interaction of fixed effects was detected, therefore this interaction was removed from the model. Length of newborns was analyzed using a linear mixed effects model, with origin and temperature as fixed effects, and clonal ID and clutch number as random factors.

Body size contributors partitioning. Using the reaction norm approach (Eq. 2, Govaert et al. 2016), we quantified the contributions of phenotypic plasticity, constitutive evolution, and evolution of plasticity in determining body size of *Daphnia galeata*

in the within-species experiment. For all calculations we used the cold control group as the basic reference. We estimated the basic phenotypic plasticity of *D. galeata* as the difference in body size of cold control *Daphnia* obtained in 20°C and 28°C treatments. We further estimated the constitutive evolution under increased temperature as the difference in body size between clones from heated and cold control lakes, as well as between clones from warm and cold control lakes, all obtained at 20°C. Finally, we calculated the evolution of phenotypic plasticity as the difference in strength of plastic response to the in lab increase in rearing temperature (20°C vs. 28°C) between *Daphnia* from heated and cold control lakes, as well as between clones from warm and cold control lakes. All analyses were performed with R version 3.5 (R Core Team 2018).

Results

Field data

Daphnia communities in heated lakes had comparable body size to control lake communities (origin effect: $F = 0.04$, $p = 0.845$, Fig. 2, top panel). In both groups of lakes, body size

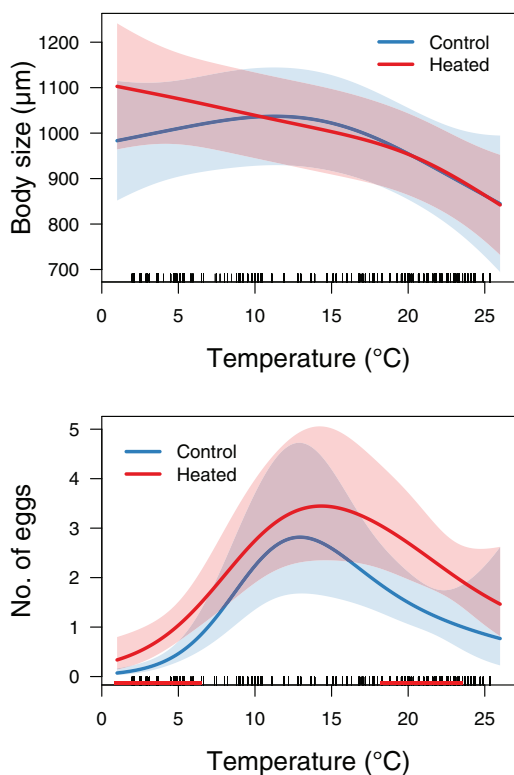


Fig 2. Body size of *Daphnia* (top panel), and number of eggs carried by *Daphnia* (bottom panel), in heated and control lakes (red and blue, respectively) in relation to epilimnion temperatures. Fitted values and 95% confidence intervals are provided (for raw data see Fig. S2). Red lines on x-axis indicate temperatures at which a significant reproductive advantage (difference between the fitted responses in reproduction of *Daphnia* community in heated and control lakes) for *Daphnia* from heated lakes occurred. Rug plot along x-axis shows distribution of data points.

of *Daphnia* was reduced in response to seasonal field temperature elevation (temperature effect in heated lakes: $F = 11.28$, $p < 0.001$; and in control lakes: $F = 4.18$, $p = 0.015$). *Daphnia* in heated lakes also displayed generally higher fecundity (origin effect: $F = 13.82$, $p < 0.001$), whereas fecundity of both groups dropped at low and high ambient field temperatures (temperature effect in heated lakes: $F = 9.27$, $p < 0.001$; and in control lakes: $F = 8.25$, $p < 0.001$; Fig. 2, bottom panel). Thermal optimum (i.e., the temperature at which the highest number of eggs was recorded) did not differ between the two groups of lakes (effect size expressed as difference in optimum temperatures was 1.5°C, 95% CI: -3.5 to 7.0), but *Daphnia* in heated lakes had a 4.8°C (95% CI: 1.2–8.2) wider thermal breadth (Fig. 2, bottom panel). Finally, a significant reproductive advantage for *Daphnia* in heated lakes occurred at very low (below 7°C), and high water temperatures (above 18°C).

Between-species experiment

In the between-species experiment we compared *D. galeata* clones that originated from heated lakes with *D. longispina* clones from nearby control lakes. *D. galeata* from heated lakes were larger than *D. longispina* from control lakes (origin: $\chi^2 = 51.47$, $p < 0.001$), but the body size of the former was significantly reduced in response to lab rearing temperature elevation (t -ratio = 3.43, $p = 0.002$). In contrast, *D. longispina* from control lakes did not express such plasticity in size (origin \times temperature: $\chi^2 = 17.99$, $p < 0.001$; Fig. 3, top panel). *D. galeata* from heated lakes generally produced more offspring (origin: $F = 4.01$, $p = 0.046$; Fig. 3, bottom panel), whereas the fecundity of all *Daphnia* was reduced under elevated lab rearing temperatures (temperature: $F = 5.29$, $p = 0.005$).

Within-species experiment

In the within-species experiment we compared *D. galeata* clones from heated lakes against conspecifics collected from cold control lakes (in a region of similar latitude), and from warm control lakes (lower latitude). *Daphnia* from heated lakes were larger than conspecifics from cold controls (origin: $\chi^2 = 7.96$, $p = 0.019$; contrast: t -ratio = 3.40, $p = 0.008$), but not those from warm control lakes (t -ratio = 1.93, $p = 0.158$; Fig. 4, top panel). There was no difference in body size between *Daphnia* from warm control and cold control lakes (t -ratio = 1.47, $p = 0.324$). Also, only *Daphnia* from heated lakes demonstrated reduced body size under elevated lab rearing temperatures (origin \times temperature: $\chi^2 = 9.40$, $p = 0.009$; contrast: t -ratio = 3.26, $p = 0.001$). *Daphnia* from heated lakes produced more offspring than *Daphnia* from cold control lakes (origin: $\chi^2 = 14.52$, $p < 0.001$; contrast: z -value = 3.58, $p < 0.001$) but not from warm control lakes (z -value = 1.89, $p = 0.059$, Fig. 4, bottom panel). Similarly to the between-species experiment, fecundity of all *Daphnia* was reduced under elevated temperatures (temperature: $\chi^2 = 64.57$, $p < 0.001$). Length of newborns was similar for all groups of

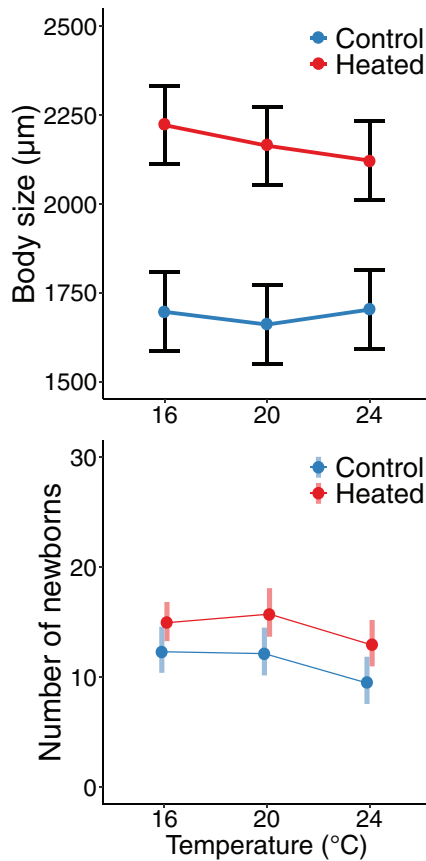


Fig 3. Body size (means and 95% confidence intervals; top panel) and fecundity (means and 95% confidence intervals; bottom panel) of *D. galeata* from heated and *D. longispina* from control lakes in the between-species experiment, under different lab rearing temperatures. Body size is shown for *Daphnia* at the third clutch.

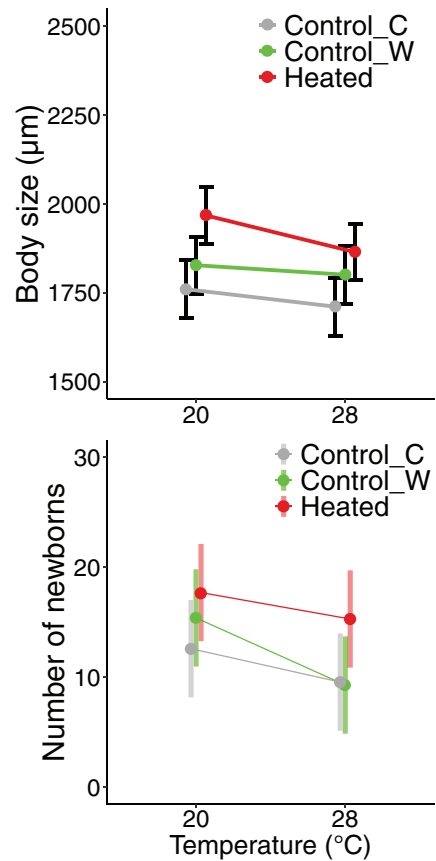


Fig 4. Body size (means and 95% confidence intervals; top panel) and fecundity (means and 95% confidence intervals; bottom panel) of *D. galeata* from heated, cold control (Control_C), and warm control lakes (Control_W) in the within-species experiment, under different temperatures. Body size is shown for *Daphnia* at the third clutch.

origin (origin: $\chi^2 = 0.81, p = 0.666$) but was generally larger in the warmer treatment (temperature: $\chi^2 = 18.59, p < 0.001$); no significant interaction between main factors was observed (Fig. S3).

Overall, *Daphnia* from heated lakes were predisposed to achieve a larger size than control *Daphnia*, as observed in both experiments (but their body size was constrained in situ). The size of these *Daphnia* was reduced in response to temperature increase, consistently with the temperature-size rule, in both field samples and experiments. In terms of fecundity, *Daphnia* from heated lakes were superior to those from controls, especially when exposed to increased temperature.

Body size contributors partitioning

On average, body size of both warm adapted groups of clones (i.e., from heated lakes and from warm controls) increased, in comparison to cold controls, and the constitutive evolution was observed to contribute the most to this change (Fig. 5). Furthermore, constitutive evolution contributed more to body size increase in *Daphnia* from heated lakes than in

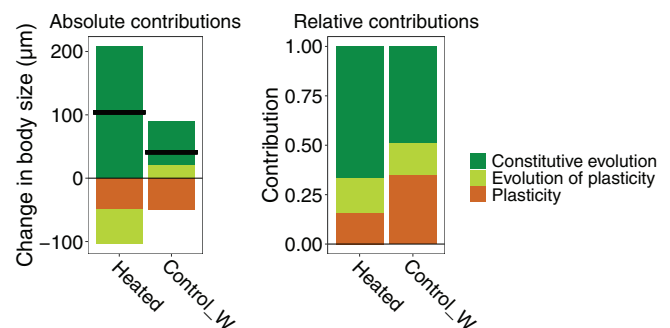


Fig 5. Absolute (left panel) and relative (right panel) contribution of constitutive evolution, phenotypic plasticity, and evolution of plasticity to differences in body size between *Daphnia* from heated lakes (Heated) and cold controls, as well as *Daphnia* from warm controls (Control_W) and cold controls. The cold control is used as a reference. The thick black line represents the average absolute change in body size (in comparison to the cold control at 20°C).

those from warm controls. Note that only the difference between *Daphnia* from heated lakes and cold controls was significant (see subsection *Within-species experiment*). The

plasticity component universally reduced body size in response to rearing temperature increase (temperature-size rule). In *Daphnia* from heated lakes evolution of plasticity additionally contributed to the body size reduction by a similar amount as the effect of plasticity. In the case of the warm controls, the absolute contribution of evolution of plasticity was relatively small, and weakened the strength of plastic size reduction. Overall, the body size contributors partitioning revealed that, while constitutive evolution promoted larger size in heated lakes, phenotypic plasticity acted in opposite direction, reducing the body size of *Daphnia* under elevated temperatures.

Discussion

Contrary to our hypothesis, *Daphnia galeata* clones isolated from warmer lakes did not evolve smaller body size than clones isolated from control lakes. Unexpectedly, our experiments suggested evolution of constitutively larger body size of *D. galeata* from heated lakes. This pattern could not have been anticipated from field data alone. In field samples, *Daphnia* from heated and control lakes had similar average body sizes, although at a low temperature range (winter conditions) there was a trend for *Daphnia* in heated lakes to be larger than in control lakes. The between-species experiment revealed that, when potentially confounding environmental factors are excluded, *Daphnia* from heated lakes become larger than *Daphnia* from control lakes. This is surprising because otherwise these two species frequently overlap in size (e.g., Stich and Lampert 1984; Kirdyasheva and Kotov 2019). As this experiment juxtaposed two different (although similar in size and hybridizing) species, we wanted to verify if within-species comparison provides similar results. The within-species experiment proved that *D. galeata* from heated lakes were constitutively larger than *D. galeata* from control habitats, indicating that long-term warming can induce evolution of larger body size in *Daphnia*.

Such a pattern seems contradictory, not only to general expectations related to global warming (e.g., Daufresne et al. 2009; Gardner et al. 2011), but also to the Metabolic Theory of Ecology (Brown et al. 2004). According to theory, metabolic rates of an organism (and related costs) increase with body mass and temperature, and since under temperature elevation resources eventually become limiting, an organism faces a trade-off, and allocates resources in a way that maximizes fitness (principle of allocation, Lloyd 1988). This usually results in body size reduction, allowing resources to be invested in reproduction (Atkinson 1994; Kozłowski et al. 2004). Evolution of larger size under elevated temperature might be perceived as a violation of temperature-size rule. However, in the case of *Daphnia*, larger body size directly correlates with efficiency in resource acquisition and general competitive strength (Brooks and Dodson 1965; Gliwicz 1990; Gliwicz et al. 2010). Assuming no resource limitation, larger

size should also become beneficial by increasing maximal reproductive investment (Bartosiewicz et al. 2015; Lühring et al. 2018), without need for a trade-off between offspring size and number. The additional metabolic requirements for larger size and reproductive investment can be balanced by the surplus of food resources in the environment, as temperature increase also boosts primary production (Gibbons and Sharitz 1981). However, temperature mediated increase in metabolic rate is bounded by an organism's physiological limitations in resource processing (Huey and Kingsolver 1989). Oxygen availability seems to be the limiting factor driving temperature-size rule, as its solubility and assimilation rate drop as water temperature rises (Pörtner et al. 2017; Walczyńska and Sobczyk 2017). Therefore, the increase in size is favorable only for temperatures at which resources are sufficient and no physiological restrictions exist. Such environmental conditions favoring large size occur in mild (ice-free) winters, when lakes are rich in resources (plenty of oxygen, less concentrated but better quality food than in summer) and predation rate is low (Sommer et al. 1986). Thus, we suggest that the body size increase observed in our study evolved because it was advantageous for active overwintering (instead of usual diapausing) during warm, iceless winters. Large-bodied *Daphnia* species and clones have higher filtering efficiency and resistance to starvation (Gliwicz 1990), allowing actively overwintering individuals to survive and reproduce effectively under winter conditions. Mild winters seem to promote active overwintering of *D. galeata*, facilitating its advantage over *D. longispina* during the subsequent season; the opposite pattern (i.e., advantage of *D. longispina*) was observed after more severe winters with ice cover (Zeis et al. 2010). The decreasing number of ephippia (diapause eggs) of *Daphnia* in heated lakes (Dziuba et al. 2020) indicates that *Daphnia* there overwinter actively, as opposed to diapausing. A similar active overwintering pattern in *D. galeata* has also been observed in other lakes during mild winters (Tsugeki et al. 2009; Zeis et al. 2010). In control lakes, where ice cover is still quite frequent, the annual production of diapause eggs remains constant (Dziuba et al. 2020), indicating that diapause is a still favored overwintering strategy. Thus, there is no advantage to being large in control lakes.

It may be argued that evolution of larger body size in *Daphnia* from heated lakes could have been triggered by hypothetically lower densities of fish in heated lakes, or by a decreased predation rate. Lower predation seems unlikely given data obtained from the same system reveals that lake heating resulted in increased fish predation pressure on zooplankton (Ejsmont-Karabin and Węgleńska 1988; Hillbricht-Ilkowska and Zdanowski 1988a), and warm water discharge generally attracts planktivorous fish resulting in increased fish density (Wierzowski and Godlewska 2001). Moreover, higher temperatures are generally known to increase fish predation on zooplankton, especially on large-bodied cladocerans (e.g., Gliwicz 1994; Brucet et al. 2010; He et al. 2018). Fish in

heated lakes have increased growth rates, and in some species increased fertility, in comparison with fish from non-heated lakes (Thorslund 1971). Therefore, the negative impact of predation on *Daphnia* body size is probably even stronger in heated than in control lakes (or at least similar). This is consistent with the lack of difference in body size of *Daphnia* in field samples from heated and control lakes throughout the warm season. In winter, however, when predatory pressure is usually relaxed (e.g., Sommer et al. 1986; Stibor and Lampert 2000), *Daphnia* from heated lakes seemed larger.

Our results indicate that extrinsic body size determinants can make actual evolutionary patterns (here increased body size) cryptic. A study of *D. magna* in an urban–rural environmental gradient (co-varying with thermal gradient) revealed an evolutionary shift toward smaller body size (Brans et al. 2017). This pattern was masked in situ by a countergradient ecological factor (probably invertebrate predation) that induced an increase in the mean body size of the population by elimination of small individuals. Cryptic evolution was also reported by Bonnet et al. (2017), who used pedigree analysis to detect selection for smaller size in a snow vole (*Chionomys nivalis*) population. Such selection for smaller size might otherwise not be observed in situ due to the masking effect of phenotypic plasticity. Inferences on trait changes based on field observations alone should be treated cautiously, and preferably supported with additional research approaches (such as experiments or genetic analyses). Observable ecological patterns and evolutionary mechanisms might be contradictory, and such countergradient variation can be easily overlooked. Thus, bringing together community ecology and evolutionary ecology will be crucial in forecasting the future fates of populations and communities (De Meester et al. 2019).

Both this study and that of Brans et al. (2017) found countergradient responses to temperature increase, but while we observed the evolution of large size in *Daphnia* from heated lakes, *D. magna* in warm urban ponds evolved to become smaller. This shows that adaptation to temperature increase can be strongly context-dependent even within the same genus. The constitutive size reduction observed in Brans et al. (2017) was explained by adaptation to the warmest periods, and specifically resource (e.g., oxygen) shortage. Conversely, the constitutive body size increase found in *Daphnia* from heated lakes is most likely beneficial during mild, iceless winters (it would not be beneficial during severe winters, when *Daphnia* switch to diapause). Nonetheless, in line with metabolic theory, the metabolic costs of large size increase with temperature. To avoid these costs, *Daphnia* from heated lakes also evolved stronger plasticity in size reduction in comparison to controls. Plastic size reduction under high lab rearing temperatures had a negligible effect on reproduction of *D. galeata* from heated lakes; they still produced more offspring than *Daphnia* from control lakes, without decreasing their quality (in terms of offspring size). This suggests that,

when facing higher metabolic costs arising from having larger size, *Daphnia* from heated lakes were able to efficiently distribute resources between reproduction and other metabolic expenditures, thus performing better than *Daphnia* from control lakes even at elevated temperatures. Since body size (and thus brood chamber capacity) of *Daphnia* was not larger in the warmer treatment, the larger size of offspring produced by all *Daphnia* (regardless of origin) at 28°C is most likely an outcome of rapid growth of newborns directly after birth and before collection.

Plasticity in resource allocation may explain how the *Daphnia* community in heated lakes is able to cope with a broader range of temperatures, obtaining a wider thermal breadth for reproduction. We do not exclude the possible role of seasonal shifts in *Daphnia* taxa composition in shaping the body size distribution in situ (e.g., Spaak and Hoekstra 1995). Nonetheless, it seems that the evolved capacity of *Daphnia* from heated lakes for strong size modulation and effective resource allocation between growth and reproduction provides an opportunity for large-bodied species to resist heat waves, or periods of more intensive fish predation. This in turn may facilitate survival of large filter-feeders during global warming. Larger body size may have far reaching consequences for functioning of ecosystems. For example, enhanced filtering efficiency of larger *Daphnia* (Gliwicz 1990) may counteract frequent algal blooms. A larger brood chamber enables *Daphnia* to produce more offspring (as in the case of *Daphnia* from heated lakes) or larger offspring (Bartosiewicz et al. 2015; Luhning et al. 2018), resulting in improved food availability at a higher trophic level. Moreover, grazing on algae while overwintering could promote the dominance of macrophytes and further improve water clarity (Scheffer et al. 1993).

We investigated the plastic and evolutionary responses of *Daphnia* to future levels of climate change, as predicted by the IPCC (2019). This was possible due to a combination of field and experimental surveys of a natural system that had experienced long-term (over 60 years) artificial temperature elevation. Taking advantage of altered ecosystems like heated lakes provides a great opportunity to address questions related to organisms' adaptation to climate change. Our results suggest that the commonly observed pattern of climate warming leading to body size decline in ectotherms (Daufresne et al. 2009; Gardner et al. 2011) might be caused by ecological pressure (e.g., increased predation) or as an effect of physiological responses (phenotypic plasticity—temperature-size rule). This pattern, however, is not necessarily underpinned by evolutionary fixation of smaller size. A recent meta-analysis found no evidence for climate warming mediated negative selection on body size, but a general (non-temperature related) trend of selection for large size (Siepielski et al. 2019). In line with our results, studies of organisms inhabiting waterbodies artificially heated by the Savannah River Plant (South Carolina, USA) have revealed that turtles and some fish species are larger in heated areas than in colder ones (Gibbons and Sharitz 1981).

Thermal enhancement of productivity in heated ecosystems improves food resources enough to enable an increased metabolic rate and thus growth rate in ectotherms. This enables the development of larger body size, which in the case of turtles (as in *Daphnia*) increases maximal clutch size (Gibbons et al. 1981). Many species tend to evolve toward larger body size (Gotanda et al. 2015) because it frequently correlates with higher fitness, and in many ectotherms it permits increased reproductive output (Kingsolver and Huey 2008). For those species that benefit from large body size, its reduction would be maladaptive, and thus should be applied only when a trade-off in resource distribution is necessary. In such cases, size reduction in response to temperature increase would be beneficial only as a reversible phenotypic plasticity, used exclusively at the upper part of an organism's thermal range and with no evolutionary fixation of smaller size. This may explain the lack of plastic size reduction in certain summer emerging insects (Cabanita and Atkinson 2006), which adapt only to warm conditions and thus may not benefit from larger size at the time of their appearance. Another interesting exception from the pattern of adaptive size reduction under temperature increase was discovered in salamanders (Luhring and Holdo 2015): larger size was found to be adaptive for survival of drought events, but otherwise the smaller species was able to outcompete the larger. The above examples all lead to the conclusion that constitutive size reduction should not be expected under climate change in those species that can benefit from large size. In such cases reversible plastic size modulation might be expected instead.

In heated lakes, long-term temperature elevation selected for larger individuals, which were nonetheless able to turn smaller when constrained by the environment. Interestingly, we found similar evolution of size increase when warm-adapted clones from lower latitudes (Italy) were compared to clones from higher latitudes (Germany), but the evolutionary component of body size determination was much stronger in *Daphnia* from heated lakes than from warm controls (Italy). We also discovered evolution of plasticity that enables efficient size reduction under thermal stress. This ability to trade-off resource allocations facilitates the wider thermal breadth of *Daphnia* from heated lakes. Our study design allowed the identification of countergradient responses in evolution and phenotypic plasticity. We argue that tracking adaptations of organisms to global warming requires the disentangling of heritable and plastic responses.

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Conflict of interest

None declared.

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