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A sterol-mediated gleaner–opportunist trade-off underlies the evolution of grazer resistance to cyanobacteria

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The human-caused proliferation of cyanobacteria severely impacts consumers in freshwater ecosystems. Toxicity is often singled out as the sole trait to which consumers can adapt, even though cyanobacteria are not necessarily toxic and the lack of nutritionally critical sterols in cyanobacteria is known to impair consumers. We studied the relative significance of toxicity and dietary sterol deficiency in driving the evolution of grazer resistance to cyanobacteria in a large lake with a well-documented history of eutrophication and oligo-trophication. Resurrecting decades-old *Daphnia* genotypes from the sediment allowed us to show that the evolution and subsequent loss of grazer resistance to cyanobacteria involved an adaptation to changes in both toxicity and dietary sterol availability. The adaptation of *Daphnia* to changes in cyanobacteria abundance revealed a sterol-mediated gleaner–opportunist trade-off. Genotypes from peak-eutrophic periods showed a higher affinity for dietary sterols at the cost of a lower maximum growth rate, whereas genotypes from more oligotrophic periods showed a lower affinity for dietary sterols in favour of a higher maximum growth rate. Our data corroborate the significance of sterols as limiting nutrients in aquatic food webs and highlight the applicability of the gleaner–opportunist trade-off for reconstructing eco-evolutionary processes.

1. Introduction

Aquatic ecosystems around the world are experiencing severe human-caused environmental changes, most notably nutrient pollution [1–3]. These anthropo-genic changes in the abiotic environment have imposed severe stress upon natural populations. Major stress that can be directly linked to lake eutrophication is the often-observed increase in the relative abundance of cyanobacteria in the phytoplankton community. Cyanobacteria often benefit from increasing dissolved nutrient (especially phosphorus) concentrations and have the potential to rapidly dominate phytoplankton communities, with far-reaching consequences for the entire food web [4,5]. Cyanobacteria are of poor food quality for zooplankton due to their morphology, production of harmful secondary metabolites, and the lack of essential nutrients, i.e. sterols and long-chain polyunsaturated fatty acids (PUFA) [6–8]. The freshwater keystone grazer *Daphnia* is incapable of synthesizing sterols *de novo* and thus depends on an adequate dietary sterol supply [9]. Labora-tory experiments have revealed that the growth of *Daphnia* on cyanobacterial diets is severely constrained by the lack of dietary sterols [8,10].

When cyanobacteria dominate the phytoplankton community, *Daphnia* is subject to strong natural selection [4,11,12], expected to favour those genotypes that are able to survive, grow and reproduce on a cyanobacteria-rich diet. In

Lake Constance, a large pre-Alpine lake in Central Europe, massive eutrophication from the 1950s to 1980 (figure 1) was accompanied by the rapid evolution of *Daphnia* resistance to cyanobacteria [13,14], as revealed by growth experiments with decades-old *Daphnia* genotypes hatched (resurrected) from dormant eggs that were chronologically deposited into the sediment. Since then, extensive restoration efforts resulted in reduced nutrient loads (oligotrophication) and a substantial decrease in cyanobacterial biomass in Lake Constance [15,16], leading to the re-emergence of *Daphnia* genotypes that are highly susceptible to cyanobacteria [17]. Previous studies on the Lake Constance *Daphnia* population have either not attributed the observed adaptation to a specific cyanobacterial trait [13,17] or have assumed that *Daphnia* adapted to cyanobacterial toxins [14]. No experimental work investigated so far to which nutritional constraint the *Daphnia* actually adapted to (toxin levels, reduced sterol availability or other constraints). The higher share of cyanobacteria in the phytoplankton community during eutrophication of Lake Constance presumably also reduced the availability of sterols for *Daphnia* nutrition. For northern boreal lakes, it has been reported that eutrophic lakes generally provide lower amounts of dietary sterols to consumers [18]. Considering that the growth of *Daphnia* is strongly influenced by the dietary sterol supply [9], this should have resulted in sterol-mediated nutritional constraints and potentially the evolution of a higher affinity for dietary sterols in the Lake Constance *Daphnia* population with eutrophication. Subsequently, with oligotrophication of the lake, the share of cyanobacteria decreased again, and therefore sterol availability should have increased, potentially resulting in relaxed selection on the ability to grow at low dietary sterol availabilities.

Here, we explore the relative significance of toxicity and sterol limitation for the evolution of resistance to cyanobacteria in the *Daphnia galeata* population of Lake Constance. In growth experiments, we exposed *D. galeata* genotypes, resurrected from dormant eggs to toxic and non-toxic cyanobacteria. The dormant eggs were produced by *Daphnia* that inhabited the lake during peak-eutrophic, post peak-eutrophic and oligotrophic conditions. This allowed us to disentangle the effects of toxicity and dietary sterol-deficiency on the adaptation of *Daphnia* to cyanobacteria. We hypothesized that *Daphnia* genotypes from peak-eutrophic periods were adapted to low dietary sterol availabilities and that this adaptation was lost again with oligotrophication and the disappearance of cyanobacteria from the lake. Using sterol-limited growth response curves, we explored whether the adaptation of *Daphnia* to trophic state-related changes in dietary sterol availability followed a gleaner–opportunist trade-off, where a lower minimum resource requirement comes at a cost of a lower maximum growth rate.

2. Methods

(a) Study site

In Lake Constance, total phosphorus (P) concentrations during winter mixing (TP_{mix}) increased more than tenfold from about 7 µg l^{-1} in the 1950s to more than 80 µg P l^{-1} in the early 1980s because of human activities [19]. Intense restoration efforts, and the subsequent reduction of phosphorus loads, resulted in a decrease in TP_{mix}, with concentrations that are currently similar
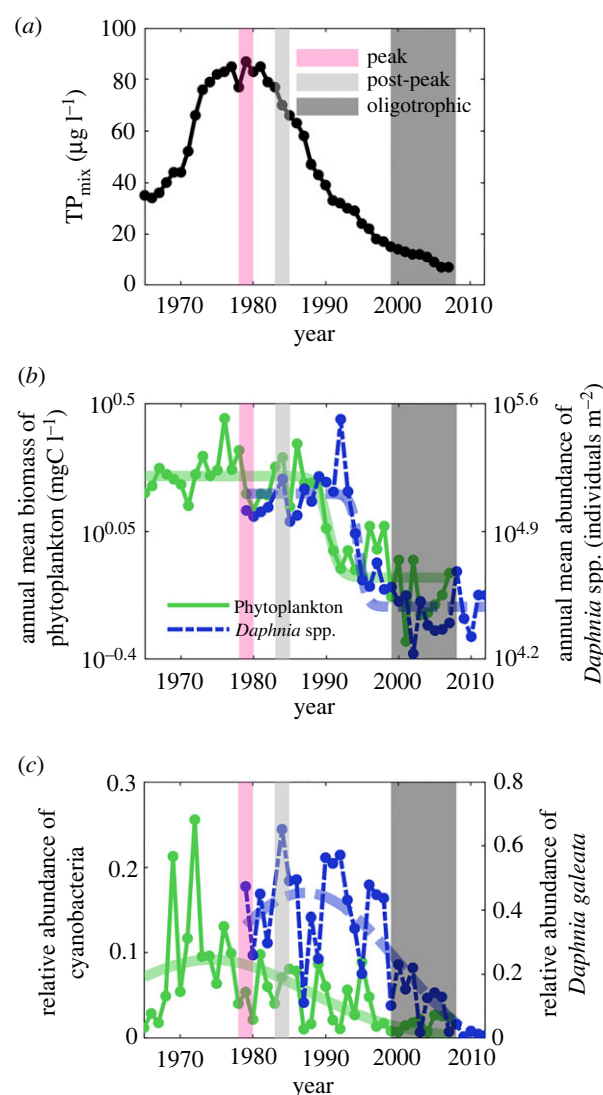

Figure 1. Temporal changes in (*a*) total phosphorus concentration during winter mixing (TP_{mix}), (*b*) mean annual biomass of phytoplankton (green) and *Daphnia* spp. (blue) and (*c*) relative abundance of cyanobacteria (green) and *D. galeata* (blue) in Upper Lake Constance from 1965 to 2012. Data points represent actual measurements, whereas thick solid lines in (*b*) and (*c*) represent appropriate curves fitted to the data capturing their overall trends. Pink, light grey and dark grey vertical shades indicate the time periods from which the different *D. galeata* genotypes were resurrected. Phyto- and zooplankton data were derived from the long-term monitoring of the IGKB (International Commission for the Protection of Lake Constance).

to those measured in the 1950s [15,20]. The phytoplankton community composition changed accordingly. The relative contribution of cyanobacteria to the total phytoplankton biomass decreased from up to 25% during the eutrophic phase (25% mean percentage; 60% maximum percentage [14]) to less than 3% at the beginning of the twenty-first century (figure 1, [15]). The phytoplankton data presented here derive from the long-term monitoring programme of the International Commission for the Protection of Lake Constance (IGKB), in which phytoplankton is sampled twice per month at the deepest part of Upper Lake Constance [20]. Cell concentrations were converted into biomasses using species-specific cell volumes [15]. Phytoplankton biomasses were averaged over the growing season (May to October) for each study year (1965–2007).

Daphnia species composition changed with eutrophication of Lake Constance [21,22]. During the first half of the twentieth century, *Daphnia longispina* (formerly referred to as *Daphnia hyalina*)

was the only *Daphnia* species occurring in Upper Lake Constance. In the late 1950s, presumably with the onset of eutrophication, *D. galeata* invaded Upper Lake Constance [22]. Recently, apparently in 2014, a third *Daphnia* species, *Daphnia cucullata*, appeared in Upper Lake Constance [20]. While *D. galeata* dominated the lake in the 1980s, the abundance of *D. galeata* has been declining in recent years [23]. At present, the *Daphnia* species assemblage in Upper Lake Constance is represented by *D. longispina*, *D. cucullata* and hybrids of *D. longispina* and *D. galeata* [20,23].

(b) Sediment cores and resurrection of *Daphnia galeata*

Sediment cores were taken in spring and autumn 2017 in.the Bay of Friedrichshafen in Upper Lake Constance (for more information please see supplementary material). Ephippia were isolated, *Daphnia* genotypes hatched, and the species identity was determined (see electronic supplementary material or [17] for more details). For the experiments, only genotypes were used that could be clearly assigned to *D. galeata*. Hybrids between *D. galeata* and *D. longispina* were not used here. Unfortunately, like in other studies [13,14], we were not able to hatch genotypes older than 40 years, resulting in no genotypes from the pre-eutrophication period. In the experiments, the peak eutrophic period was represented by four genotypes from 1978 to 1980, the post-peak eutrophic period by four genotypes from 1983 to 1985, and the oligotrophic period by nine genotypes from 1999 to 2008. The trophic status classification refers to the total phosphorus concentration and not to the abundance of cyanobacteria in the lake.

(c) Growth experiments

A first growth experiment was conducted to assess the relative significance of toxicity and sterol limitation in driving the evolution of grazer resistance to cyanobacteria. This experiment comprised three different food environments. (1) The green alga *Acutodesmus obliquus* (SAG 276–3a, 100% of total provided carbon), which is a good food for *Daphnia* (hereafter 'good' food, sterol content 8.7 µg mg C^{-1}). (2) A mixture of *A. obliquus* (80% of the total provided carbon, providing 7.0 µg mg C^{-1} of sterols) and the toxic cyanobacterium *Microcystis aeruginosa* (PCC 7806; 20% of total provided carbon), which produces various microcystins and cyanopeptolins [24]. This relative food composition has been used previously to assess the evolution of resistance to cyanobacteria [13,17]. A sterol limitation, potentially resulting from the exchange of 20% of the total provided carbon by *M. aeruginosa*, is superimposed by the toxicity of the strain used here [8] (i.e. growth-limiting effects can be attributed mostly to toxicity; hereafter 'toxic' food). (3) The cyanobacterium *Synechococcus elongatus* (SAG 89.79; 100% of total provided carbon), which is well assimilable and known to cause severe sterol-limitation in *Daphnia*. The *S. elongatus* strain used here does not provoke any symptoms of intoxication in *Daphnia* and thus has become a model food for studying sterol-limited growth responses [9,25–27]. It was provided as the sole food source to be able to explore the full range of sterol limitation (hereafter 'non-toxic' food). Sterols were not detected in either of the cyanobacteria used here. Third-clutch neonates born within less than 12 h were isolated from age-standardized cohorts of each genotype and placed individually into jars containing 80 ml of filtered (less than 0.2 µm) Lake Constance water and 2 mg C l^{-1} of the respective food suspension. A subsample of 20 neonates was taken from the isolated cohort of each clone for initial dry mass determination after freeze-drying. Each treatment consisted of 10 jars (food × clone combination × jar; three food treatments × 17 genotypes × 10 jars = a total of 510 jars). Daphniids were transferred every other day into new jars with freshly prepared food suspensions. At day

five of the experiment, shortly before the first animals released their first-clutch offspring, daphniids were taken out of their jars, rinsed with ultra-pure water, transferred into pre-weighed aluminium boats (three individuals were pooled to obtain one dry mass, resulting in $n = 3$), and stored at $-80°C$ until they were freeze-dried and weighed (±0.1 µg) for dry mass determination.

In a second growth experiment, sterol-limited growth responses were recorded for all genotypes. For this experiment, second-clutch neonates born within <12 h were isolated and randomly transferred to jars containing 80 ml of filtered lake water (<0.2 µm) and 2 mg C l^{-1} of *S. elongatus* (three individuals per jar). Dietary sterol gradients were established by adding increasing amounts of cholesterol-containing liposomes to the jars. Liposomes were prepared as described previously [10]. To account for the increasing amounts of carbon provided through the addition of liposomes, sterol-free control liposomes were added so that each jar was supplemented in total with the same amount of liposomes (for details, see [26]). Each dietary sterol concentration was provided in triplicates ($n = 3$). Daphniids were transferred daily into new jars containing freshly prepared food suspensions. At day five, daphniids were taken out of their jars for dry mass determination as described above (the three individuals from one jar were pooled for one dry mass). *A. obliquus*, *M. aeruginosa* and *S. elongatus* were cultured semi-continuously in Cyano medium [28] at 20°C and illumination at 120 µmol $m^{-2} s^{-1}$ in aerated 5-l vessels. The vessels were filled with 4 l of medium of which 1 l was replaced with fresh medium every other day. This culturing method resulted in single-cell growth, i.e. there was no colony formation in none of the cultures. All three species are known to be well ingestible by *Daphnia*. The same *S. elongatus* culture was used for both experiments. Food suspensions were obtained from the respective cultures by centrifugation and resuspension of cells in filtered (<0.2 µm) lake water. The carbon concentrations of the food suspensions were estimated from previously established carbon-extinction equations (480 nm).

(d) Data analysis

To illustrate the general trends in our time series, we fitted a sigmoidal function to the absolute abundances of the phytoplankton community and the *Daphnia* complex, respectively (figure 1*b*), and a *Gaussian* function to the relative abundances of cyanobacteria and *Daphnia galeata*, respectively (figure 1*c*).

Mass-specific juvenile somatic growth rates (g, d^{-1}) were calculated from the increase in dry mass from the start of the experiment (M_0) until day five of the experiment (M_t) with time (t) expressed as age in days:

$$g = \frac{\ln(M_t) - \ln(M_0)}{t}$$

The reduction in somatic growth rates (GRR) caused by the exposure to cyanobacteria was determined according to Hairston *et al.* [13], using the equation:

$$GRR_T = \frac{(g_{good} - g_{toxic})}{g_{good}}$$

or

$$GRR_{NT} = \frac{(g_{good} - g_{non-toxic})}{g_{good}}$$

with g_{good}, g_{toxic} and $g_{non-toxic}$ representing the juvenile somatic growth rates (d^{-1}) on pure *A. obliquus*, 80% *A. obliquus* and 20% *M. aeruginosa*, and pure *S. elongatus*, respectively. Juvenile somatic growth rates and GRRs were analysed using two-factorial ANOVAs, followed by Tukey's HSD post hoc tests, using the functions *anovan()* and *multcompare()* from *MATLAB*. We did not

Table 1. Statistical analysis of somatic growth rates of *Daphnia* genotypes on the different food sources (figure 2*a*,*b*) and growth rate reductions (figure 2*c*,*d*). To test for significant differences among somatic growth rates, a two-way ANOVA with the factors 'food' and 'time' was performed, followed by a multiple comparison test (Tukey's HSD; electronic supplementary material tables S1 and S2).

two-way ANOVA					
figure 2*a* and *b*					
source	sum of squares	degrees of freedom	mean squared differences	*F*-value	*p*-value
food	0.338	2	0.169	48.75	**<0.001**
time	0.135	2	0.068	19.5	**<0.001**
error	0.159	46	0.003		
total	0.632	50			
figure 2*c* and *d*					
source	sum of squares	degrees of freedom	mean squared differences	*F*-value	*p*-value
food	0.024	1	0.024	0.86	0.36
time	0.607	2	0.304	11.14	**<0.001**
error	0.817	30	0.027		
total	1.448	33			

find a significant interaction between the two different factors 'food' and 'age', and so omitted the interaction term from further analysis.

Somatic growth rates of the different *Daphnia* clones increased nonlinearly with the dietary sterol concentration. We fitted modified Bertalanffy growth curves [10] to three subsets of the dataset representing three different groups of *Daphnia* clones, that co-occurred either under peak eutrophic, post-peak eutrophic or oligotrophic conditions (figure 1*a*), using the following representation of the Bertalanffy growth function (*g*):

$$g(s) = g_{max}(1 - 4^{-s/H})$$

with *s* [µg mg C^{-1}], g_{max} [1 d^{-1}] and H [µg mg C^{-1}] denoting the supplemented sterol concentration, the maximum somatic growth rate and the threshold value at which the maximum growth rate is reduced by 25%, respectively [29]. Since sterols are essential for somatic growth of *Daphnia*, we assumed $g(0) = 0$. To estimate g_{max} and H for a given dataset we used the function *nlmfit()* from *MATLAB*. We tested for significant differences between the parameter estimates of the different treatments using *Welch's t*-test [30], assuming a significance level alpha of 0.05 (for further details please see supplementary material). Data were analysed using *MATLAB*, v. 9.6.0.

3. Results

Juvenile somatic growth rates of *Daphnia* were reduced on both cyanobacterial diets, i.e. irrespective of toxicity. However, genotypes from peak and post-peak eutrophic periods were generally less susceptible to cyanobacteria than genotypes from oligotrophic periods (table 1 and figure 2*a*). On both cyanobacterial diets, *Daphnia* genotypes from peak and post-peak eutrophic periods had similar growth rates, which were significantly higher than those of genotypes from the oligotrophic period (table 1 and figure 2*a*). The slopes of the reaction norms obtained for the toxic and the non-toxic cyanobacterial food both increased gradually with oligotrophication, i.e. from peak eutrophic to oligotrophic

conditions (figure 2*a*,*b*), which was also reflected in the proportion by which growth rate was reduced on the cyanobacterial diets (growth rate reduction, GRR; see Methods; figure 2*c*,*d*). On both cyanobacterial diets, genotypes from the peak eutrophic period showed a significantly lower GRR (=higher resistance) than genotypes from the oligotrophic period, whereas genotypes from the post-peak eutrophic period showed intermediate GRRs (table 1 and figure 2*b*,*c*). Irrespective of the period from which the genotypes were resurrected, the variance of resistance among *Daphnia* clones tended to be higher for the non-toxic than for the toxic cyanobacterial food (two-sample *F*-test for equal variances: $F = 8.93$, d.f.1 = d.f.2 = 3, $p = 0.11$ (peak eutrophic); $F = 13.04$, d.f.1 = d.f.2 = 3, $p = 0.06$ (post-peak eutrophic); $F = 7.44$, d.f.1 = d.f.2 = 8, $p = 0.01$ (oligotrophic); figure 2*d*). The GRRs obtained on the toxic and the non-toxic cyanobacterial diet were significantly correlated with each other (Spearman's rank correlation coefficient = 0.609; $p < 0.05$; figure 2*e*).

Sterol-limited growth responses, established by adding increasing amounts of cholesterol-containing liposomes to the non-toxic cyanobacterium *S. elongatus*, differed among *Daphnia* genotypes originating from the different periods (figure 3). Maximum somatic growth rates of genotypes from the peak eutrophic period (0.14 d^{-1}) were on average lower than the maximum somatic growth rates of genotypes from the post-peak eutrophic period (0.21 d^{-1}; $p = 0.021$) and those of genotypes from the oligotrophic period (0.19 d^{-1}; $p = 0.026$; figure 3*d*). The dietary sterol content at which maximum somatic growth rates were reduced by 25% (defined here as threshold level for sterol-limited somatic growth) was on average significantly lower for genotypes from the peak eutrophic period (3.7 µg mg C^{-1}) than for genotypes from the post-peak eutrophic (25.4 µg mg C^{-1}; $p = 0.022$) and the oligotrophic period (26.7 µg mg C^{-1}; $p = 0.004$; figure 3*d*). Genotypes from the post-peak eutrophic and the oligotrophic period did not differ significantly in their
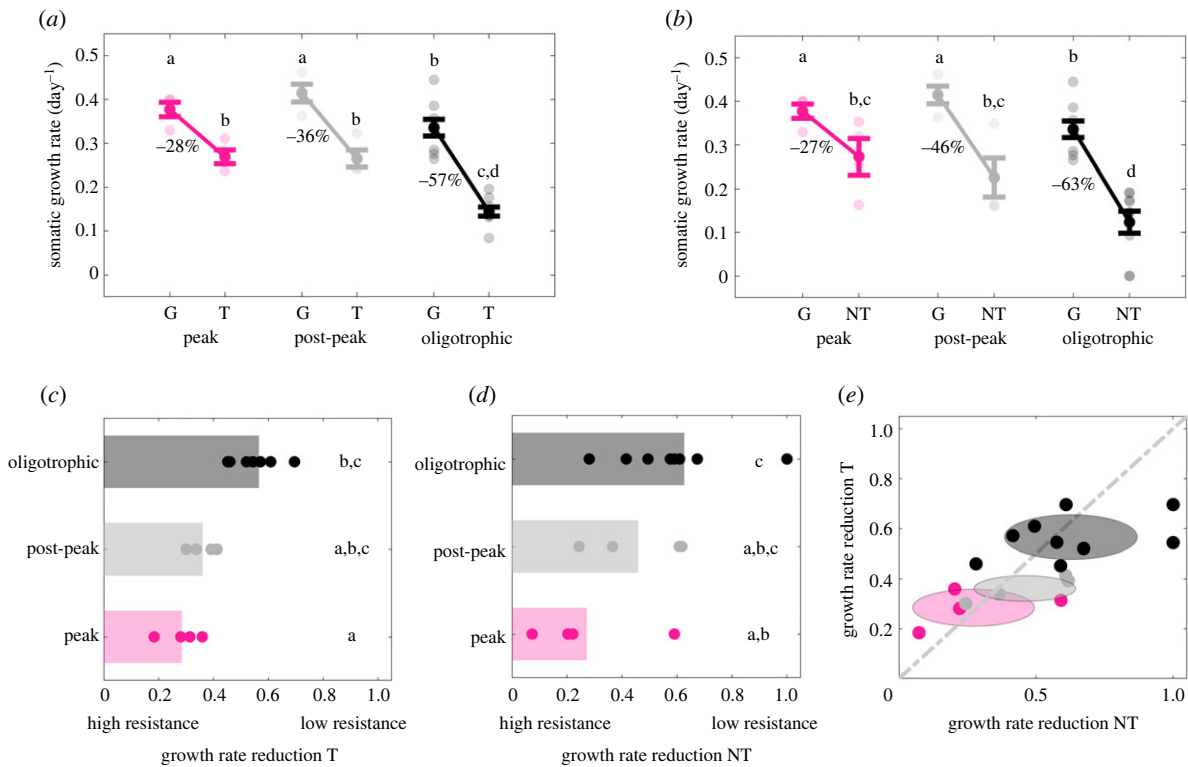
Figure 2. Reaction norms (juvenile somatic growth rates as a function of food quality) of *Daphnia* genotypes inhabiting the lake during different periods (peak eutrophic, post-peak eutrophic, oligotrophic): (*a*) reaction norms on good food (G) versus toxic cyanobacterial food (T), (*b*) reactions norms on good food (G) versus non-toxic cyanobacterial food (NT). Resistance of *Daphnia* genotypes to (*c*) toxic cyanobacteria and (*d*) non-toxic cyanobacteria. Single data points depict average ($n = 3$) growth rate reductions (GRRs) for individual genotypes, bars show the average GRR of all genotypes within a single period (pink = peak eutrophic, light grey = post-peak eutrophic and dark grey = oligotrophic; high GRR = low resistance). (*e*) Relationship between GRRs for individual genotypes on the two cyanobacteria treatments (T and NT). For each period, the spread of data points is visualized by an ellipse. The center of an ellipse is given by the arithmetic means of the GRR_{NT} values (*x*-coordinate) and the GRR_T values (*y*-coordinate) of the corresponding period. Accordingly, the semi-major and the semi-minor axes of the ellipse are further given by the corresponding standard deviations of the GRR_{NT} and GRR_T values, respectively. Note that the oligotrophic period has nine genotypes, however, the data points are partly overlapping so they cannot be distinguished clearly in all graphs.

maximum somatic growth rates ($p = 0.632$) and their threshold levels for sterol-limited somatic growth ($p = 0.908$). Hence, there was a negative relationship between the initial slope (affinity for sterols) and the maximum somatic growth rate of the different *Daphnia* genotypes originating from the three different periods.

4. Discussion

(a) Toxic versus non-toxic cyanobacteria

The adaptation of *Daphnia* to toxic cyanobacteria has been studied intensively in past decades [11,13,31]. However, no food quality aspect other than the production of harmful secondary metabolites was taken into consideration as a factor driving the evolution of resistance. We demonstrate here that temporal changes in the abundance of cyanobacteria in a large lake with a distinct eutrophication and oligotrophication history were associated with changes in dietary sterol-requirements in the lake's *D. galeata* population.

On cyanobacteria-containing diets, genotypes from eutrophic periods (peak and post-peak) achieved higher somatic growth rates than genotypes from the oligotrophic period, irrespective of whether a toxic or a non-toxic cyanobacterium was used as food. The *M. aeruginosa* strain used here is well-known to produce microcystins and several other harmful secondary metabolites [24] causing reduced growth and high mortality in *Daphnia* [8,32]. Sterol

supplementation does not improve the quality of this *M. aeruginosa* strain as food for *Daphnia*, indicating that sterol-mediated nutritional constraints are superimposed by the toxicity of this strain [8]. In our experiment, only 20% of the total provided carbon was represented by *M. aeruginosa*. This share has been used previously to assess the expression and subsequent loss of grazer resistance to cyanobacteria in Lake Constance [13,14,17]. A similar percentage of cyanobacteria occurred in Lake Constance a few years before the peak-eutrophic period from which genotypes where resurrected from. During the peak-eutrophic period, the share of cyanobacteria was already decreasing. The adaptation of populations to changing environmental conditions generally occurs with a certain time lag, mostly depending on the strength of natural selection and the generation time of the organisms. Thus, the adaptive state of a natural population to a previously encountered selection pressure can be observed for some time after the relaxation or reversal of a selection pressure, which is also evident in our data.

To test our hypothesis that the higher resistance of genotypes from eutrophic periods [13,17] was not exclusively mediated through a higher resistance to cyanobacterial toxins, we additionally used a non-toxic cyanobacterium (*S. elongatus*) as food. The *S. elongatus* strain used here has become a model for studying sterol-limited growth responses, it is well assimilated by *Daphnia* and does not provoke any signs of toxicity in sterol supplementation experiments [10,25,26]. The reaction norms obtained for the
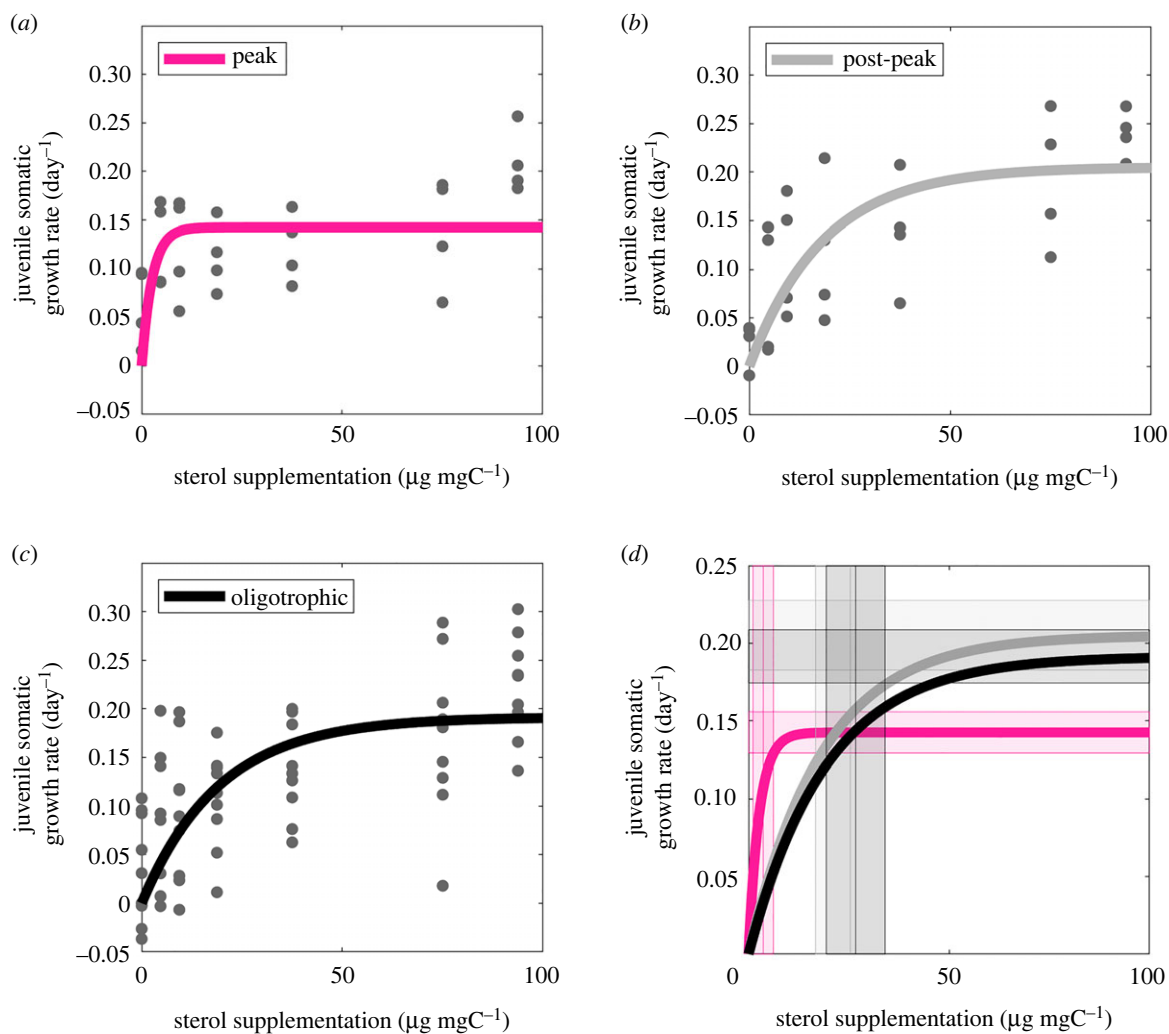
Figure 3. Sterol-limited growth responses of the different *D. galeata* genotypes originating from peak eutrophic (*a*), post-peak eutrophic (*b*) and oligotrophic (*c*) conditions. Functional response curves that were fitted to the growth responses in panels (*a*,*c*) are combined in panel (*d*) (pink: peak eutrophic; grey: post-peak eutrophic; black: oligotrophic conditions), with vertical and horizontal lines showing estimates of parameters H and g, respectively (*d*). Shaded areas indicate standard errors. Note: The oligotrophic period is represented by nine genotypes. However, the data points are partly overlapping so they cannot be distinguished clearly in all graphs.

toxic and the non-toxic cyanobacterial food both revealed a gradual increase in slope with oligotrophication (figure 2*a*,*b*). This pattern was also reflected in the GRRs, indicating a gradual decrease in resistance to cyanobacteria with oligotrophication for both the toxic and the non-toxic food (figure 2*c*, *d*). The fact that the toxic and the non-toxic cyanobacterial food induced similar growth response patterns indicates that the evolution and subsequent loss of grazer resistance to cyanobacteria was not exclusively driven by toxicity. It rather suggests that the two food quality constraints, 'toxicity' and 'sterol deficiency', imposed independent selection pressures. The higher inter-clonal variability of GRRs on the non-toxic food implies greater trait variation for sterol sensitivity than for toxin resistance in the studied genotypes (figure 2), which also indicates that the mechanisms underlying both adaptations were different. A higher share of toxic cyanobacteria is intrinsically linked to a lower dietary sterol supply, thus requiring adaptations to both food quality constraints. However, cyanobacteria are not necessarily toxic, emphasizing the significance of sterol-mediated nutritional constraints for the evolution of grazer resistance to cyanobacteria. Our data suggest that the *D. galeata* population of Lake Constance has responded to eutrophication by increasing its tolerance to

toxic cyanobacteria and, concomitantly, by increasing its ability to grow at low dietary sterol availabilities.

(b) Gleaner–opportunist trade-off

In *Daphnia*, juvenile somatic growth rate is often strongly correlated with population growth rate [9,33–36]. For *D. galeata* specifically, this correlation, in response to changes in dietary sterol content, has been shown to be outstandingly strong ($r = 0.9941$, $p < 10^{-13}$; for further details see supplementary material) [9]. The juvenile somatic growth rates reported here may thus serve as surrogates for population growth rate of *D. galeata*. The sterol-limited growth response curves we present here therefore suggest that the adaptation of *Daphnia* to trophic state-related changes in dietary sterol availability followed a gleaner–opportunist trade-off. The gleaner–opportunist trade-off was originally defined as one between maximum growth rate and minimum resource requirement with the organism showing minimum resource requirements being the gleaner and the organism showing maximum growth being the opportunist [37,38]. Originally described for population growth in phytoplankton, the gleaner–opportunist trade-off is commonly used in ecology

to explain differences in performance at low and high resource concentrations or light availability [39–41]. In our dataset, *Daphnia* genotypes from the peak-eutrophic period showed a 'gleaner' response, manifested in their ability to exploit low dietary sterol levels, while genotypes from the post-peak eutrophic and the oligotrophic period showed an 'opportunist' response, manifested in their higher maximum growth rates. This trade-off implies that the genotypes from peak-eutrophic conditions (gleaner) achieved a higher affinity for dietary sterols at the cost of a lower maximum growth rate. By contrast, genotypes from post-peak eutrophic and oligotrophic conditions (opportunists) expressed a lower affinity for dietary sterols, and thus a weaker performance at low dietary sterol concentrations, allowing for a higher maximum growth rate at saturating sterol concentrations. The adaptation to low dietary sterol concentrations presumably involved significant changes in the overall energy budget. The possible energetic costs of a higher sterol affinity will likely not be visible during low dietary sterol supply, because sterol limitation will prevent energy allocation into somatic growth. At non-limiting dietary sterol supply, however, energetic costs that are potentially associated with a higher sterol affinity may constrain maximum growth rate. Genotypes that exhibit a lower sterol affinity should thus have a growth advantage under oligotrophic conditions (high sterol supply), whereas those exhibiting a higher sterol affinity should be more competitive under eutrophic conditions (low sterol supply; figure 3). Laboratory experiments have shown that carbon pathways in *Daphnia* are strongly influenced by sterol-mediated nutritional constraints: at high food quantity, a low dietary sterol supply has been shown to decrease the overall (bulk) carbon assimilation efficiency and to increase carbon losses due to egestion, excretion, and respiration, resulting in a lower net growth efficiency [25]. At the same time, the sterol assimilation efficiency of *Daphnia* has been shown to increase at low dietary sterol supply [25]. Taken together, this suggest that optimizing sterol uptake mechanisms at low dietary sterol supply is costly, thus providing a possible mechanistic explanation for the gleaner–opportunist trade-off we describe here.

Our data add to the current discussion about the general validity and applicability of the gleaner–opportunist trade-off, which is a critical assumption in many ecological models [42–44]. Based on a positive relationship between maximum clearance rate and maximum ingestion rate across a wide range of heterotrophic eukaryotes, Kiørboe & Thomas [42] concluded that there is no empirical evidence for the existence of a gleaner–opportunist trade-off among heterotrophs. By contrast, Letten & Yamamichi [44] argued that this interpretation is inconsistent with the original definition of the gleaner–opportunist trade-off, describing the trade-off between maximum population growth rate and minimum resource requirement [37], and contingent on a close relationship between ingestion rate and population growth rate. The latter is a critical assumption given the various factors that additionally may affect population growth, such as conversion efficiency or mortality [45–47]. Based on this complexity and thus the various ways in which a gleaner–opportunist trade-off may establish itself at the population and community level, Letten & Yamamichi [44] concluded that it is premature to rule out the importance of the gleaner–opportunist trade-off for the maintenance of species diversity in natural ecosystems. Our results support

this reasoning by demonstrating the existence of a novel gleaner–opportunist trade-off for heterotrophs with respect to food quality. The overall relationship between handling time and attack rate of a consumers' functional response with respect to the density of a resource that Kiørboe & Thomas [42] emphasize is not relevant for the food quality-mediated gleaner–opportunist trade-off we describe here. Instead, the observed trade-off is akin to the gleaner–opportunist trade-off observed among autotrophic phytoplankton species that are competing for essential mineral nutrients, such as nitrogen or phosphorus [48–50].

A gleaner–opportunist trade-off is expected to promote the coexistence of functionally similar species or genotypes because it requires the different players to share common resources [51]. Experimental evidence comes from studies on rotifers, where the competitive outcome between two very similar species has been found to depend on food quantity [52]. By contrast, the resurrection ecology approach we applied here revealed a gleaner–opportunist trade-off in a natural *Daphnia* population (i.e. among different genotypes of the same species) that shaped its evolutionary response to trophic state-related changes in food quality. While the 'gleaner genotype' had a competitive advantage at low dietary sterol availabilities under eutrophic conditions, it was later outcompeted by the 'opportunist genotype' at increasing dietary sterol availabilities that were linked to oligotrophication. The sterol-limited growth response curves suggest that the adaptation to low dietary sterol levels was already lost in the mid-1980s (post-peak eutrophic conditions). Assuming one sexual reproduction cycle per year, which is reasonable for natural *Daphnia* populations in large temperate lakes, it therefore took a maximum of five sexual generations for the gleaner adaptation to be lost due to relaxed selection. This reversed evolution appears to have happened even faster than the previously described reversed evolution of grazer resistance to toxicity, which was reported to be lost between the 1990s and 2000s [13,14,17]. Historical data on toxin or sterol concentrations are not available for Lake Constance, but the decreasing share of potentially toxic cyanobacteria in the phytoplankton community implies that cyanobacterial toxin concentrations decreased while dietary sterol concentrations increased with oligotrophication. Detrimental effects of toxicity likely occur at lower dietary proportions of cyanobacteria than detrimental effects resulting from sterol limitation, potentially explaining why the resistance to toxicity persisted longer in the population than the higher sterol affinity.

(c) Fate of *D. galeata* in Lake Constance

Daphnia galeata invaded the Lake in the early 1950s, presumably with the onset of eutrophication. The exact mode of invasion, as well as the origin of the species, remain unknown; possible explanations can be found in the literature [21,23,36]. *Daphnia galeata* was the dominant species in routine zooplankton samples during the eutrophic period [23,53], and also in the resting egg bank [21]. To minimize any effects of hybridization with the native *D. longispina* on our study results we used only those genotypes for our experiments that could be clearly assigned to the species *D. galeata*. Our results demonstrate that the *D. galeata* population in Lake Constance adapted its trait composition to the altered food quality conditions, i.e. the decrease in toxin concentrations

and the increase in sterol availability with oligotrophication. It appears, however, that these trait adaptations were not sufficient for the long-term persistence of *D. galeata* in the lake, as indicated by the constantly decreasing numbers of *D. galeata* in routine zooplankton samples (figure 1*c*). This evolutionary failure may reflect other potentially detrimental environmental changes with which *D. galeata* was not able to cope. Compared with the co-occurring species *D. longispina*, *D. galeata* is presumably less competitive at low food quantity [36] and does not perform diel vertical migration to escape vertebrate and invertebrate predation [54,55]. Other changes that were observed with oligotrophication of Lake Constance include the incursion of the three spined stickleback into the pelagic zone in about 2013 [56], and the appearance of a third *Daphnia* species, *Daphnia cucullata*, in 2014 [23].

Experimental evidence from other ecosystems has shown that genetic variation in natural *Daphnia* populations allows adaptation to various environmental changes [57–59], such as alterations in elemental food quality [60,61], temperature [62], metal concentrations [63] or predation intensity [64]. While natural changes in lake trophic state are generally slow [65], human-caused changes in lake trophic state typically proceed at much higher rates. In Lake Constance, it roughly took 20 years from peak-eutrophic to restored oligotrophic conditions. It appears that the trait adaptations we observed here in the *D. galeata* population of Lake Constance have been insufficient to face the multiple changes that were associated with changes in trophic state and to prevent competitive replacement by other *Daphnia* species. Although *D. galeata* successively disappeared from Lake Constance, its hybrids with *D. longispina* are still present [23]. The role of hybridization for the long-term survival of the *D. galeata complex* in Lake Constance is unclear. Future research should explore the effects of hybridization on eco-evolutionary dynamics [36] and its potential to prevent the terminal extinction of *D. galeata* through evolutionary rescue in Lake Constance.

4. Conclusion

Lake ecosystems around the globe are suffering from nutrient pollution and the associated proliferation of harmful cyanobacteria. In past decades, eutrophication has been reversed in many lake ecosystems through extensive restoration measures. In Lake Constance, trophic state-related changes in the relative abundance of cyanobacteria resulted in the evolution and the subsequent loss of grazer resistance to cyanobacteria. We demonstrate here that this evolution of grazer resistance involved changes in dietary sterol requirements, challenging the common assumption that changes in the ability to cope with cyanobacteria are exclusively mediated through an adaptation to cyanobacterial toxins. Sterol-limited growth responses of *Daphnia* genotypes inhabiting the lake during eutrophic and oligotrophic periods revealed a novel gleaner–opportunist trade-off between sterol affinity and maximum growth rate. Genotypes from eutrophic periods expressed a higher sterol affinity at the cost of a lower maximum growth rate, whereas genotypes from oligotrophic periods expressed a lower sterol affinity in favour of a higher maximum growth rate, which is consistent with the observed temporal changes in the relative abundance of cyanobacteria and thus dietary sterol availability. Even though the significance of dietary sterols for the performance of zooplankton has been demonstrated in numerous laboratory studies, the role of sterols in mediating the evolution of grazer resistance to cyanobacteria had not yet been explored. Our data suggest that this character trait will be crucial for understanding ecological as well as evolutionary processes and ultimately for maintaining biodiversity and ecosystem functioning.

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