



Prior exposure of a fungal parasite to cyanobacterial extracts does not impair infection of its *Daphnia* host

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Abstract Cyanobacteria periodically dominate phytoplankton composition in lakes, and produce a wide array of toxic secondary metabolites. Blooms of cyanobacteria often coincide with infections of zooplankton by microparasites (such as *Metschnikowia bicuspidata*, a parasitic yeast of *Daphnia*), and prior research has shown that cyanobacteria-based diets could mitigate fungal infections of the host. Here, we tested whether cyanotoxins could exert detrimental effects against free-living parasite stages: we inoculated two genotypes of the host *Daphnia galeata* × *longispina* with fungal spores, which were previously exposed to cyanobacterial extracts or to a

placebo solution. Additionally, to test for interactive effects of cyanotoxins through environmental exposure and host consumption, *Daphnia* from each treatment were fed using either green algae or the same cyanobacterium. Exposing spores to cyanobacterial extracts did not reduce their infectivity; instead, parasite infectivity was increased, but only on one host genotype. The effect of host diet on parasite growth was also host-genotype dependent, with only one *Daphnia* genotype showing impaired spore production under a toxic diet. Our results suggest that dissolved cyanobacterial compounds released during blooms may not exert any detrimental effect on fungal spore banks, but likely influence transmission of the parasite when incorporated as part of the host's diet.

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Introduction

Cyanobacteria are one of the most ancient organisms on Earth (Garcia-Pichel, 1998; Sánchez-Baracaldo & Cardona, 2020). With an evolutionary origin dating back to at least 2000 Ma, their photosynthetic and nitrogen-fixing abilities have contributed to greatly affecting the Earth's atmospheric composition and carbon cycle at a global scale (Knoll, 2008). Acting as main primary producers throughout most of

the Proterozoic, cyanobacteria have since remained important—and sometimes dominant—components of phytoplankton communities within both marine and freshwater ecosystems (Sergeev et al., 2002). The seasonal ability of cyanobacteria to proliferate and dominate phytoplankton species assemblages during short periods of time is commonly referred to as ‘blooms’, which have been recorded increasingly throughout the twentieth century (Hallegraeff, 1993; Gobler, 2020) and elevated to a greater level of concern throughout the past three decades (Van Dolah et al., 2001; Moore et al., 2008; Grattan et al., 2016; Sukenik & Kaplan, 2021). In part thanks to their ancient evolutionary history, cyanobacteria exhibit a number of competitive traits, allowing them to outcompete other phytoplankton (such as green algae) when conditions are favourable. This includes their buoyancy regulation—allowing them to occupy superficial niches in the water column, thus optimizing light harvesting while shading competitors (Carey et al., 2012)—and faster growth under conditions of elevated temperatures, high concentrations of inorganic carbon (CO₂) or replete nutrient availability (Mantzouki et al., 2016; Ji et al., 2017). Incidentally, such environmental conditions have been associated with contemporary ecological shifts of anthropogenic origin: human activity is causing harmful blooms to become more frequent, due to a combination of greenhouse gas effects and the eutrophication of aquatic ecosystems (Mantzouki et al., 2016), although increasing evidence show that cyanobacteria can also thrive in relatively low-nutrients environments (Reinl et al., 2021).

Besides the inherent phenology and population dynamics of cyanobacteria, their interactions with primary consumers (i.e. zooplankton) are also expected to change in a warming, increasingly eutrophic world (Benndorf & Henning, 1989; Ger et al., 2014). Cyanobacteria act as a relatively poor food source for common zooplankton taxa, such as copepods (DeMott & Moxter, 1991) or various species of cladocerans (Hanazato & Yasuno, 1987; Lundstedt & Brett, 1991; Smith & Gilbert, 1995). This is commonly imputed to low nutritional value, due to a lack of poly-unsaturated fatty acids (PUFAs) and/or sterols (DeMott & Müller-Navarra 1997; Ravet et al., 2003), in addition to their toxicity (DeMott et al., 1991). A major concern regarding the increasing occurrence of harmful algal blooms, cyanobacteria

are renowned for their exceptional bioactivity and toxin production (Namikoshi & Rinehart, 1996; Huang & Zimba, 2019). Over time, cyanobacteria have evolved a variety of secondary metabolites and bioactive compounds (Welker & Von Döhren, 2006; Agha & Quesada, 2014), some of which can cause adverse effects to other organisms (including humans) and have thus been referred to as cyanotoxins (Carmichael, 1992; Chorus, 2012). These are proposed to have evolved as biochemical defences against invertebrate grazers (Ghadouani et al., 2004; Rohrlack et al., 2004; Czarnecki et al., 2006), following an evolutionary pathway akin to that of terrestrial plants (Kirk & Gilbert, 1992). Indeed, it was shown that cyanobacterial dominance can lead to seasonal decreases in zooplankton biomass (Hegg et al., 2022), while long-term exposure to high concentrations of cyanotoxins may promote the selection of more resistant species or genotypes (Wojtal-Frankiewicz et al., 2013).

In addition to their anti-grazing properties, a wider allelopathic activity was also documented in several compounds produced by cyanobacteria, reportedly efficient against algae and other bacteria (Berry et al., 2008). Associating such properties with the ancient evolutionary origin of cyanobacteria (Knoll, 2008), Sánchez et al. (2019) hypothesized that cyanotoxins could have evolved under the selective pressure of more ancestral antagonists, predating the apparition of their zooplanktonic (e.g. metazoan) grazers. Seeing as some cyanobacteria are infected by highly specialized parasites, such as fungi of the order Chytridiomycota (Canter, 1950) and viruses (i.e. cyanophages; Suttle, 2000), it is not surprising that a number of cyanobacterial secondary metabolites also display strong antifungal and antiviral properties (Volk & Furkert, 2006; Shishido et al., 2015; Marrez & Sultan, 2016). While the maintenance of cyanotoxins and other bioactive compounds may be driven by the selective pressure of such parasites (Rohrlack et al., 2013), several other hypotheses have been put forward regarding the primary biological functions of these metabolites, including: nutrient (Fe) scavenging (Utkilen & Gjølme, 1995), protection against oxidative stress (Zilliges et al., 2011), allelopathy (Schagerl et al., 2002; Sukenik et al., 2002; Leão et al., 2010) and quorum sensing (Kehr et al., 2006; Schatz et al., 2007). Given a somewhat lax binding specificity and their ability to act as inhibitors for a wide spectrum of enzymes (Teta et al., 2015), cyanotoxins could

have later gained an adaptive value when competitors (prokaryotes, algae) and grazers (metazoan) emerged, thus promoting their conservation and diversification in some extant taxa.

Among the many grazers involved in the consumption of cyanobacteria in freshwater ecosystems, cladocerans of the genus *Daphnia* have been suggested as a potential source of control for their proliferation in eutrophic ponds and lakes (Chislock et al., 2013; Urrutia-Cordero et al., 2016). As nonselective filter feeders (DeMott, 1990), *Daphnia* are likely to feed on a mixed diet seston, whose composition is expected to be dominated by cyanobacterial cells during blooms (Ferrão-Filho et al., 2000). Of particular interest, in this regard, is the temporal co-occurrence of cyanobacterial blooms, in temperate lakes of the Northern hemisphere, with seasonal epidemics of the yeast *Metschnikowia bicuspidata* (Metschnikoff) Kamienski, a generalist parasite of *Daphnia* causing typical outbreaks in the late summer to early autumn (Duffy et al., 2009; Hall et al., 2011; Wolinska et al., 2011). Thus far, information on how cyanobacteria-based diets could modulate fungal infection in *Daphnia* have provided fairly consistent observations, often resulting in impaired parasite transmission (Penczykowski et al., 2014; Sánchez et al., 2019; Manzi et al., 2020). However, it is not always clear whether such observations stem from cyanobacteria's poor nutritional value – leading to lowered transmission rate and/or parasite growth within the host – or rather involve effects on host immunity and possibly direct antagonistic effects on the fungus.

Considering the effects of cyanobacterial diets reported thus far in the *Daphnia-Metschnikowia* system, the following prospective mechanisms may be considered: (i) cyanobacteria reduce the success of infection through indirect consequences of their low nutritional value on host growth (i.e. reduced body size and filtering rate), leading to decreased rates of exposure as well as reduced parasite growth in successful infections; (ii) when incorporated as part of the host's diet, cyanobacteria could affect functions of host immunity (similar to how toxic stress induced by blooms of marine dinoflagellates was shown to increase haemocyte activity in oysters; Hégaret & Wikfors, 2005); or (iii) by way of their suspected antifungal properties, secondary metabolites of cyanobacteria might directly interfere with ingested spores, either in the gut lumen or the haemolymph, thus acting as 'medicines' against

the parasite. If the latter is true, antagonistic processes which usually occur at the within-host level may also be replicated outside of the host's internal environment. To our knowledge, this prediction was not previously tested in experimental infections of *Daphnia*. Yet, a number of environmental contaminants (e.g. heavy metals) and persistent organic pollutants have been shown to influence the longevity and infectivity of environmental stages of parasites, particularly in aquatic ecosystems (reviewed in Pietrock & Marcogliese, 2003; Morley et al., 2006). Moreover, other external factors were proven to influence the infectivity and environmental survival of *Metschnikowia*, independently of later encounters with the host, such as solar radiation (Overholt et al., 2012) or extreme temperatures (Shocket et al., 2019; Duffy & Hunsberger, 2019).

To explore the possibility of direct antagonistic interactions between cyanobacteria and a fungal parasite of zooplankton, we inoculated two genotypes of the common lake hybrid *Daphnia galeata* × *longispina* with spore suspensions of the parasitic yeast *Metschnikowia bicuspidata*. Prior to their inoculation, fungal spores were either pre-exposed to (i) cyanobacterial extracts or (ii) a placebo solution consisting of cyanobacterial culture medium; exposing spores to extracts allowed us to test whether secondary metabolites produced by cyanobacteria could affect fungal spores outside of the host's internal environment. In addition, to disentangle such effects from the limited nutritional value of cyanobacteria, *Daphnia* from each treatment were later maintained under two separate diets, one of which consisted of a high-quality, algal-based diet and the other of a mixture of a green alga and the same toxic cyanobacterium. We measured three variables influencing the overall transmission success of the parasite. Based on the putative antifungal properties of the cyanobacterium, we hypothesized that (i) prior exposure of the parasite to cyanobacterial toxins will impair the success of later infections and (ii) combining prior exposure to toxins with a toxic host diet will further reduce infection success.

Methods

Study system

Two genotypes of *Daphnia galeata* × *longispina* hybrids (AMME_12 and AMME_51) were selected

from a wider collection of clones isolated from Lake Ammersee, Germany; the same two host genotypes were previously used in a life-table experiment involving mixed cyanobacterial diets, which revealed distinct clonal responses to such treatments (Manzi et al., 2020). Within the *D. longispina* species complex, hybrid genotypes commonly occur, and sometimes even dominate community composition in permanent lakes (Keller et al., 2008). Hybrids may also occupy intermediate habitats beyond the ecological niche of their respective progenitor species (Griebel et al., 2015; Ma et al., 2018). *Daphnia* were maintained in synthetic SSS culture medium (Saebelfeld et al., 2017) at 19°C, under a 12:12 light–dark photoperiod and fed three times per week with 1 mg C/l of green algae *Scenedesmus obliquus* (Turpin) Kützing.

The yeast *Metschnikowia bicuspidata* (hereafter referred to as *Metschnikowia*) is a generalist parasite infecting several *Daphnia* species (Ebert, 2005; Dallas et al., 2016). Infections of *Daphnia* hosts by *Metschnikowia* are common in nature. Epidemics typically start in late summer/early autumn (Wolinska et al., 2011) and the parasite can reach high prevalence (i.e. up to 60%) in lake *Daphnia* populations (Cáceres et al., 2006). Infection takes place upon ingestion of spores by hosts when filter feeding. Mature, needle-shaped spores pierce the gut wall before reaching the haemolymph (Metschnikoff, 1884). Infection symptoms become clearly visible after 9 to 10 days; at this point, the parasite's final developmental stage (elongated asci) can be seen throughout the entire body cavity (Stewart Merrill & Cáceres, 2018). As an obligate killer, damage to the cuticle or decomposition of the host's corpse is necessary for the parasite to be released into the environment; infective stages can then encounter new hosts, or build up as spore banks in the sediment. During this 'environmental' phase of the parasite's life-cycle, spores could potentially be exposed to dissolved cyanobacterial toxins. A single *M. bicuspidata* strain was used (METS_AMME_2008), isolated from the same lake as the host. This strain was later propagated on lab-reared *Daphnia magna* Straus, 1820 (genotype E17:07) for long-term maintenance (Hesse et al., 2012). Due to its low host specificity, the parasite can be raised on *D. magna*—a larger host species, providing high spore outputs—and later used to infect other *Daphnia* species (Hesse et al., 2012; Manzi et al., 2020).

Two phytoplankton species were used as different food sources for the host: the unicellular green alga *Scenedesmus obliquus* (long-standing laboratory culture, used as standard food for *Daphnia*) and the coccoid cyanobacterium *Microcystis aeruginosa* (Kützing) Kützing (strain MaGr01, isolated from Greifensee, Switzerland; Tellenbach et al., 2016), one of the most common bloom-forming taxa in freshwater lakes (Reynolds & Walsby, 1975). Laboratory cultures of MaGr01 lost their colonial morphology, thus displaying an optimal shape and size range for *Daphnia* ingestion. This strain was confirmed to produce microcystin, with a reported concentration of 696 fg per cell (Tellenbach et al., 2016): a family of heptapeptides, microcystins are the main group of cyanobacterial toxins associated with the toxicity to vertebrates and invertebrates (Carmichael, 1992). *Scenedesmus* cultures were maintained in modified Z-medium (Zehnder & Gorham, 1960), under constant light exposure at 19°C. *Microcystis* were maintained as long-standing batch cultures in Z8 medium (Kótai, 1972), exposed to constant light at 16°C. Prior to the experiment, a subset of *Microcystis* cultures were brought up to the same conditions as *Scenedesmus* (19°C) to accelerate growth and equate the rearing temperature of *Daphnia*.

Experimental setup

Prior to the start of the experiment, the two *D. galeata* × *longispina* genotypes were maintained for two generations under standard conditions (19°C, 12:12 light–dark photoperiod, fed daily with 1 mg C/l of *S. obliquus*). 19°C is the standard rearing temperature of stock cultures in the laboratory and matches the typical August/September epilimnion temperature in Ammersee, when infection by *Metschnikowia* is usually first observed (Wolinska, personal observation). Individual *Daphnia* from two parthenogenetic lines (AMME_12/AMME_51) were used in a full factorial design, including two food sources of varying quality (*Scenedesmus*/*Microcystis*), and two infection treatments ('METS': spores exposed to a placebo solution/ 'METS + Extract': spores exposed to *Microcystis* extracts). Either 20 replicates (AMME_12) or 16 replicates (AMME_51) were set up for each combination of food and infection treatments, accounting for a total of 144 experimental units. All 144 jars

containing individual *Daphnia* were inoculated with the parasite.

Experimental *Daphnia* were born within a 48-h time span, after which mothers were removed from the common jars (day 1). On day 4, spore suspensions of the fungal parasite *Metschnikowia* were prepared; to determine spore concentrations, the suspension obtained from crushed infected *D. magna* was homogenized and loaded twice ($2 \times 10 \mu\text{l}$) on an Improved Neubauer counting chamber. Total spore yield was estimated from the number of mature spores counted in four squares of 1×10^{-4} ml capacity, across two independent loads. Two Eppendorf tubes were prepared (each containing 500 000 spores in 1 ml medium), which were either completed with a *Microcystis* extract (1 ml) or with a placebo solution (Z8 medium, 1 ml). *Microcystis aeruginosa* (strain MaGr01) were taken from an exponentially growing stock culture (OD at 750 nm = 0.181; approx. 7,500,000 cells/ml), which was then diluted (using Z8 medium) to reach an optical density of 0.09 (approx. 3 750 000 cells/ml) in 5 ml. This whole volume was briefly submerged into a solution of liquid nitrogen, to induce cell lysis (Zheng et al., 2011). The solution was then incubated at 4°C for a period of 48 h, to maximize extraction. Following this incubation period, the extracted cell content was left to thaw at room temperature for two hours and later filtrated using a GF/F glass fiber filter, to exclude any cell debris. From the resulting filtrate, 1.0 ml was added to the spore suspension used in the ‘METS + Extract’ treatment, while the ‘METS’ treatment received 1.0 ml of sterile Z8 medium (placebo solution).

Based on the level of microcystin production for strain MaGr01 assessed by Tellenbach et al. (2016) and the average absorbance / biomass ratio of *Microcystis aeruginosa* (Kaebernick et al., 2000), we aimed for an incubation concentration approximating 1300 $\mu\text{g/l}$ of microcystin. Assuming a 50% to 80% extraction success granted by the liquid nitrogen method (Zheng et al., 2011), however, we estimated a final incubation concentration in the range of 650–1040 $\mu\text{g/l}$ in the ‘METS + Extract’ treatment (for detailed calculations, see Supplementary Material). Such a concentration exceeds usual levels of microcystin observed during blooms in open water ($\leq 240 \mu\text{g/L}$, Francy et al., 2015), though higher concentrations are occasionally reported upon termination of large blooms in enclosed sections of

freshwater bodies ($\leq 1800 \mu\text{g/L}$, Jones & Orr, 1994). The two spore solutions were kept at 4°C for 24 h, allowing for infective stages of the parasite to be directly exposed to cyanobacterial cell content, prior to their encounter with the host. On day 5, all experimental *Daphnia* were transferred to individual jars containing 5 ml of fresh culture medium. Experimental jars were then inoculated with a dose of 1000 spores/ml (i.e. 5000 spores per individual *Daphnia*), from either of these two suspensions. As the present protocol only allowed for a single inoculation event, this concentration was chosen to roughly equate the amount of spores previously introduced in Manzi et al., 2020 (i.e. 1250 spores/ml split across two separate events of parasite exposure). *Daphnia* were exposed to spore-inoculated medium for a total of two days: no food was provided on the day of inoculation, as low food supplies were shown to promote spore uptake (Hall et al., 2007); on the second exposure day, all experimental units were fed with 0.5 mg C/l of *Scenedesmus*.

Throughout the first six days of the experiment, all *Daphnia* were fed daily with 0.5 mg C/l of *S. obliquus* (with the exception of inoculation day, see above). On day 7, *Daphnia* were transferred to 15 ml of fresh medium and split into their respective diets (*Scenedesmus* or *Microcystis*). At this point in time (i.e. 48 h after inoculation), the parasite should have been able to reach and settle into the haemolymph (Stewart Merrill & Cáceres, 2018), and the further ingestion of infective stages would not be possible, due to the change in medium. In the *Microcystis* treatment, a food mixture was used in which *Microcystis* contributed 75% of the total amount of carbon, with *Scenedesmus* making up the remaining 25% (similarly as in Manzi et al., 2020). The correlation between optical density and carbon content for each phytoplankton taxon was established and used to prepare food suspensions accordingly. According to Ferrão-Filho et al. (2000), mixed diets are a suitable approach to estimate the toxicity of cyanobacteria, since *Daphnia* are likely to feed on seston of mixed origin, even in lakes temporally dominated by cyanobacteria.

Daphnia were transferred to fresh medium every 4 days. Neonates were counted and removed daily. Starting from day 12 (i.e. 7 days after parasite inoculation), all animals that died were fixed in 3.7% formaldehyde, to score them later for the presence of parasite spores. The earliest record of infection symptom

in this system is day 8 post-inoculation (Manzi et al., 2020). As no further deaths were observed following 16 days after parasite inoculation, the experiment was terminated on day 21; all surviving individuals were then fixed for later inspection.

Recorded parameters

Parasite infectivity (calculated as the proportion of successfully infected *Daphnia*) was assessed by checking fixed animals for the presence of infection symptoms. The presence of mature spores was confirmed in suspensions obtained from crushed individuals (Nikon SMZ25 stereomicroscope, 200× magnification). Parasite reproduction (the total number of spores accumulated upon host death, calculated individually per infected host) was estimated from a suspension of crushed infected *Daphnia* using a counting chamber (see *Experimental setup*). Age at death was recorded for each individual *Daphnia* that died starting from day 6 (one day after the introduction of the parasite); animals that were fixed in formaldehyde on the last experimental day were considered to have died at the age of 21 days (no natural death occurred on that day).

Data analysis

Data were analyzed using R version 4.1.0 (R Core Team, 2021). Graphical outputs were produced using the ‘ggplot2’ (Wickham, 2016) and ‘Hmisc’ (Harrell & Harrell, 2019) packages. Analysis of variance (*F*-test or χ^2 test) was performed with the ‘car’ package (Fox et al., 2012) using type III sums-of-squares for ‘parasite infectivity’ and ‘parasite growth’, and type II for ‘host lifespan’ (as no significant interaction was detected for this variable). Model selection was then performed by a stepwise regression approach based on Akaike’s Information Criterion (AIC).

Parasite infectivity was analyzed by performing a binomial logistic regression (0 = not infected, 1 = infected) with *Exposure*, *Diet* and *Clone* as explanatory variables. Only those individuals, which survived until at least day 8 post-inoculation (i.e. earliest observation of fungal asci in this experiment and one previous study, Manzi et al., 2020) were considered for infectivity, as reliable detection of infection symptoms was not possible prior to that day. To control for the potential influence of host lifespan on the

cumulative number of spores produced by the parasite, parasite growth was estimated as the ratio of total spore yield over the number of days survived post-inoculation. Parasite growth and host lifespan post-inoculation were analyzed using generalized linear models with *Exposure*, *Diet* and *Clone* as explanatory variables. Normal distribution and homoscedasticity of the residuals were verified by visual inspection of quantile–quantile plots and residuals against fitted values, respectively. Only animals which became successfully infected by the parasite were included in these analyses.

Results

Parasite infectivity

Out of 144 *Daphnia* exposed to *Metschnikowia* spores, 63 individuals died before day 8 post-inoculation (categorized as *early death*). As infection could only be confirmed starting from day 8 post-inoculation, these individuals were not included in the determination of parasite infectivity. Two individuals were lost due to handling error, prior to their inspection under the microscope. Among the 79 remaining individuals, 53 were confirmed infected (67%) and 26 remained uninfected.

Spores which were pre-exposed to cyanobacterial toxins (‘METS + Extract’) had higher infectivity on clone AMME_51, as compared with the placebo treatment (‘METS’). However, no such effect was found on AMME_12 (significant *Exposure* × *Clone* interaction, Table 1). Specifically, pre-exposed spores led to 100% infection on clone AMME_51, while non-exposed spores infected less than 60% of this genotype (Fig. 1a). Otherwise, there was a tendency towards higher proportion of infected hosts under a *Microcystis* diet (Table 1, Fig. 1a).

Parasite growth

Pre-exposure of *Metschnikowia* to cyanobacterial toxins did not influence the parasite’s ability to produce spores (there were neither a main effect nor any significant interaction involving this factor, Table 1). Parasite growth was reduced by about two-fold when AMME_12 were fed with *Microcystis*, as compared with the *Scenedesmus* diet (Fig. 1b). Within clone

Table 1 Three-way ANOVA (χ^2 test or F -test) testing for fixed effects of spore *Exposure*, host *Diet*, host *Clone* and their interactions on fitness components of the host and parasite

Response variable	Distribution (link function)	Explanatory variables	Statistic _(df)	<i>P</i> -value
Parasite infectivity	Binomial (link: logit)	Exposure	$\chi^2_{(1,74)} = 0.034$	0.853
		Diet	$\chi^2_{(1,74)} = 3.001$	0.083
		Clone	$\chi^2_{(1,74)} = 1.841$	0.175
		Exposure \times Clone	$\chi^2_{(1,74)} = 9.234$	0.002
Parasite growth	Normal	Exposure	$F_{(1,48)} = 0.023$	0.881
		Diet	$F_{(1,48)} = 23.243$	< 0.001
		Clone	$F_{(1,48)} = 7.883$	0.007
		Diet \times Clone	$F_{(1,48)} = 4.898$	0.032
Host lifespan post-inoculation	Normal	Exposure	$F_{(1,49)} = 0.014$	0.908
		Diet	$F_{(1,49)} = 11.244$	0.002
		Clone	$F_{(1,49)} = 0.097$	0.757

Model selection was performed by stepwise regression based on Akaike's Information Criterion (AIC); only the final model is reported here. Significant *P*-values (≤ 0.05) are highlighted in bold

AMME_51, however, parasite reproduction was comparable under both diets (significant *Diet* \times *Clone* interaction, Table 1). In addition, the parasite's ability to produce spores under a *Microcystis* diet was significantly higher on clone AMME_51 than AMME_12 (Tukey's HSD test, $P = 0.032$).

Host lifespan post-inoculation

The maximum lifespan recorded among successfully infected individuals was 15 days post-inoculation. Pre-exposure of the parasite did not influence the lifespan of infected hosts (Table 1). However, those maintained on a *Microcystis* diet survived on average 2 days longer, compared to the standard diet (Fig. 1c).

Discussion

Given the complex evolutionary history and putative antifungal properties of cyanobacteria, we expected isolated cyanotoxins to exert direct antagonistic effects against a fungal parasite of *Daphnia*, possibly lowering its infection success upon later encounter with the host. While our experiment was not the first attempt to incorporate extracted cyanobacterial toxins into infection assays using the *Daphnia-Metschnikowia* system (Penczykowski et al., 2014; Sánchez et al., 2019), our protocol differed from previous studies by one key aspect. Specifically, the

inclusion of cyanobacterial toxins was performed in such a way that would guarantee prolonged exposure of the parasite (i.e. 24 h), while limiting potential adverse effects on the host. By contrast, previous studies incorporated cyanobacterial compounds into the host's diet, thus exposing both antagonists at the same time. This specificity allowed us to test for possible negative effects of secondary metabolites against infective stages of a fungal parasite, independently of their consumption by infected *Daphnia*.

Effects of parasite pre-exposure

Contrary to our expectations, pre-exposure to cyanobacterial toxins did not impair the parasite's ability to infect in any treatment. Instead, we report a significant increase in infectivity for one of the tested *Daphnia* genotypes (AMME_51). While *Daphnia* from the 'METS + Extract' treatment were technically exposed to cyanobacterial toxins through the parasite inoculate (i.e. estimated concentration of microcystin in the experimental vial ranging from 0.00259 to 0.00418 $\mu\text{g/ml}$, see Supplementary Material), such a concentration remains largely inferior to the 48-h LC50 described for *Daphnia hyalina* Leidig, 1860 (11.6 $\mu\text{g/ml}$; DeMott et al. 1991); thus, the amount of cyanobacterial toxins introduced as part of the inoculate is unlikely to have caused direct detrimental effects on *Daphnia* upon exposure. However, we cannot rule out that some toxic compounds extracted

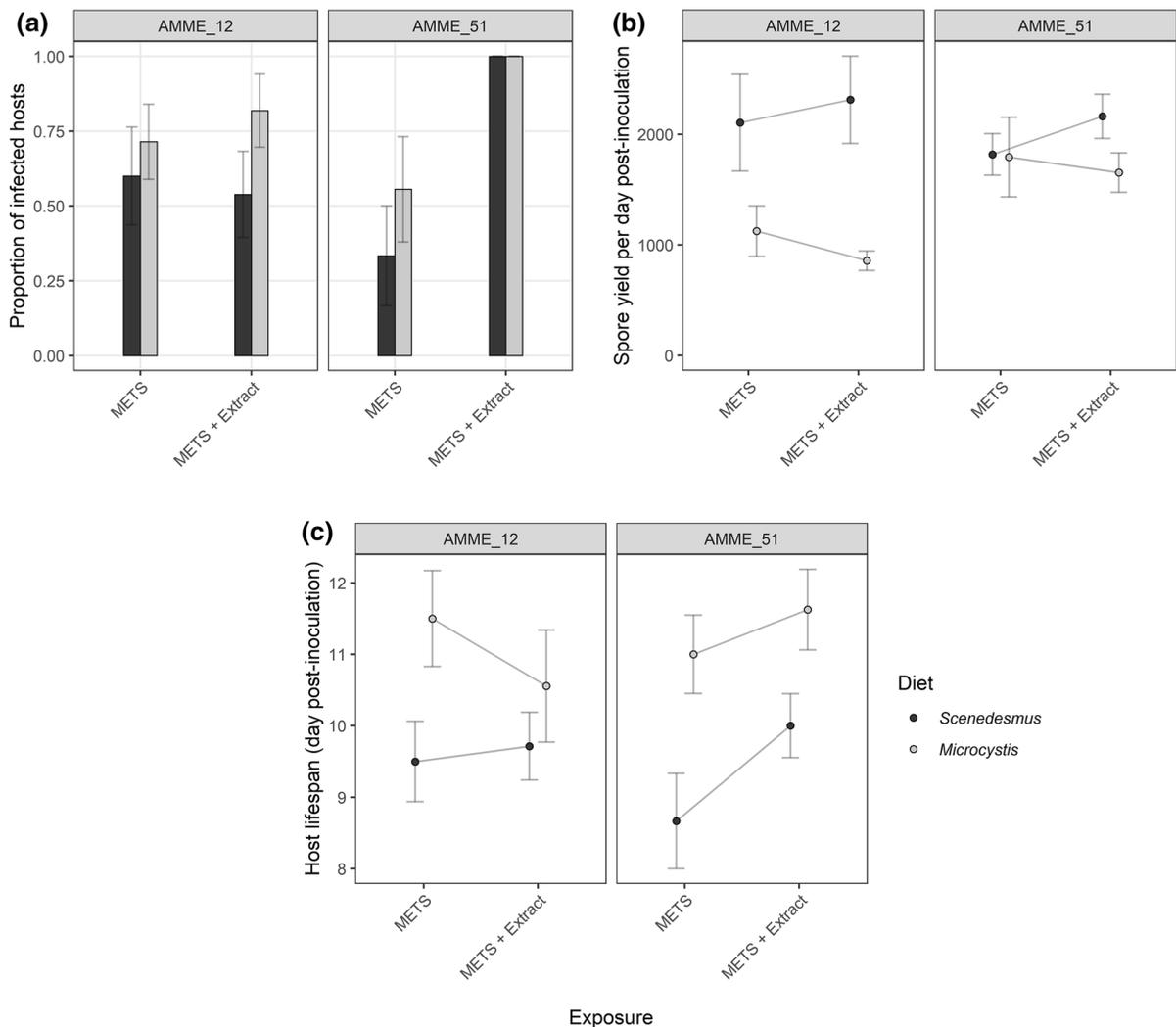


Fig. 1 Graphical representation of **a** parasite infectivity, computed as the proportion of infected hosts among those which survived long enough to allow for completion of the parasite's life-cycle, **b** parasite growth, computed as the ratio of total spore yield upon host death over the number of days survived after inoculation, and **c** host lifespan post-inoculation, computed as the number of days survived by the host, following introduction of the parasite on day 5. Two genotypes of *Daphnia galeata* × *longispina* hybrids (AMME_12; AMME_51) were inoculated with 1000 spores/ml of the para-

sitic yeast *Metschnikowia bicuspidata*. These spore solutions were previously exposed to an extract of *Microcystis aeruginosa* ('METS + Extract'), or to a placebo solution ('METS'). Following completion of the inoculation process, individual *Daphnia* were then fed either with a standard diet, consisting of the green alga *Scenedesmus obliquus* or with a mixed diet, where the cyanobacterium *Microcystis aeruginosa* contributed 75% of the total carbon content. Error bars depict the standard error of the mean

from *Microcystis* could have adhered to the surface of fungal spores. Upon passing through the gut epithelium, lingering cyanotoxins may have thwarted the immune response of the host (i.e. mobilization of the haemocytes; Metschnikoff, 1884), thus directly acting against defence mechanisms of the host, rather than

'enhancing' the infectious potential of the parasite's propagules. Although previous studies did not report such an increase in host susceptibility, the apparent lack of detrimental effect on the parasite remains consistent with the results of Penczykowski et al. (2014): the authors artificially coated green algal

cells with cyanobacterial extracts, showing no reduction in either the transmission rate or spore yield of *Metschnikowia*.

Effects of the *Microcystis*-based diet

Based on previous results using this system, we expected a two- to three-fold reduction in parasite reproduction, when the host is maintained under a sustained *Microcystis* diet (Manzi et al., 2020). Surprisingly, this effect only applied to infections of the *Daphnia* genotype AMME_12. In genotype AMME_51, the *Microcystis* diet allowed the parasite to produce spores at a rate comparable to that of the high-quality diet. These types of host-genotype-by-environment ($G_H \times E$) interactions affecting parasitic infections are commonly reported in the *Daphnia* system, meaning that the outcome of infection can depend on environmental conditions, in a genotype-specific way (Wolinska & King, 2009). For instance, it was found that the infectivity and virulence of the bacterium *Pasteuria ramosa* Metschnikoff, 1888 would vary interactively between clonal identity of the host and either nutrition (Mitchell & Read, 2005; Little et al., 2007) or temperature (Mitchell et al., 2005; Little et al., 2007). Previously, we observed that the reduction in parasite reproduction induced by a *Microcystis* diet applied to both *Daphnia* genotypes, but was stronger on AMME_12 than AMME_51 (Manzi et al. 2020). While the disparity was more pronounced here, this reflects a similar interaction and thus remains consistent with our previous findings.

Additionally, we observed that a *Microcystis*-based diet allowed infected hosts to survive slightly longer than the *Scenedesmus* treatment (by about two days). A similar increase in the longevity of infected hosts was previously observed on a *Microcystis*-based diet, although this was found exclusively at 23°C (Manzi et al., 2020). Paradoxically, what could be interpreted as a protective effect for the host might in fact increase the parasite's transmission success: in spite of a less-nutritious diet, the parasite can benefit from an extended infection time, allowing it to accumulate a higher spore yield upon host death; a paradox that has long been described as the virulence-transmission trade-off (Anderson & May, 1982; Acevedo et al., 2019). Because *Microcystis* strongly reduced the rate of spore production in AMME_12 hosts, the small

increase in host longevity granted by this diet was not sufficient to outcompete spore outputs in the *Scenedesmus* treatment (Figure S1). By contrast, the mitigated effects of a toxic diet on AMME_51 allowed the parasite to perform equally under both levels of food quality (Figure S2).

Microcystis has been reported to induce feeding inhibition in cladocerans (even when integrated as low as 5% of a mixed diet), which may be imputed in part to behavioural avoidance of toxic cells (Ferrão-Filho et al., 2000) or diet-induced reduction of the host's body size, leading to lower filtering rates (Penczykowski et al., 2014). In the latter study, a drastic reduction in the infection rate of *Metschnikowia* was attributed to both behavioural and size-related effects. In our experimental assay, we found an opposite trend, with systematically higher proportion of infected hosts under a *Microcystis* diet, although this tendency was not statistically significant ($P=0.08$). However, this was expected, due to a different layout in our experimental design: because the *Microcystis* diet was only introduced after parasite exposure, any potential effect of food quality on parasite encounter per se can be ruled out. Thus, within the context of our study, the *Microcystis* diet could only have influenced within-host processes involved in the parasite's infectivity, such as its ability to overcome the host's immune response. Our results thereby suggest that maintaining the host on a toxic diet throughout the progression of the parasite's development cycle does not impair the parasite's infectivity. Instead, the greatest consequence of a poor diet under natural conditions should consist of limited exposure to the parasite, due to behavioural and/or physiological decreases in feeding rates, constraining the overall success of infection (Ferrão-Filho et al., 2000; Penczykowski et al., 2014).

Environmental relevance

When cyanobacteria are incorporated as part of *Daphnia*'s diet, endotoxins are delivered upon digestion of bacterial cells, during their passage through the gut lumen (Carmichael, 1992). Considering that *Microcystis* compounds are capable of binding to (thus inhibiting) digestive proteases within the gut lumen of *Daphnia* (Agrawal et al., 2005; von Elert et al., 2012), in the context of parasitic infections, the digested cell content of cyanobacteria may also get in

contact with ingested spores. Therefore, cyanobacteria are likely to interact with spores of horizontally transmitted parasites, when both are consumed by the host and concurrently passing through the host's gut.

Throughout the course of harmful algal blooms, cell lysis of dominant cyanobacteria results in the release of large amounts of (otherwise intracellular) bioactive compounds in the water column (Jones & Orr, 1994; Ha et al., 2009). The levels of cyanotoxins released during blooms have been remarked to approximate half the LC50 for *Daphnia* (DeMott et al., 1991; Sánchez et al., 2019), with concentrations of microcystins reportedly ranging from <0.10 to 240 µg/l in freshwater lakes and recreational sites (Francy et al., 2015). Upon termination of a large bloom within enclosed water bodies, particularly high concentrations of microcystins (1300–1800 µg/l) have been noted to persist for up to nine days, prior to a rapid phase of degradation (Jones & Orr, 1994). Thus, such concentrations of dissolved compounds may be high enough for cyanobacteria to exert adverse effects on cladoceran hosts, independently of their consumption as live cells (Ibelings et al., 2005). As far as cyanobacterial toxins directly interfering with free-floating spores or spore banks in the sediment, however, the likelihood of such event is more difficult to infer. On the one hand, some waterborne parasites (e.g. fish helminths) are able to bioconcentrate chemicals and pollutants at a much higher rate than free-living species, even when these are present at very low concentrations in the environment (Sures, 2003; Nachev & Sures, 2016). However, it can be argued that cyanobacteria and parasite spore banks normally occupy different layers in the stratified water column. While cyanobacteria's high buoyancy allows them to maintain close proximity to the surface, spores of the fungal parasite *Metschnikowia* are commonly recruited from the sediment, as infected hosts tend to sink to the bottom upon their death (Cáceres et al., 2006; Duffy & Hunsberger, 2019). Thus, by filter-feeding onto higher parts of the water column, cladocerans may sequester large amounts of dissolved cyanobacterial toxins and prevent those from reaching the sediment.

While our results did not provide support for our starting hypothesis (i.e. that the putative antifungal effects of *Microcystis aeruginosa* may not be limited to 'medicinal effects' mediated by diet but could instead directly interfere with the parasite, prior to

their ingestion by *Daphnia* hosts), the specificity of our experimental design allowed us to raise the question of the parasite's vulnerability to an environmental stress of its own. Considering that outbreaks of this parasite typically occur for the span of a few months (i.e. from late summer to early winter) in temperate lakes, infective propagules contributing to the next seasonal epidemic will remain for considerable amounts of time buried in the sediment (Decaestecker et al., 2004). In temporary ponds susceptible to draining, spore banks of the parasite can even survive extended periods of complete host absence, such as during the dry seasons (Ebert, 2005). This effectively means that most of the parasite's lifespan (i.e. from release into the environment, to completion of a new reproductive cycle) is effectively spent outside of the host's internal environment, where infective propagules of parasites may be prone to a number of environmental disturbances (Pietroock & Marcogliese, 2003; Sures et al., 2017). Specifically, resting stages of *Metschnikowia* were previously shown to degrade or decrease in infectivity, following their exposure to solar UV (Overholt et al., 2012), high temperatures (Shocket et al., 2019) or near-freezing conditions (Duffy & Hunsberger, 2019). Such studies demonstrate that the transmission potential of this parasite can indeed be compromised by external environmental factors acting prior to—and independently of—its encounter with the host. Our results suggest, however, that exposure to dissolved cyanobacterial toxins during blooms does not constitute one such mechanism. While cyanobacterial dominance may not directly interfere with the infectivity of fungal spore banks, other parasites of cladocerans belonging to distant taxa, such as the endospore-forming bacterium *Pasteuria ramosa* (Ebert et al., 1996) or the viral agent for White Fat Cell Disease (Toenshoff et al., 2018) may be worthy of investigation, in light of the wider allelopathic properties of cyanobacteria.

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Author contributions FM, MM, JW and RA conceptualized the study. FM and MM performed the experiment, data analysis and results visualization. FM wrote the article, with help from RA, JW and MM. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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Data availability The dataset supporting this study can be found at: <https://doi.org/10.5281/zenodo.5841637>.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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