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A test for microbiome-mediated rescue via host phenotypic plasticity in *Daphnia*

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Phenotypic plasticity is a primary mechanism by which organismal phenotypes shift in response to the environment. Host-associated microbiomes often change considerably in response to environmental variation, and these shifts could facilitate host phenotypic plasticity, adaptation, or rescue populations from extinction. However, it is unclear whether changes in microbiome composition contribute to host phenotypic plasticity, limiting our knowledge of the underlying mechanisms of plasticity and, ultimately, the fate of populations inhabiting changing environments. In this study, we examined the phenotypic responses and microbiome composition of 20 genetically distinct *Daphnia magna* genotypes exposed to non-toxic and toxic diets containing *Microcystis*, a cosmopolitan cyanobacterium and common stressor for *Daphnia*. *Daphnia* exhibited significant plasticity in survival, reproduction and population growth rates upon exposure to *Microcystis*. However, the effects of *Microcystis* exposure on the *Daphnia* microbiome were limited, with the primary effect being differences in abundance observed across five bacterial families. Moreover, there was no significant correlation between the magnitude of microbiome shifts and host phenotypic plasticity. Our results suggest that microbiome composition played a negligible role in driving host phenotypic plasticity or microbiome-mediated rescue.

1. Introduction

Projected biodiversity losses from anthropogenic global change this century [1,2] necessitate an understanding of the magnitude and mechanisms of adaptive phenotypic responses to environmental change [3,4]. Although organisms can rapidly evolve in response to global change [5–7], environmentally induced phenotypic plasticity—defined as the capacity for a given genotype to express different phenotypes across different environments [8]—is a primary mechanism by which organisms respond to environmental changes [9,10]. Phenotypic plasticity is considered adaptive when environmentally induced trait changes improve an organism's fitness, thereby reducing phenotype–environment mismatches that could lead to extinction [11,12]. Hence, the mechanisms that produce patterns of adaptive phenotypic plasticity are critical to the maintenance of biodiversity.

The microbiome impacts nearly all aspects of host phenotype [13–16] and provides additional functional traits that extend the host's capacity to respond to environmental challenges [17]. Host-associated microbiomes both show sensitivity to environmental changes and often have profound impacts on host fitness [17], even outside of cases of obligate symbiosis [18]. The composition of host-associated microbial communities can influence host performance and relative fitness [19], and intraspecific variation in microbiome composition has been observed to affect host physiology

and performance in various taxa [20–22]. The dual observations of both the environmental responsiveness of the microbiota and the substantial impact shifts in microbiome composition can have on host physiology have led to considerable speculation about the microbiome's potential contribution as a mechanism driving adaptive phenotypic plasticity in host populations [23].

Microbiome-mediated plasticity—defined as changes in microbiome composition or function that influence host phenotype—has been proposed as a general mechanism that facilitates evolutionary adaptation by reducing mismatches between host phenotype and the environment [23,24]. Despite considerable interest, empirical tests of microbiome-mediated plasticity outside of obligate symbioses are rare (but see [25]). A foundational extension is cases where microbiome-mediated plasticity facilitates host population growth and persistence. This addition of positive demographic effects is termed microbiome rescue and is defined as changes in microbiome abundance, composition or activity that improve host fitness and decrease the likelihood of host extinction [26,27]. Microbiomes can play a critical role in helping hosts cope with various environmental challenges, including toxicants, by potentially modulating detoxification mechanisms and stress responses [28–30]. Yet, outside of cases of obligate symbiosis (e.g. coral–algal symbiosis [31], aphid–*Buchnera* [32] and squid–*Vibrio* [33]), there is limited empirical evidence linking microbiome composition and effects on host population dynamics, leaving considerable uncertainty about the strength of any such beneficial effects or the potential for microbiome rescue [34].

Empirical tests of the contributions of shifts in the microbiome to adaptive plasticity are needed to determine whether microbiome plasticity facilitates population persistence or rescue in response to environmental stress. Such tests should meet the following criteria: (i) determine whether environmental change is associated with a shift in microbiome composition, (ii) demonstrate whether host populations exhibit phenotypic plasticity in response to the environmental change, (iii) determine whether host phenotypic plasticity has an overall adaptive or maladaptive effect and (iv) assay whether the magnitude of the host plastic response is associated with changes in microbiome composition. In contrast to other emerging areas of hypothesis testing in microbiome science, these aims cannot be achieved through transplant experiments as the experiment directly induces changes in microbiome composition instead of allowing microbiomes to respond to environmental change.

Daphnia (water fleas) are a tractable system to investigate both microbiome-mediated plasticity and microbiome rescue. *Daphnia* exhibit observable and quantifiable phenotypic changes in response to environmental fluctuations that are crucial to population dynamics, including alterations in survival rates, morphology and reproductive strategies [35]. The interaction between *Daphnia* and harmful algal blooms of the cyanobacterium *Microcystis*—a global environmental challenge [36]—is both important for host fitness and environmental health [37–40]. *Microcystis* species, such as the well-studied species *Microcystis aeruginosa*, can produce toxic secondary metabolites that negatively affect zooplankton. As non-selective feeders, *Daphnia* are known to ingest *M. aeruginosa* and accumulate the cyanobacterial toxin, microcystin, which has been documented to negatively impact zooplankton life-history traits such as survival and reproduction [39,41–43]. Microbiome transplant experiments have demonstrated that both host genotype and gut microbiota can mediate tolerance in *Daphnia* to *M. aeruginosa* [44]; host genotypic variation in tolerance disappears when *Daphnia* are made germ-free and inoculated with a standardized microbiome [44]. In another reciprocal transplant experiment, *Daphnia* performed better when receiving a microbiome from their source region when exposed to toxic *M. aeruginosa*, indicating microbiome-mediated local adaptation in stress tolerance [45]. This effect was most pronounced when donor microbiomes were pre-exposed to toxic cyanobacteria, and it also depended on the pond and genotype of the *Daphnia* [45]. More broadly, the gut microbiome of *Daphnia* exhibits considerable plasticity in both diversity and composition and is influenced by the environmental bacterial community and host genotype, among other potential environmental drivers [45–47].

Prior work on *Daphnia–Microcystis* interactions has been instrumental in revealing the mechanisms by which microbiomes can impact hosts, but understanding the implications of these interactions for host plasticity and population dynamics requires different approaches. First, determining the extent of *Daphnia* microbiome shifts, and whether these shifts are repeated and deterministic, is critical to understanding how robust the effects of microbiome changes may be on host phenotypes [48,49]. Second, explicitly testing whether the magnitude or direction of microbiome shifts are correlated with overall phenotypic plasticity is key to understanding the contribution of microbiome change to host phenotypic response [24]. Finally, measuring the magnitude of an observed shift on host fitness due specifically to microbiome alteration is critical in evaluating the microbiome rescue hypothesis [26,27]. Doing so requires measurements of the relationship between microbiome shifts and parameters central to population dynamics. Together these data can provide a test of the role of microbiome shifts in host phenotypic plasticity and population dynamics in changing environments.

To address key questions regarding the role of the microbiome in facilitating host plasticity and rescue, we conducted an algal toxicity and microbiome study on 20 genetically distinct genotypes of *Daphnia magna* collected from a single lake. We first catalogued the impacts of *M. aeruginosa* exposure on *D. magna* phenotypes and population growth rate for each genotype. We then tested the following questions: (i) What is the magnitude of *D. magna* phenotypic response to *M. aeruginosa* exposure? (ii) Does *M. aeruginosa* exposure alter *D. magna* microbiome composition and abundance and, if so, are these changes parallel and deterministic across different genotypes, under varying treatments? (iii) Is the magnitude and/or direction of shifts in the microbiome in response to *M. aeruginosa* correlated with the magnitude of adaptive phenotypic plasticity in *D. magna*? To answer these questions, we conducted a 21-day chronic toxicity exposure experiment across two common gardens (non-toxic, *Chlorella vulgaris* only; and toxic, 3 : 1 ratio of *C. vulgaris* to *M. aeruginosa*) using 20 *D. magna* genotypes. Clonal replication allows for an assessment of the effects of microbiome shifts across multiple genetic backgrounds and enables projections of population-level responses. We use these fitness-associated phenotypes in each of 20 genotypes to parameterize population projection models and test for the effects of shifts in the microbiome on host plasticity.

2. Methods

(a) *Daphnia magna* field collection and culturing

In late spring and early summer, 20 genotypes of *D. magna* were obtained from 'Langerodevijver' (LRV; 50° 49' 42.08'', 04° 38' 20.60''), a small lake situated within the nature reserve of Doode Bemde, Vlaams-Brabant, Belgium [50]. LRV, with a surface area of 140 000 m^2 and a maximum depth of 1 m, features a single basin and experiences seasonal blooms of *Microcystis*. Additionally, LRV harbours a large population of *D. magna*. Parthenogenetic lines of each genotype were collected from the field and maintained as separate clonal lineages for over 5 years (approximately 300 generations) in continuous cultures at 20°C, utilizing UV-filtered dechlorinated municipal tap water enriched with 2 mg C l^{-1} of the green alga *C. vulgaris* (strain CPCC 90; Canadian Phycological Culture Centre, Waterloo, ON, Canada). Each lineage was confirmed to represent a unique genotype (see [51]). Culturing of *C. vulgaris* was carried out using COMBO medium [52]. Filters with a pore size of 0.22 µm were placed at both the input and output of the aeration system to prevent any bacterial contamination.

(b) *Microcystis aeruginosa* culturing

In accordance with our previously outlined methodology [53], *M. aeruginosa* (strain CPCC 300; Canadian Phycological Culture Centre, Waterloo, ON, Canada) was cultivated in BG-11 media and maintained in a growth chamber under sterile conditions at a constant temperature of 21 ± 1°C, illuminated with cool-white fluorescent light at an intensity of 600 ± 15 lx, and subjected to a photoperiod of 16 : 8 h light : dark. The culture was allowed to grow undisturbed for at least one month before being prepared for the 21-day chronic study. *M. aeruginosa* CPCC 300 is known to produce microcystin-LR (CAS: 101043-37-2, $C_{49}H_{74}N_{10}O_{12}$) and its desmethylated form [D-Asp^3]-microcystin-LR (CAS: 120011-66-7, $C_{48}H_{72}N_{10}O_{12}$).

To facilitate testing on *D. magna*, an aliquot of the stock was inoculated into 100% COMBO medium 2 weeks prior to test initiation, where it was cultured until reaching a cell density of 1.25 ± 0.02 × 10^7 cells ml^{-1}. This medium was chosen because it supports the growth of both algae and cyanobacteria while remaining non-hazardous to zooplankton [52]. Filters with a pore size of 0.22 µm were placed at both the input and output of the aeration system to prevent any bacterial contamination.

(c) Gut microbiome experiment

To evaluate shifts in the microbiome and phenotypic responses of *D. magna* to *M. aeruginosa*, we used 20 unique genotypes derived from one lake. From each of the 20 *D. magna* genotypes, 10 offspring were randomly selected to establish 10 replicate founding mothers per genotype (200 founding mothers in total). Each adult *D. magna* female was isolated in a 50 ml glass tube containing COMBO medium and *C. vulgaris* (2 mg C l^{-1}) and monitored daily for reproduction. These founding mothers were maintained under controlled conditions (temperature: 21 ± 1°C, photoperiod: 16 : 8 h light : dark, light intensity: 600 ± 15 lx) to generate neonates for the feeding experiment.

A 21-day chronic toxicity study was performed following previously described methods [45,54]. Neonates born within a 24 h period from these founding mothers were randomly assigned to experimental groups. For each genotype, a total of 90 neonates (15 neonates per replicate jar) were placed in six 1 l glass jars containing 750 ml of UV-filtered water. These six jars per genotype were divided evenly between two common gardens: (i) non-toxic diet (*C. vulgaris* only; control), and (ii) toxic diet (3 : 1 mixture of *C. vulgaris* to *M. aeruginosa*; toxic diet). Thus, this design included three replicate jars per feeding treatment for each genotype. All animals were fed 2 mg C l^{-1} daily (3 × 10^6 cells total), consistent with previous studies exposing daphnids to dietary combinations of green algae and cyanobacteria [38,39,41]. In total, 1800 animals were used for this study (900 per treatment).

As this was a semi-static test, solutions were refreshed 3 × per week on Mondays, Wednesdays and Fridays. This process involved transferring *D. magna* from the old to the new glass jar and providing *D. magna* with a food supply consisting of 3 × 10^6 cells (i.e. the 3 : 1 treatment received 2 × 10^6 *C. vulgaris* cells and 1 × 10^6 *M. aeruginosa* cells, corresponding to 2 mg C l^{-1}). On a daily basis, survival, reproductive output and the timing of broods were recorded to evaluate potential interactions between genotype and treatment effects. Offspring production was tracked separately from maternal survival; offspring were removed after each brood and recorded for precise measurement of reproductive output. The study was conducted under 400–800 lx cool-white fluorescent light at a temperature of 20 ± 1°C with a 16 : 8 h light : dark cycle. Phenotypic responses included survival, reproduction (number of offspring produced until the end of the experiment) and the timing of first brood.

At 21 days, *D. magna* replicates were transferred to sterile-filtered tap water for 24 h to eliminate food particles from the gut and environmental bacteria from the carapace and filter apparatus [45]. Subsequently, *D. magna* guts were extracted using dissection needles under a stereomicroscope and transferred into an Eppendorf tube filled with 10 µl sterile milliQ water, after which they were stored in −80°C for future DNA extraction.

(d) Library preparation and sequencing

To analyse the gut microbial communities of *D. magna* at the conclusion of the 21-day experiment, DNA extractions were performed using the NucleoSpin Soil DNA kit (Macherey-Nagel, Düren, Germany). Each *D. magna* gut was pressed using a stainless steel probe and collective contents were bead beaten to increase DNA extraction efficiency. DNA was eluted using 50 µl of 5 mM Tris/HCl at pH 8.5. Subsequent DNA quantification was conducted with 5 µl per sample of eluted DNA on a Qubit

(Invitrogen, Massachusetts, United States) using a dsDNA broad-range assay. Samples that had low DNA concentrations were vacufuged before sequencing to increase concentration. Isolated DNA was quantified (Equalbit 1 × dsDNA HS Assay kit) and amplified using primers covering the V3–V4 hypervariable 16S rRNA region. Library quality was assessed and libraries were dual indexed before Equimolar pooling based on QC values. Pooled libraries were sequenced on an Illumina MiSeq with a 250 bp read length configuration to a depth of 0.3M reads for each sample.

Data were imported using demux single-end forward reads with initialization of primer trimming followed by high-resolution DADA2 [55] filtering at truncation quality 20 to remove low-quality reads and chimeric sequences via QIIME2 [56]. No taxonomic groups were selectively removed. Post-filtering with an average input of 88 473 reads, we retained a mean of 16 941 filtered and non-chimeric sequences (quantile range: 1% = 7237, 25% = 14 943, 50% = 17 006, 75% = 19 835, 100% = 25 629). Taxonomy was assigned using greenegene full-length 16S rRNA backbone database with a scikit-learn naive Bayes machine-learning classifier [57]. Before transitioning into the *phyloseq* package in R [58], we generated an unrooted phylogenetic tree to improve the accuracy of the downstream analysis. In *phyloseq*, the combined dataset was first filtered by amplicon sequence variants (ASVs) that were represented with fewer than five total observations in the whole sample set. Technical replicates (20 genotypes × 3 replicates × 2 treatments) of the same biological genotype were then combined and rarefied at a depth of 20 000 reads. Data were aggregated to bacterial class for analyses of relative abundance and differential abundance analysis graphs.

(e) Water sampling for analysis

Water samples were collected from each treatment at the beginning of the test, during solution changes, and at the conclusion to measure standard water parameters (Dataset S1, electronic supplementary material). Briefly, water parameters recorded before and after solution renewals and showed no indication of hypoxia-induced stress on *D. magna* behaviour. To ensure consistent cell concentrations, measurements were taken from the main reservoirs of both treatments (non-toxic: 100% *C. vulgaris*; toxic: 3 : 1 *C. vulgaris* to *M. aeruginosa*) at test initiation and before each solution renewal. Additionally, triplicate water samples were collected from the main reservoir of the toxic treatment (3 : 1 *C. vulgaris* to *M. aeruginosa*) at these same time points for cyanobacterial toxin analysis (Dataset S1, electronic supplementary material). These samples were quick-frozen at −80°C and subsequently analysed for cyanobacterial toxins, the methods of which have been reported previously [51]. Mortality and immobilization were initially detected within 48 h after exposure to the toxic treatments and persisted throughout the 21-day test period. This outcome was anticipated, given that microcystin concentrations measured in this test were 1.56 ± 0.0003 µg l^{-1}, which is considered sublethal to lethal in *Daphnia* laboratory assays and is commonly encountered in freshwater ecosystems affected by harmful algal blooms [39,42,59,60].

(f) Statistical analysis

All analyses were completed in R version 4.2.2 [58]. To test the magnitude of clonal variation across treatments, we used a linear mixed effects (LME) model for each *D. magna* phenotypic response with 'genotype' treated as a random effect and 'treatment' treated as a fixed effect. The exponential rate of increase (r) was calculated based on the total number of neonates produced by each genotype, taking into account both survival and output, to estimate the reproductive output of *D. magna* that were exposed to toxic and non-toxic diets. The mean difference in r between toxic and non-toxic diets was calculated for each genotype-treatment combination. This allowed us to assess whether *Microcystis* exposure had a net positive ($r > 0$), neutral ($r = 0$), or negative ($r < 0$) effect on population dynamics relative to rearing on a non-toxic diet. These calculations were constructed using the full 21-day data on *D. magna*. The difference in neonate production between toxic and non-toxic diets was calculated for each 'genotype' and 'treatment' combination and plotted. *Daphnia* phenotypic plasticity in response to *Microcystis* exposure has been documented for several specific traits [43,51,61], here we focus on r to address plasticity in a key parameter linked with population dynamics.

Downstream microbiome analyses were conducted in the *phyloseq* package in R. We calculated α-diversity (per sample ASVs and Shannon diversity index) and tested for the effects of algal treatment and genotype on α-diversity using the *lme4* package in R. Bray–Curtis, weighted UniFrac and Jaccard Index ß-diversity metrics were used to assess the multivariate effects of algal treatment and genotype in a permutational multivariate analysis (PERMANOVA) *adonis2* function from the *vegan* package in R. ß-diversity distance matrices were treated as response variables with 'treatment' + 'genotype' treated as fixed effects and the sampled guts in each replicate treated as a random effect. To identify bacterial differences on the family level between samples associated with algal treatment, we used a differential abundance analysis from the *DESeq2* package in R, where ASVs representing less than 1% of the reads were discarded and p-values were adjusted using Benjamini–Hochberg procedure [62]. To support our findings from *DESeq2*, we additionally employed the *phylofactor* package in R as an alternative which utilizes phylogenetic data to assess the differences in microbial community composition [63].

To examine the relationship between microbiome shifts and phenotypic plasticity, we compared the microbiome composition with and without *Microcystis* exposure in each of the 20 genotypes to assess microbiome plasticity. This approach accounts for the starting microbiota of each genotype while allowing for tests of the correlations between microbiome shifts and host phenotypic plasticity. We used the *multivarvector* package in R [49] to generate multivariate vectors and angles by connecting the population means of principal component analysis (PCA) scores with genotypes across different algal treatments (20 total vectors and angles) as an empirical measure of microbiome shifts in response to *Microcystis* exposure. The relationship between

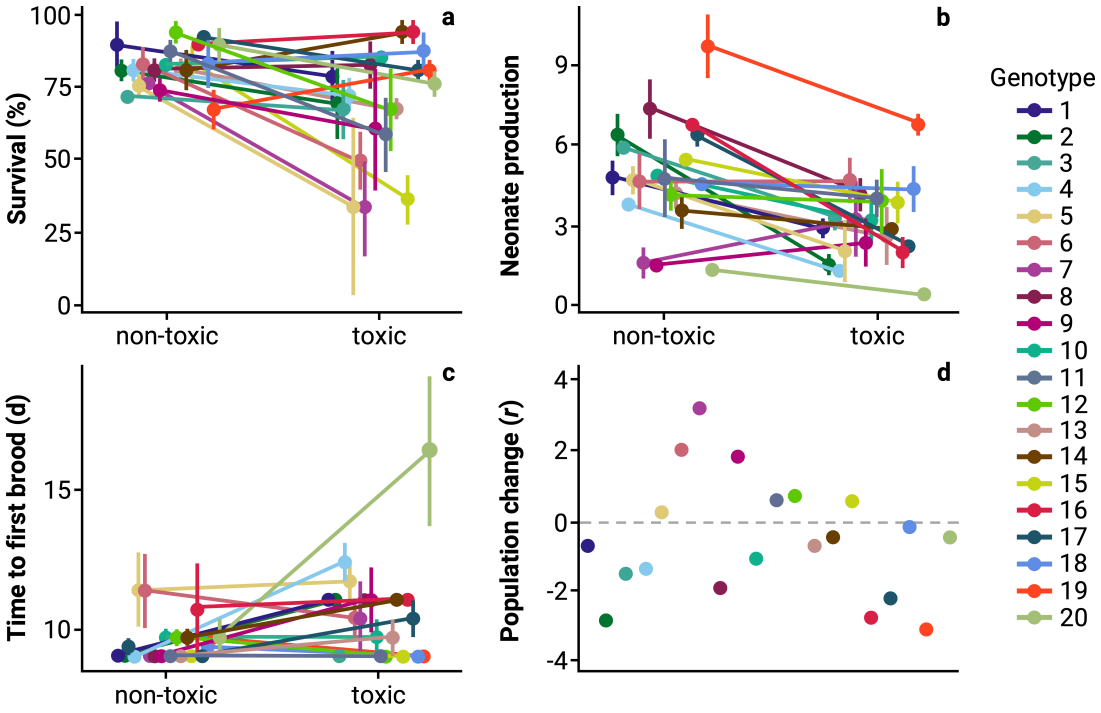

Figure 1. Phenotypic variation among the 20 *Daphnia magna* genotypes at the end of the 21-day gut microbiome chronic experiment across two treatments: non-toxic diet (*Chlorella* only) and toxic diet (3 : 1 *Chlorella* : *Microcystis*). Phenotypes measured were (a) survival (%), (b) mean number of neonates produced per *D. magna* and (c) mean time to first brood per *D. magna* (d). Panel (d) is the exponential rate of population change (*r*) between treatments across each of 20 *D. magna* clonal populations at the end of the 21-day gut microbiome chronic experiment. Positive values (>0) indicate a beneficial effect on population dynamics, while negative values (<0) indicate a detrimental effect.

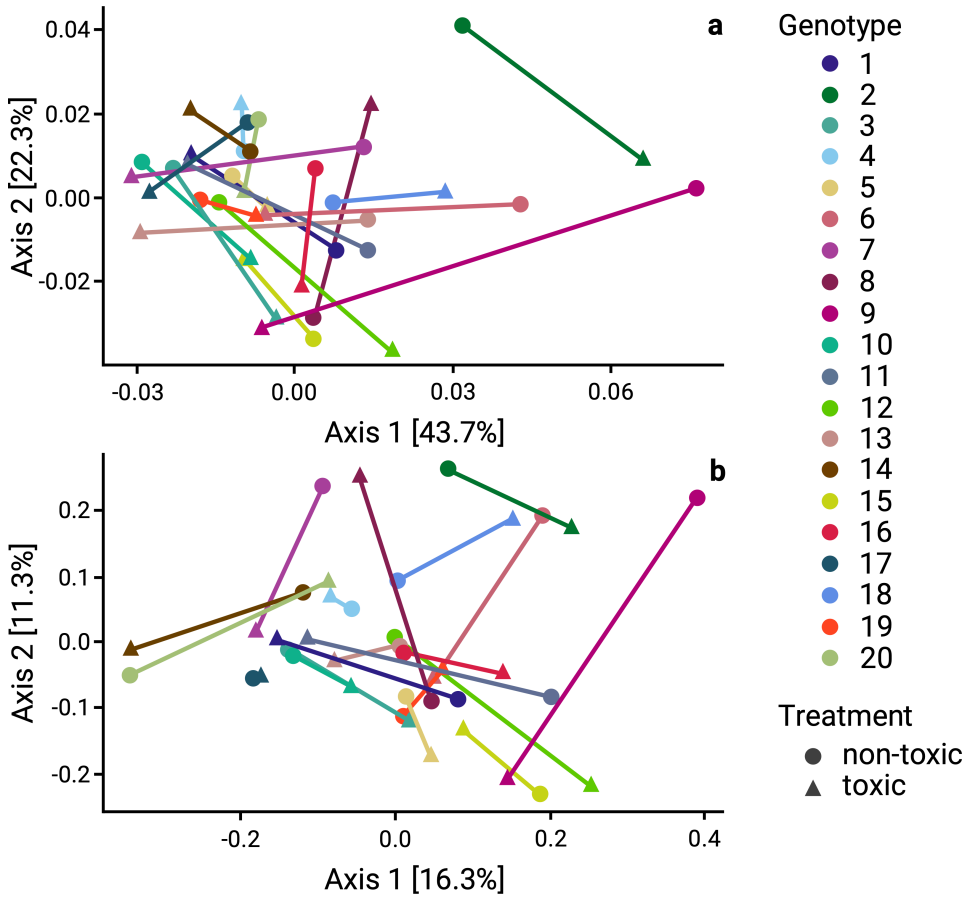

Figure 2. Principal component of analysis (PCoA) in (a) weighted UniFrac, and (b) Bray–Curtis distances in relation to the gut microbiome across the 20 *Daphnia magna* genotypes subjected to non-toxic (*Chlorella*-only; ▲) and toxic (3 : 1 *Chlorella* : *Microcystis*; ●) treatments. Each genotype is connected by a line to show the shift in host response across treatments.

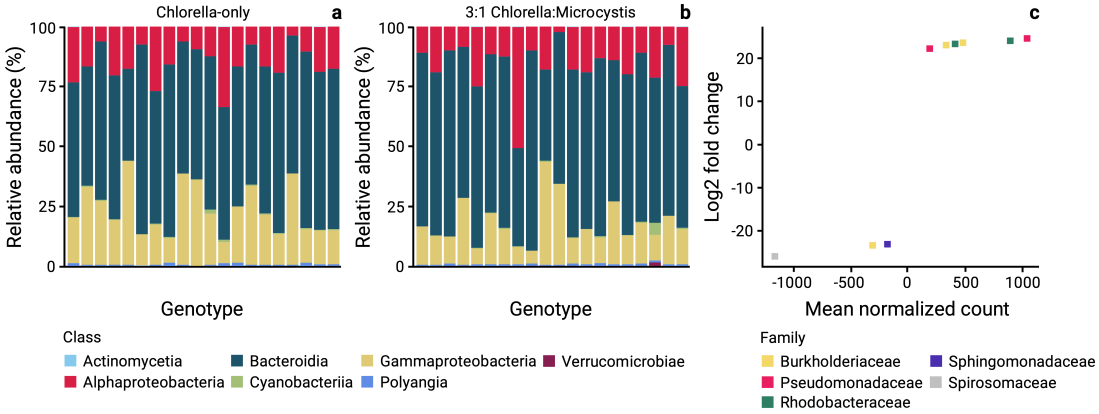

Figure 3. The average relative abundance of bacterial classes in the gut microbiomes of 20 *Daphnia magna* genotypes across (a) non-toxic (*Chlorella*-only) and (b) toxic (3 : 1 *Chlorella* : *Microcystis*) treatments at the end of the 21-day gut microbiome chronic experiment, colour coated based on bacterial classes. (c) The *DESeq2* differential abundance analysis, colour coated based on family.

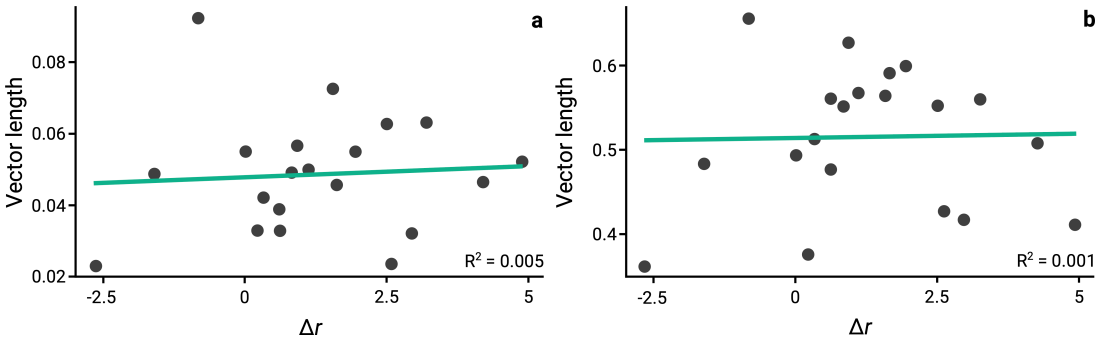

Figure 4. Relationship between the magnitude of microbial plasticity, measured using (a) weighted UniFrac and (b) Bray–Curtis distances, and changes in population growth rate (*r*) across 20 *Daphnia magna* genotypes exposed to non-toxic (*Chlorella*-only) and toxic (3 : 1 *Chlorella* : *Microcystis*) treatments. The mean vector is shown as a blue-green line.

the magnitude of plasticity in microbial composition and host performance, *r* and delta mean neonate production, was assessed using the goodness of fit of R^2 extracted from a linear model.

To assess the degree of parallelism in the microbiome shift associated with algal treatment, we used *multivarvector* package, generating the angular value of the parallelism of replicates given toxic and non-toxic diets to then assess the parallel, non-parallel or anti-parallel relationships of our data. In addition to *multivarvector,* we implemented betadisper from *vegan* to assess the multivariate homogeneity of group dispersions on our ß-diversity metrics.

3. Results

(a) *Daphnia magna* plasticity in life-history traits and population projection

Exposure to the toxic diet (3 : 1 *Chlorella* : *Microcystis*) had widespread plastic effects on *Daphnia,* including a significant decrease in survival ($F_{1,19}$ = 10.81, p = 0.0039; figure 1a); *Daphnia* from the non-toxic diet had a 81.5 ± 1.3% survival rate compared to *Daphnia* from the toxic diet who had a 68.13 ± 3.2% survival rate to the cessation of the study at 21 days. Similarly, we observed a significant effect on neonate production per *Daphnia* ($F_{1,19}$ = 19.56, p = 0.00029; figure 1b); *Daphnia* reared on non-toxic diets produced 4.76 ± 0.29 neonates per *Daphnia* compared to 3.02 ± 0.23 neonates per *Daphnia* for those reared on toxic diets. Lastly, we observed a significant delay in time to the first brood ($F_{1,19}$ = 6.66, p = 0.018; figure 1c); *Daphnia* reared on non-toxic diets reproduced at 9.52 ± 0.15 days compared to 10.53 ± 0.26 days for those reared on toxic diets. We did not observe significant effects on the total number of broods per *Daphnia* ($F_{1,19}$ = 0.25, p = 0.62) by 21 days.

Microcystis exposure negatively impacted the population dynamics of most genotypes. Across the 20 *Daphnia* genotypes, 13 showed a decrease in *r*, while seven showed an increase (figure 1d). On average, *Microcystis* exposure led to a net reduction in population growth, with a mean difference in *r* between toxic (2.38) and non-toxic diets (2.88) of −0.49. These findings suggest that *Microcystis* exposure generally had a negative impact on *Daphnia* population dynamics, though the extent of this effect varied across genotypes.

(b) Testing for effects of toxic exposure on *Daphnia* microbiomes

Within individual genotypes, we observed considerable shifts in microbiome composition associated with algal treatment. However, we found no significant effect of algal treatment ($F_{1,38} = 1.417$, $p = 0.241$; $F_{1,38} = 0.763$, $p = 0.388$) or *Daphnia* genotype ($F_{19,20} = 0.864$, $p = 0.623$; $F_{19,20} = 0.985$, $p = 0.511$) on microbiome α-diversity (total ASVs and Shannon index, respectively) when averaging across all clones. Effects of algal treatment and genotype were likewise modest on ß-diversity; we observed no significant effect of algal treatment ($F_{1,39} = 1.53$, $p = 0.195$) or genotype ($F_{19,39} = 1.22$, $p = 0.173$) using weighted UniFrac (figure 2a), a marginally significant effect of genotype ($F_{19,39} = 1.17$, $p = 0.049$), but not algal treatment ($F_{1,39} = 0.96$, $p = 0.505$), when assessed using Bray–Curtis (figure 2b) and no effect of algal treatment ($F_{1,39} = 1.12$, $p = 0.11$) or genotype ($F_{19,39} = 1.05$, $p = 0.087$) with Jaccard Index. Using a permutation test for homogeneity of multivariate dispersions, we likewise saw no significant difference in variance in community composition associated with algal treatments (weighted UniFrac: $F_{1,39} = 0.601$, $p = 0.443$; and Bray–Curtis: $F_{1,39} = 0.009$, $p = 0.152$).

Microbiome composition shifts in response to dietary treatments were not parallel across genotypes. Using multivarvector analysis, we evaluated directional shifts in microbiome composition for each genotype and found no significant departure from non-parallel patterns. The angular relationships between shifts in microbiome composition, tests against the null hypothesis of 90° (indicative of non-parallel shifts), showed mean angles of 89.73° (weighted UniFrac; $t_{19} = -0.231$, df = 19, $p = 0.82$) and 90.14° (Bray–Curtis; $t_{19} = 0.34$, df = 19, $p = 0.74$). These results suggest that microbiome responses to dietary perturbation vary across genotypes, highlighting genotype-dependent patterns of microbiome plasticity.

Although ß-diversity metrics demonstrated no significant community-wide differences in microbiome composition, there were particular microbial groups that differed in their abundance across algal diets (figure 3). A *DESeq2*-based analysis of differential read abundance found five differentially abundant bacterial families (figure 3c): Spirosomaceae (log2FC = −25.57, $p < 0.001$), Sphingomonadaceae (log2FC = −23.02, $p < 0.001$), Rhodobacteraceae (log2FC = 23.03, $p < 0.001$), Pseudomonadaceae (log2FC = 23.57, $p < 0.001$) and Burkholderiaceae (log2FC = 23.67, $p < 0.001$). A *Phylofactor*-based analysis identified only Spirosomaceae ($F_2 = 11.28$, $p = 0.001$) as a differentially abundant taxa.

(c) Magnitude of microbial plasticity and degree of parallelism

We next tested whether the magnitude of microbiome shifts in response to toxic diets was associated with the magnitude of host phenotypic plasticity. No significant correlation was observed between the degree of microbiome shifts, measured as changes between the starting microbiome and the microbiome after *Microcystis* exposure, and changes in *r* across diet treatments for any genotype. Specifically, results for both weighted UniFrac ($F_{18} = 0.083$, $p = 0.776$, $R^2 = 0.005$; figure 4a) and Bray–Curtis ($F_{18} = 0.009$, $p = 0.923$, $R^2 = 0.0005$; figure 4b) metrics indicated no significant relationship.

4. Discussion

(a) Relationship between phenotypic responses, shifts in the microbiome and population-level effects

Overall patterns of host phenotypic plasticity associated with *Microcystis* dietary exposure were considerable, with mean shifts in survival, number of offspring produced and time to first brood generally trending toward a negative response across the 20 *Daphnia* genotypes (figure 1a–c). Within these mean shifts, there was considerable variation among genotypes in the magnitude and direction of plasticity, creating a strong scenario to examine the potential for microbiome-mediated plasticity. When phenotypes were combined to parameterize a population projection, dietary exposure to *Microcystis* produced considerable clonal variation and led to an overall decrease in population growth rates (figure 1d). This response aligns with prior work [64], which has shown significant mortality in *Daphnia* due to *Microcystis* [36,38,39,65], along with evidence of adaptive responses to *Microcystis* blooms [37,65,66].

In contrast to the pronounced effects of *Microcystis* on host phenotypic plasticity, microbiome shifts were modest (figure 3a–c), with no parallel changes across *Daphnia* genotypes (figure 2) and no correlation between microbiome shifts and phenotypic plasticity (figure 4). Prior studies using microbiome transplants and axenic rearing have demonstrated that a few dominant microbial taxa in the *Daphnia* gut microbiome [67] can strongly influence host fitness when exposed to diets containing *Microcystis* [44,68–70]. However, we find these potentially beneficial changes in microbiome composition do not occur in response to *Microcystis*. Our findings demonstrate that microbiome shifts occur naturally in response to toxic stress, but these shifts did not mediate phenotypic plasticity or stabilize population growth rates in this system. This distinction demonstrates the context-dependence of microbiome-mediated effects and highlights the importance of approaches that capture natural host–microbiome interactions.

(b) Microbiomes and adaptive plasticity revisited

The role of the microbiome in host responses to environmental stress has been a topic of considerable interest [23,24,71,72]. Spurred by early work documenting shifts in microbiome composition associated with environmental stress [73,74], there has been considerable speculation about the role of the microbiome in host acclimation and adaptation [22,26]. Transplantation

studies have been invaluable for testing the functional roles of specific microbial taxa, but they fundamentally differ from approaches like the present work by imposing artificial constraints on microbiome composition. Such studies reveal what major changes in the microbiome could theoretically do for host fitness but cannot test how microbiomes respond naturally to environmental stressors. Our findings raise questions about the circumstances and generality of microbiomes as a mechanism underlying adaptive plasticity in hosts. We show that the *Daphnia* microbiome only exhibits significant shifts in the abundance of five bacterial families. Though it has been proposed that shifts in microbiome composition play a crucial role in influencing the fitness of hosts and stabilizing growth rates to reduce the probability of extinction [24,26,27], we find no correlation between the degree of shift in the microbiome and host adaptive plasticity (figure 2) or population growth rate (figure 1d). Future mechanistic work on the link between specific microbial taxa and *Daphnia* fitness could enable investigations into the role of specific ASVs in driving host plasticity.

Prior work demonstrating a role of adaptive plasticity has often studied microbiome contributions in systems without explicit consideration of host genetic diversity [25] or through complete microbiome transplants [44–46,70,75]. However, recent work [76] has revealed that microbiota plasticity can be influenced by genotype-by-environment interactions, demonstrating the complexity of host–microbiome relationships. While these studies provide valuable insights under controlled conditions, they may overestimate the importance of microbiome shifts in more natural settings [47].

An important caveat of our work is that it was conducted under laboratory conditions, where *D. magna* gut bacterial communities, and local species pools, can differ significantly from those in natural environments. Recent work shows that microbiome diversity and composition can change rapidly after transfer to the lab, with ongoing shifts even after 2 years [77]. Despite these differences, key bacterial classes [47,77]—including Burkholderiaceae, Pseudomonadaceae and Sphingomonadaceae—were dominant across field and lab rearing environments and present in high abundances in our study. Our analysis of parallelism (figure 4) revealed that the relative abundances of microbiota were not consistently altered by exposure to *Microcystis*. This finding aligns with prior work conducted under semi-natural conditions, which found no evidence linking natural variation in microbiome diversity or composition to cyanobacteria tolerance [47]. It is possible that microbiomes contribute to host plasticity through cryptic changes, perhaps through shifts in the abundance of a few specific ASVs; yet we find no compelling evidence to support this, and disentangling any such true associations at the strain-level from random processes would require either strong parallelism in responses or a very high degree of statistical power. Future work that extends our general experimental framework to field conditions, or rears hosts in environments with a natural microbiota, is needed to determine whether hosts can modulate microbiome composition through horizontal acquisitions, thereby producing adaptive plasticity and positive effects on population growth.

While it is possible that certain traits or environments might benefit from microbiome-mediate rescue [26,27] or 'microbiome flexibility' [78], our findings suggest this was not the case here. Moreover, field exposures similarly recover limited evidence for *Daphnia* microbiome shifts in response to *Microcystis*, which does not indicate a strong pathway for adaptive plasticity via microbiome shifts or microbiome rescue [47]. Although concepts like microbiome plasticity and rescue were initially motivated by findings in organisms with strong host–microbiome symbiosis, it has been explicitly posited that these phenomena are likely to be broad and widespread [26,27].

In the absence of microbiome mediate rescue, the relative importance of other mechanisms, including phenotypic plasticity and adaptation from standing genetic variation, in reducing phenotype-environment mismatches is even greater [7,8,79,80]. These mechanisms shape the phenotypic response of organisms to environmental challenges [8,9,81]. It is plausible that microbiome-driven adaptive plasticity is also a component of adaptive plasticity but operates on longer timescales [24] or requires transgenerational effects [25], as suggested by the microbiota-mediated transgenerational acclimatization concept [82]. These effects might be limited to systems with high microbiome heritability [83]. However, given the modest heritabilities of many host-associated microbiomes [84,85], these may not be general mechanisms. More broadly, advancing microbiome studies beyond tightly controlled mechanistic investigations to include genetically, environmentally and phenotypically diverse populations will be critical for understanding the role of microbiomes in organismal responses to environmental change [86].

(c) Quantitative and qualitative replication of host–microbiome studies

Despite observing significant shifts in *Daphnia* life-history traits and population growth associated with *Microcystis* exposure, we observed no overall community-level differences in gut microbiome composition between *Daphnia* reared on toxic and non-toxic diets when averaging across all 20 genotypes. This result aligns with findings from outdoor mesocosms, which demonstrated that despite significant seasonal changes in *D. magna* gut microbiome diversity and composition there was surprisingly no evidence linking natural variation in microbiome diversity or composition with exposure to *Microcystis* [47].

Our results suggest that earlier mechanistic studies—which used axenic cultures and reciprocal microbiome transplants to test the effects of microbiome composition on *Microcystis* toxin tolerance [44,45,75]—may not capture the dynamics of host–microbiome interactions in natural trophic interactions and prompt further investigation into the ecological relevance of such mechanistic findings [86]. These differences highlight major questions about reproducibility and repeatability in microbiome research, due both to underlying stochasticity and inherent differences between experimental environments [87]. In planning this study, we consulted authors on the prior work and they generously shared methodological details which we made efforts to replicate. However, there are experimental differences that could contribute to the considerable variation seen between studies. These include: previous studies did not detail the concentration of *Microcystis* cells or amount of microcystin toxins, and hence exposures may not have been comparable [88]; cyanobacteria-based diets may differ in nutritional composition, including fatty acid and carbohydrate profiles, which could have additional effects on studied populations [89]; biological variation in *Daphnia*

test populations and *Microcystis* strains; and inherent differences in lab microbiomes. Whether these differences are sufficient to lead to qualitatively different conclusions about the role of microbiome composition in host fitness is beyond our scope to determine. The differences observed highlight the need to consider not only genetic and environmental factors but also methodological consistency when interpreting microbiome research results. Given the observed differences between studies and the inherent repeatability challenges in microbiome research, our findings should be interpreted within the context of these broader uncertainties. While our results contribute valuable insights, they also underscore the importance of continued investigation and methodological rigor in this field.

5. Conclusion

Our findings provide an empirical test of the contribution of the microbiome to host plasticity and population dynamics. Although significant phenotypic changes were observed in *Daphnia* exposed to *Microcystis*, there was no correlation between this response and the degree of change in the composition of the microbiome or any pattern observed indicative of microbiome-mediated rescue. These results suggest that the microbiome's role in host plasticity may be more context-dependent than previously thought. However, given the scope of this single study, further research is needed to explore how shifts in the microbiome influence reaction norms in fitness-associated phenotypes across species and environmental contexts. Future studies in natural conditions, where hosts can acquire a wider range of microbes, will be critical to developing a more comprehensive understanding of how host–microbiome interactions shape natural populations. Such investigations may help resolve long-standing questions about the microbiome's potential to direct host evolution and serve as a mechanism of rescue in populations facing novel environmental challenges.

Ethics. No specific ethical approval or permits were required for this study, as *Daphnia magna* is an invertebrate species not subject to institutional or national animal ethics guidelines.

Data accessibility. The data supporting the results is archived in the public repository, Dryad [90].

Supplementary material is available online [91].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. R.S.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing—original draft, writing—review and editing; A.R.G.: formal analysis, software, writing—original draft, writing—review and editing; E.R.A.: methodology, writing—original draft, writing—review and editing; J.F.: funding acquisition, project administration, resources, writing—review and editing; S.M.R.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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