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Can parasites adapt to pollutants? A multigenerational experiment with a *Daphnia* × *Metschnikowia* model system exposed to the fungicide tebuconazole

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ABSTRACT

There is increasing evidence about negative effects of fungicides on non-target organisms, including parasitic species, which are key elements in food webs. Previous experiments showed that environmentally relevant concentrations of fungicide tebuconazole are toxic to the microparasite *Metschnikowia bicuspidata*, a yeast species that infects the planktonic crustacean *Daphnia* spp. However, due to their short-term nature, this and other experimental studies were not able to test if parasites could potentially adapt to these contaminants. Here, we tested if *M. bicuspidata* parasite can adapt to tebuconazole selective pressure. Infected *D. magna* lineages were reared under control conditions (no tebuconazole) and environmentally realistic tebuconazole concentrations, for four generations, and their performance was compared in a follow-up reciprocal assay. Additionally, we assessed whether the observed effects were transient (phenotypic) or permanent (genetic), by reassessing parasite fitness after the removal of selective pressure. Parasite fitness was negatively affected throughout the multigenerational exposure to the fungicide: prevalence of infection and spore load decreased, whereas host longevity increased, in comparison to control (naive) parasite lineages. In a follow-up reciprocal assay, tebuconazole-conditioned (TEB) lineages performed worse than naive parasite lineages, both in treatments without and with tebuconazole, confirming the cumulative negative effect of tebuconazole. The underperformance of TEB lineages was rapidly reversed after removing the influence of the selective pressure (tebuconazole), demonstrating that the costs of prolonged exposure to tebuconazole were phenotypic and transient. The microparasitic yeast *M. bicuspidata* did not reveal potential for rapid evolution to an anthropogenic selective pressure; instead, the long-term exposure to tebuconazole was hazardous to this non-target species. These findings highlight the potential environmental risks of azole fungicides on non-target parasitic fungi. The underperformance of these microbes and their inability to adapt to such stressors can interfere with the key processes where they intervene. Further research is needed to rank fungicides based on the hazard to non-target fungi (parasites, but also symbionts and decomposers), towards more effective management and protective legislation.

1. Introduction

Environmental stressors are known to affect and modulate disease spread (Altizer et al., 2013; Sures et al., 2017; Wolinska and King, 2009). Among anthropogenic factors that may affect parasite and pathogen success, pollution stands out as one of the most relevant factor (Blanar et al., 2009; Sures, 2008). Chemical pollution can enhance disease incidence by increasing host susceptibility or parasite virulence (Coors et al., 2008; Coors and De Meester, 2011; Kelly et al., 2010), but it can also decrease the incidence of disease if parasites are more susceptible to pollutants than hosts or the transmission of the parasite is compromised (Blanar et al., 2010; Civitello et al., 2012; Hanlon et al.,

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2012). Whereas interactive scenarios between pollution and parasitism are usually synergistic (Coors and De Meester, 2008; Kelly et al., 2010; Lafferty and Kuris, 1999), recent studies have demonstrated anti-parasitic effects of contaminants on some host-parasite systems (Cuco et al., 2017b; De Coninck et al., 2013; Hall et al., 2013; Hanlon et al., 2012).

Environmentally relevant concentrations of some fungicides seem capable of complete suppression of non-target fungal parasites, that otherwise cause disease in cyanobacteria (Ortiz-Cañavate et al., 2019), invertebrate (Cuco et al., 2017a) or vertebrate (Hanlon et al., 2012) hosts. For example, we showed that common fungicide tebuconazole completely suppressed infection signs and transmission stages of the microparasitic yeast *Metschnikowia bicuspidata*, a common parasite of the planktonic crustacean *Daphnia* (Cuco et al., 2017a, 2017b, 2018). Suppression of disease should be seen as an important ecological disturbance, because parasites control host populations, play important roles in the energy flow in food webs, and are engaged in a co-evolutionary arms race with their hosts, mutually promoting genetic diversity (Anderson, 1978; Lafferty et al., 2008; Turko et al., 2018). With increasing application of anti-fungal compounds to improve productivity in agricultural areas (Kahle et al., 2008; Stoate et al., 2009), these negative effects on non-target parasitic fungi may be more widespread than currently recognised. Given our present dependence on agrochemicals (and particularly fungicides) to grow and store food (Liu et al., 2015; Zubrod et al., 2019), further insight on such disturbances of host-parasite relationships is thus necessary. In particular, the ecological consequences of agrochemical fungicides to non-target parasites (and their hosts) are still not fully understood, as long-term (multigenerational) studies are missing.

As fungicides are usually applied over long time periods (in multiple applications) and many have moderate persistence in aquatic ecosystems (Bereswill et al., 2012; Kahle et al., 2008), this leads to chronic (i.e. multigenerational) exposure scenarios to the pollutants. Long-term exposure to pollution might result in parasite (and host) adaptation. Although both antagonists have the potential to adapt to pollutants (Medina et al., 2007), this is more likely to happen first to parasites, given their shorter generations and larger populations sizes. Indeed, the development of resistance to fungicides has been observed in the wild for some fungal pathogens (Gil et al., 2014; Tlili et al., 2016). Nevertheless, previous laboratory studies often disregarded this possibility, because of the short-term nature of experimental assays.

Our goal was to determine if the yeast *M. bicuspidata* can adapt to tebuconazole during a long-term exposure, following our previous data on the *Daphnia-Metschnikowia* experimental system (Cuco et al., 2017a, 2017b, 2018). Parasite lineages were grown in the absence (naive lineages) or presence (conditioned lineages) of tebuconazole for four generations, and their performance was then compared in a reciprocal assay. Three possible outcomes were expected: (i) conditioned *M. bicuspidata* lineages perform better than naive lineages under tebuconazole exposure, confirming phenotypic adjustment (acclimation) or genetic adaptation to the chemical stressor; (ii) conditioned *M. bicuspidata* perform worse than naive lineages under control (no tebuconazole) conditions, demonstrating costs of prolonged (multigenerational) exposure to tebuconazole, or (iii) conditioned lineages outperform naive lineages under stressful conditions (tebuconazole presence) but perform worse under control conditions (no tebuconazole), which supports adaptation at some cost. Additionally, we assessed whether the observed effects were transient (phenotypic) or permanent (genetic), by reassessing parasite fitness after the removal of selective pressure.

2. Methods

2.1. Host-parasite system: origin and maintenance

The host-parasite system selected for this study included *Daphnia magna* as the host (clone E17:07, Saebelfeld et al., 2017) and the microparasitic yeast *Metschnikowia bicuspidata* as the parasite (strain AMME, Lohr et al., 2010). *Daphnia* sp. are small freshwater crustaceans that are commonly used in aquatic ecology and ecotoxicology studies as experimental models, due to their central position in aquatic food webs and their advantageous life cycle characteristics, such as cyclic parthenogenetic reproduction and short generation time (Lampert, 2006; Miner et al., 2012). Daphnid populations are affected by various ecto- and endoparasites (Ebert, 2005), including the yeast *M. bicuspidata*, which grows inside the host body. The infection process starts when needle-shape ascospores of *M. bicuspidata* are filtered by the host, pierce the gut wall and germinate, filling the entire body cavity of the host and impairing host fecundity, ultimately causing its death (Ebert, 2005; Stewart Merrill and Cáceres, 2018). A logistical advantage of this particular host-parasite model system is to allow the control of host and parasite features (e.g., genetic variability), as well as a strict control of parasite transmission and spore load (Cuco et al., 2017a; Ebert et al., 2000).

Uninfected and infected asexual *D. magna* cultures (clone E17:07) were cyclically reared in standard laboratory conditions, maintained in moderately hard reconstituted water (123 mg l^{-1} $MgSO_4{\cdot}7H_2O$, 96 mg l^{-1} $NaHCO_3$, 60 mg l^{-1} $CaSO_4{\cdot}2H_2O$, 4 mg l^{-1} KCl) supplemented with a standard organic additive (algal extract) and vitamins (see Loureiro et al., 2012, 2011). Medium was renewed three times per week and, at each medium renewal, cultures were fed with a *Raphidocelis subcapitata* (see Krienitz et al., 2011) suspension at a final density of 3.0×10^5 cells ml^{-1}. Infected cultures were maintained by adding uninfected *D. magna* every other week, ensuring a continuous supply of hosts and promoting parasite transmission. Both cultures were kept in a controlled temperature room (at 20 ± 1 °C) with a $16h^L$:$8h^D$ photoperiod. More details about culture maintenance and host-parasite model system can be found in Cuco et al. (2017a, 2017b).

2.2. Test chemical

Tebuconazole (Tebuconazol PESTANAL®, CAS nr. 107534-96-3) [(RS)-1-p-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl) pentan-3-ol] was purchased from Sigma Aldrich (Munich, Germany). Concentrated stock solutions were prepared in ethanol, which was used as a carrier into aqueous test solutions; ethanol final concentration was equal in all treatments (0.01 ml l^{-1}). Final test solutions were obtained by dissolving the proper amount of the concentrated stock solution in reconstituted water (see *Host-parasite system: origin and maintenance*). In order to check the validity of the nominal concentrations tested, two random aliquots of freshly prepared test solutions were collected and sent to an independent analytical laboratory (certified according to ISO/IEC 17025:2005). Quantification of tebuconazole was performed by liquid chromatography mass spectrometry (LC–MS/MS), according to an internal method adapted from ISO 11369:1997.

2.3. Evolution experiment

Parasite was exposed to tebuconazole for four consecutive generations. Because *M. bicuspidata* is an obligatory parasite, the Evolution experiment was carried out by growing the yeast in its host, *D. magna*, and performing the serial passage of spores to new hosts from stock cultures at each generation. The use of healthy hosts from stock cultures at each serial passage allowed controlling long-term effects of tebuconazole on host performance; also, a single host clone was used to exclude any genetic diversity of the host (Brockhurst and Koskella, 2013). The experiment lasted 3.5 months, which roughly corresponds to the maximum duration of *M. bicuspidata* epidemics in the field (Caceres et al., 2006; Duffy and Sivars-Becker, 2007).

Four treatments were applied: a naive treatment (raising host-parasite system at 0.00 µg l^{-1} of tebuconazole) and three conditioning treatments (raising host-parasite system at 3.65, 4.40 and 5.31 µg $l^{-1}$of tebuconazole). In the naive treatment, ethanol was added to a final

concentration of 0.01 ml l^{-1}, to assure comparability with the tebuconazole treatments (see *Test chemical*). The levels of tebuconazole exposure were chosen based on infection profiles (and IC_{50} values) for *M. bicuspidata* as a function of tebuconazole concentration for three distinct host species (including *D. magna* E017:07 – Supporting Information, Fig. S1). Whilst the selected concentrations are on the onset of the antiparasitic action of tebuconazole, they are innocuous to the host. Specifically, these concentrations are ca. ten times lower than toxic levels reported for *Daphnia* reproduction (Cuco et al., 2017b, 2016; Qi et al., 2015); moreover, exposure to these concentrations did not increase host susceptibility to the parasite (Cuco et al., 2017a, 2017b, 2018). Because the two highest tebuconazole treatments led to the partial or total loss of lineages throughout the Evolution experiment (Supporting Information, Fig. S2), only the 3.65 μg l^{-1} conditioned lineages (hereafter designated as TEB lineages) were used in all remaining setups and analyses.

Each treatment consisted of five independent parasite lineages (nested within treatment). The experiment started by placing groups of 20 *D. magna* neonates (< 24 h old, born between the 3rd and 5th clutch) into each glass vessel, which contained 300 ml of the correspondent test solution. On day 4, test solution volume was reduced to 100 ml and spores from a single initial spore suspension (obtained from infected *Daphnia* crushed in distilled water) were added to each experimental unit to reach a concentration of 2500 spores ml^{-1} (according to Engelbrecht et al., 2012; Hesse et al., 2012). Spores were counted with a Neubauer improved counting chamber. No food was added on that day to promote higher filtration. On day 5, feeding was restored, and test solution volume was progressively increased to 200 ml (day 5) and 300 ml (day 6), by adding fresh test solutions. From day 7 onwards, daily feeding was resumed, and medium renewal was carried out every three days.

The serial passage of *M. bicuspidata* spores of each replicate lineage from one generation to the following was performed without ever mixing the spores between the lineages (Fig. 1). At each parasite transfer (always performed at day 28), five infected hosts from each replicate lineage were ground up to obtain a spore suspension; infected hosts that had recently died or that were dying were used to make the suspensions, to assure that *M. bicuspidata* ascospores were fully mature. Spore suspensions were adjusted to match a final density of 2500 spores ml^{-1}, and then used to inoculate a new generation of hosts following the procedure described in the previous paragraph (inoculation at day 4, along with volume reduction and starving of the host). Neonates from uninfected *D. magna* cultures were the source of hosts for the new *M. bicuspidata* generation. These *M. bicuspidata* passages were performed three times, which corresponds to a total of four growth and sporulation cycles of *M. bicuspidata*. Spores from the F_4 generation were used in a follow-up reciprocal assay (see Fig. 1).

Vessels were checked daily for the presence of *Daphnia* offspring (which were discarded) and visibly infected or dead adult *Daphnia* (infected mothers became opaque and died within a few days; Ebert, 2005). Upon death, the presence of spores inside the body cavity was confirmed with an Olympus CKX41 inverted microscope. In each generation, the time of death (in days) of all infected mothers was calculated per experimental unit, as well as prevalence (proportion of infected *Daphnia*). Estimates of prevalence (as a measure of infectivity) excluded the few cases where *Daphnia* died before infection could be confirmed (i.e., before day 14). Spore load per infected host was also assessed in each replicate lineage by counting the spore suspensions (made from five infected hosts) at the end of each generation.

2.4. Reset experiment

To assess the reversibility of the multigenerational tebuconazole effects in *M. bicuspidata*, the serial passage of the parasite spores was continued for two more generations in the naive and TEB lineages, after removing the influence of the selective pressure in TEB lineages (Fig. 1). This experiment was analogous to the Evolution experiment, using 20 *Daphnia* in 300 ml vessels as experimental units, challenged with spores from the previous generation (F_4 naive vs. F_4 TEB spores), except that both naive and TEB lineages were now reared in the absence of tebuconazole. The Reset experiment involved one serial passage of *M. bicuspidata* spores, resulting in two growth and sporulation cycles of *M. bicuspidata* (Fig. 1). Vessels were checked daily for visibly infected or dead *D. magna*, and prevalence and spore load were quantified (see *Evolution experiment*). Spores from the F'_2 generation were used in a follow-up reciprocal assay (Fig. 1).

2.5. Follow-up reciprocal assays

The purpose of the two follow-up reciprocal assays was to compare the performance of naive and TEB lineages in environments with and without tebuconazole. These assays were performed independently at two distinct moments (Fig. 1): directly after the Evolution experiment and later after the Reset experiment. To do so, parasite spores from naive and TEB lineages were used to infect *D. magna* along a gradient of tebuconazole concentrations: 0.0, 3.65 and 4.4 μg l^{-1}. Specifically, *D. magna* neonates (< 24 h old, born between the 3rd and 5th clutch) were exposed to the three tebuconazole levels, and then half of these juveniles were infected with the naive parasite lineages and the other half with TEB parasite lineages, in a balanced design with 300 experimental units: 2 treatments (TEB vs. naive) × 5 lineages (nested within treatments) × 3 tebuconazole concentrations × 10 individual hosts.

In both follow-up reciprocal assays, hosts were individually raised in 50 ml vessels. Parasite challenge was carried out on day 4, similarly to the infection procedure described in the Evolution and Reset experiments, by stopping adding food and reducing test volume to 15 ml while exposing the hosts to naive or TEB spores (final spore density of 2500 spores ml^{-1}). On the following days, feeding was resumed, and volume was increased to 30, 45 and 50 ml on days 5, 6 and 7, respectively. Vessels were checked daily for offspring and mortality. The proportion of infected hosts was calculated at day 21 (pooling the 10 individual hosts in each combination of lineage and concentration), after excluding the few cases where *Daphnia* died before infection could be confirmed (i.e. before day 14). Spore load per host was assessed as the average spore density in three randomly chosen infected hosts, for each combination of lineage and concentration. The experiments lasted until the last infected host died.

2.6. Statistical analysis

Data were analysed using generalized linear mixed models (GLMM), with prevalence being modelled as a binomially distributed variable with a logit link function (i.e., logistic model) and spore load as a normally distributed variable (i.e., linear model). To improve homoskedasticity, spore load data were log_{10}-transformed. All analyses were run in *R* software, version 3.6.1 (R Core Team, 2019), with GLMM models being fit via function "*glmer*" (for prevalence) or "*lmer*" (for spore load) from R package "*lme4*" (Bates et al., 2015). Plots were also produced in *R* using packages "*ggplot2*" (Wickham, 2016) and "*cowplot*" (Wilke, 2019).

In the case of the Evolution experiment and Reset experiment, GLMMs were used to investigate the effect of the evolution treatment (naive vs. TEB) through time (generation). Analogously, GLMMs were used to investigate the effects of treatment and tebuconazole concentration in the follow-up reciprocal assays. In both cases, the random variation across individual lineages (which are nested within treatment) was included in the models. Significance of non-random effects was tested using Wald χ^2 tests (for prevalence) or F tests with Kenward-Rodger adjustment (for spore load), via the "*Anova*" function from *R* package "*car*" (Fox and Weisberg, 2019).

In the presence of a significant treatment × generation or treatment × concentration interaction, simple main effects of treatment (Quinn

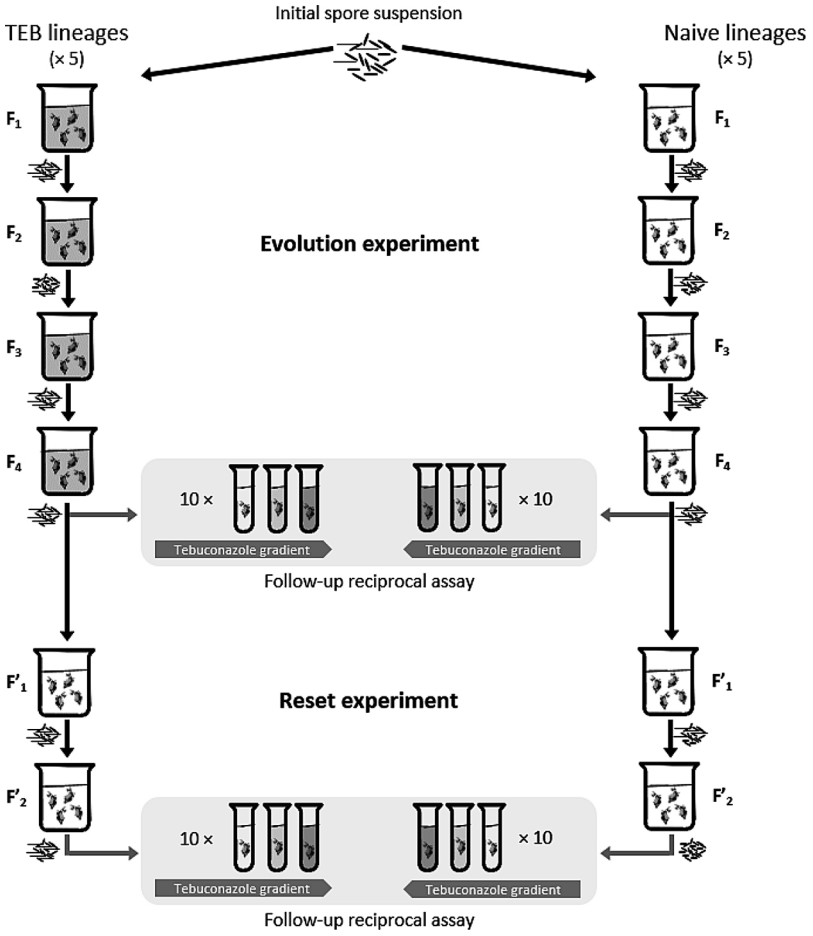

Fig. 1. Conceptual framework of the various experimental steps undertaken in this study. Lineages of the microparasitic yeast *Metschnikowia bicuspidata* were reared *in vivo* (on *Daphnia magna* host) during four growth and sporulation cycles (Evolution experiment, top), under multigenerational tebuconazole conditioning (TEB lineages) or control conditions (naive lineages). Then, naive and TEB lineages were reared for two additional growth and sporulation cycles under control conditions (Reset experiment, bottom). Black arrows represent the serial passage of *M. bicuspidata* spores of a single lineage from one generation to the following; this was replicated independently five times (i.e., 5 naive lineages + 5 TEB lineages). At the end of each experiment (Evolution and Reset), follow-up reciprocal assays were carried out with naive and TEB spores to test parasite fitness.

and Keough, 2002) were tested within each generation or concentration, after adjusting the false discovery rate (Benjamini and Hochberg, 1995). This was performed via the function "*testInteractions*" from R package "*phia*" (De Rosario-Martinez, 2015).

3. Results

3.1. Test chemical analysis

Analytical data confirmed the validity of the nominal concentrations, despite some variation in the samples: nominal tebuconazole concentrations of 3.65, 4.40 and 5.31 μg l^{-1} were estimated to be 4.05 ± 0.35 μg l^{-1}, 5.45 ± 0.21 μg $l^{-1}$and 6.5 ± 0.56 μg l^{-1} (n = 2), i.e. 111 %, 124 % and 122 % of nominal concentrations, respectively. We will refer to nominal concentrations for coherence.

3.2. Evolution experiment

Tebuconazole-conditioned parasite lineages became less infective and virulent through time (i.e. across generations), when compared to naive parasite lineages. Specifically, prevalence was significantly reduced in TEB lineages from F_2 generation onwards whereas spore load was reduced from F_3 onwards (Fig. 2). This temporal (transgenerational) effect of tebuconazole exposure was confirmed by a significant treatment × generation interaction (Prevalence: $\chi^2_{(3,24)} = 16.22$, P = 0.001; Spore load: $F_{(3,24)} = 9.48$, P < 0.001). Similarly, a delay in the time of death of hosts (a measure of parasite virulence) from TEB lineages also became more apparent through time (Supporting Information, Fig. S3).

3.3. Reset experiment

After removal of selective pressure, tebuconazole-conditioned parasites became as infective as naive parasite lineages (Fig. 3). Initially (i.e. in the F'_1 generation), prevalence and spore load in the Reset experiment remained lower in the TEB lineages relatively to the naive lineages, but both variables became comparable across naive and TEB lineages in the second generation, F'_2 (significant treatment × generation interaction; Prevalence: $\chi^2_{(1,8)} = 16.66$, P < 0.001; Spore load: $F_{(1,8)} = 6.30$, P = 0.037).

3.4. Follow-up reciprocal assays

The two follow-up reciprocal assays allowed assessing parasite fitness after the Evolution and Reset experiments. In both cases, parasite prevalence and spore load were reduced by tebuconazole, and the effect of the tebuconazole concentration gradient was consistent across naive and TEB lineages (no significant treatment × concentration interaction; Fig. 4). However, prevalence data from the reciprocal assay conducted after the Evolution experiment showed that TEB lineages consistently performed worse than naive lineages across all tebuconazole concentrations, including control (Fig. 4). This treatment effect (naive vs. TEB) was no longer present after the Reset experiment. These results confirm above observations for the overall underperformance of TEB lineages but their fast recovery after removing of selective pressure.

4. Discussion

The parasitic yeast *M. bicuspidata* did not reveal the potential for rapid evolution or acclimation to a common fungicide. Instead, costs of prolonged exposure to the fungicide were observed, with tebuconazole-

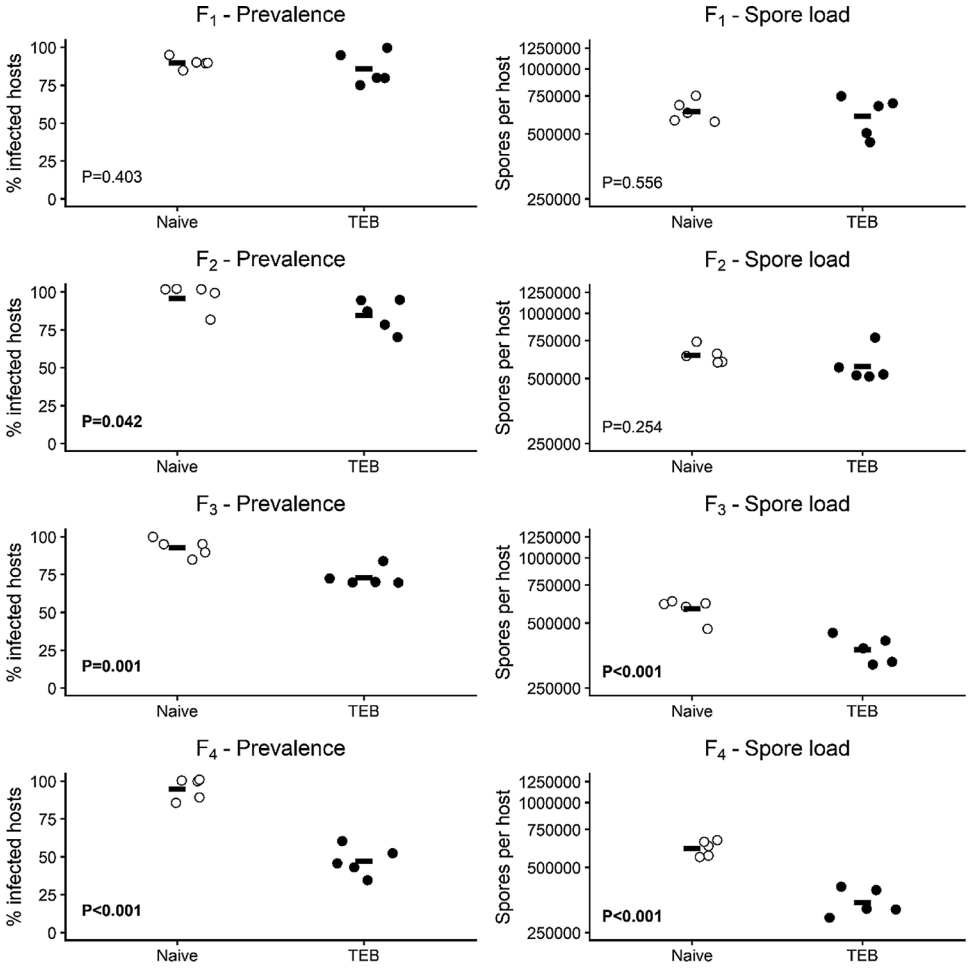

Fig. 2. Prevalence (% infected hosts) and intensity (spore load per infected host) of *M. bicuspidata* infection during the multigenerational exposure to tebuconazole (Evolution experiment). P-values are shown for simple main effects between naive (open circles) and TEB lineages (closed circles) within each generation (significant differences are highlighted in bold).

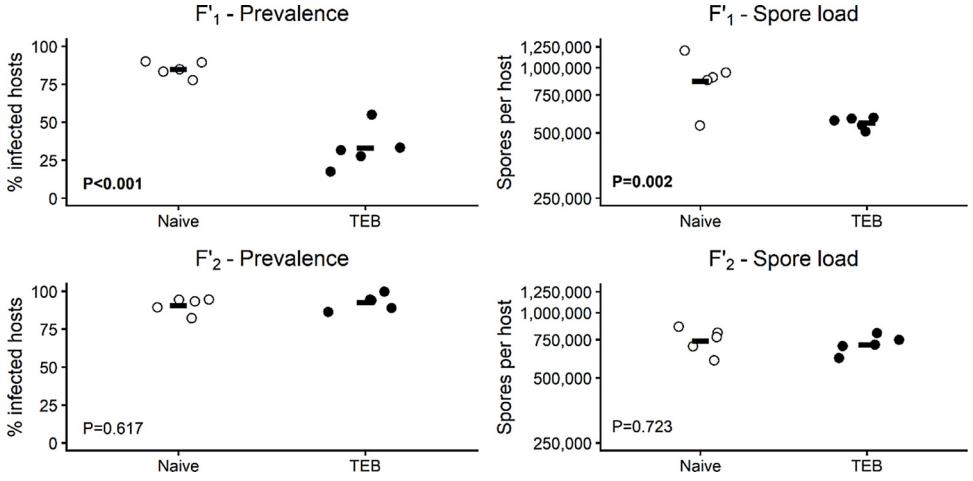

Fig. 3. Prevalence (% infected hosts) and intensity (spore load per infected host) of *M. bicuspidata* infection during removal of the selective pressure (Reset experiment). P-values are shown for simple main effects between naive (open circles) and TEB lineages (closed circles) within each generation (significant differences are highlighted in bold).

exposed (TEB) parasite lineages performing worse (in terms of infectivity and virulence) than naive lineages. Given the extensive use of fungicides (particularly azoles) and their persistence in the environment, our results reinforce the need to develop more studies with these compounds, to better understand their effects on non-target fungi and the important processes where they intervene.

The negative effects of tebuconazole on the microparasitic yeast *M. bicuspidata* were first noticeable by the lower prevalence and spore load of TEB parasite lineages when compared to naive lineages throughout the Evolution experiment, and by the loss of lineages exposed to the higher tebuconazole concentrations (4.40 and 5.31 $\mu g\ l^{-1}$ – Supporting Information, Fig. S2). The underperformance of TEB lineages in the naive environment, as observed in the first reciprocal assay, indicates costs of prolonged (multigenerational) exposure to tebuconazole. Other studies have also demonstrated a direct negative effect of toxicants on parasites: exposure to a mixture of zinc and copper decreased the fitness

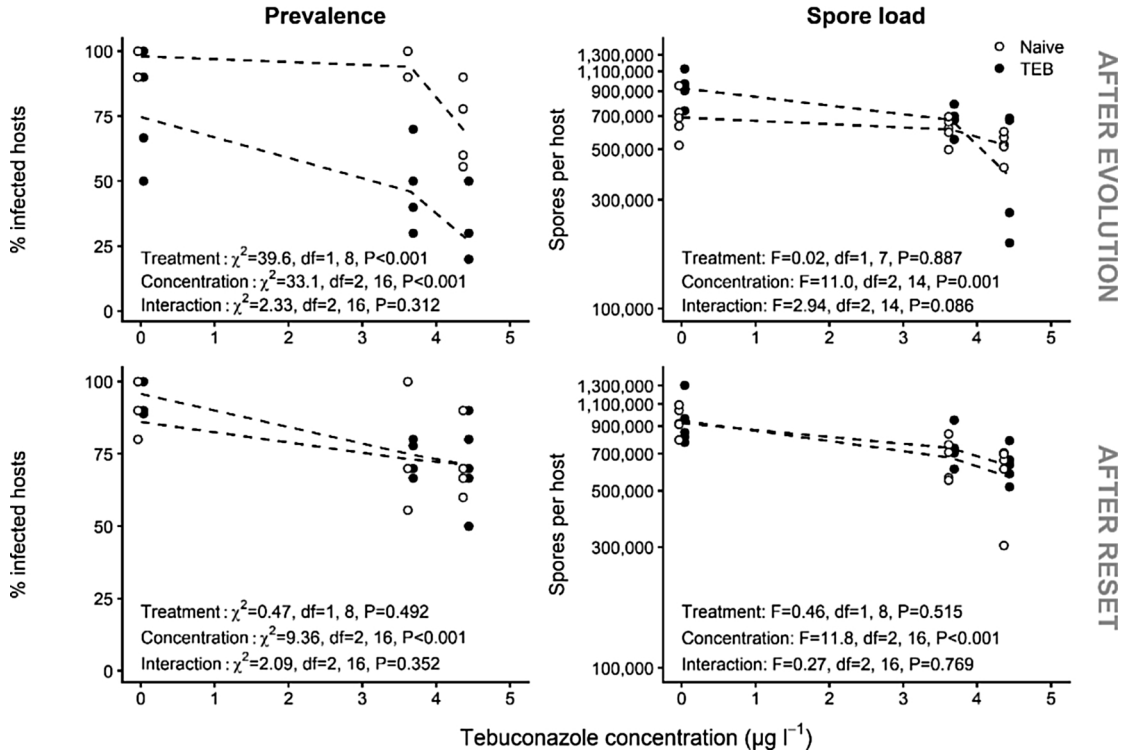

Fig. 4. Prevalence (% infected hosts) and intensity (spore load per infected host) of *M. bicuspidata* infection in naive (open circles) and TEB lineages (closed circles) exposed to three tebuconazole levels (0.0, 3.65 and 4.40 µg l^{-1}). Dashed lines between data points are used to facilitate visualization of the response pattern across tebuconazole concentrations. Experiment was conducted after the multigenerational exposure to tebuconazole (Evolution experiment, top panels) and after removal of the selective pressure (Reset experiment, bottom panels). Bifactorial GLMM summaries are shown with associated test statistic and significance.

of the parasite *Discocotyle sagittata* infecting *Salmo salar* L. (Blanar et al., 2010), whereas the fungicide thiophanate-methyl cleared the infection caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) in larval southern leopard frogs (Hanlon et al., 2012). However, these studies reported short-term effects, which may not always allow extrapolation to the response pattern observed in lengthier exposures. For example, the short-term exposure of a *Daphnia-Metschnikowia* system to copper enhanced parasite transmission (via increased filtering rate of the host caused by copper), whilst a multigenerational exposure depressed parasite reproduction, leading to contrasting outcomes in terms of disease spread (Civitello et al., 2012).

The decrease in prevalence and spore load over generations demonstrates that long-lasting stressful environments can affect the quality and quantity of transmission stages (spores) in the *Daphnia-Metschnikowia* system. Analogously, host nutritional status influenced parasite fitness in the subsequent generations in a *Daphnia magna-Pasteuria ramosa* system (Little et al., 2007). In the *Daphnia-Metschnikowia* system, the quality of the hosts as the environment for the parasite seems to be the main determinant of parasite fitness, at least in terms of infectivity (Searle et al., 2015). The decrease in prevalence and virulence due to environmental factors can lead to a scenario where the parasite is not able to successfully pass its transmission stages to new hosts, compromising the development of epidemics (Duffy et al., 2012; Ortiz-Cañavate et al., 2019). In our study, the transmission procedure was artificially controlled, and spore dose was normalized in all generations to maximize the possibility of infection. By doing so, the transgenerational impact in terms of spore quantity was buffered, meaning that the observed effect across generations was due to a reduction in spore quality only. Together, effects on both spore quantity and quality would likely result in even more pronounced negative effects on disease spread.

After removal of tebuconazole (Reset experiment), prevalence and spore load of the parasite recovered to values comparable to the initial generation, confirming that the parasite response to tebuconazole was phenotypic and transient. Analogously, *Metschnikowia* is capable of rapidly responding to its host "environment" (e.g., host genotype), and such host effects can be reversed in a single generation (Searle et al., 2015). Since *M. bicuspidata* shows overall low genetic diversity (Searle et al., 2015; Wolinska et al., 2009) and little evidence for genetic variation in infectivity or virulence (Duffy and Sivars-Becker, 2007), the observed changes are plastic and not genetically determined. In part, this can be explained by the parasexual mode of reproduction of this yeast, which leads to very low genetic change across reproductive events (Naumov, 2011; Pitt and Miller, 1970). Our results confirmed the plastic response of this microparasitic yeast to the fungicide tebuconazole, demonstrating that the infectivity and virulence of *M. bicuspidata* are not only determined by its within-host environment (as shown by Searle et al., 2015) but also by waterborne pollution.

Our study intended to mimic a likely environmental scenario; we exposed *M. bicuspidata* to tebuconazole concentrations that can occur in the field (< 10 µg l^{-1}; Berenzen et al., 2005), and exposure length simulated the duration of a *M. bicuspidata* epidemic in the field. Furthermore, the selected concentrations allowed exploring the window where infection is negatively affected but still successful (according to Cuco et al., 2017b). Previous results (Cuco et al., 2017a, Cuco et al., 2017b, 2018) consistently showed that environmentally-relevant concentrations of tebuconazole cause (i) inhibition of sporulation of the parasite, (ii) clearance of infection signs in the host, and (iii) suppression of transmission stages (spores). The present study additionally shows that tebuconazole cumulatively decreases parasite virulence and infectivity on the long term, at concentrations lower than those reported to cause complete suppression of infection.

Although *M. bicuspidata* did not reveal potential to adapt under contaminant stress, the micro-evolutionary potential of other non-target organisms exposed to pollution should not be undervalued (see review by Medina et al., 2007). Some pathogens, such as *Aspergillus* and

Candida species, have already demonstrated their capacity to develop resistance to the antifungal drugs used in fungal disease therapy, which poses an emergent threat (Sanglard, 2016). These scenarios can be further exacerbated by cross-resistance scenarios, where fungicides used in agriculture may induce resistance in human pathogens if they share the same mode of action than their pharmaceutical counterparts. Azole compounds, in particular, are extensively used in agriculture and clinical therapy due to their effectiveness against target fungi, and are thus potential targets for cross resistance (Faria-Ramos et al., 2014; Stensvold et al., 2012; Verweij et al., 2016). Studies on fungicide resistance with other aquatic fungi are still scarce.

Given the persistence of fungicides, alongside with their frequent and widespread use, long-term exposure scenarios such as the ones used here are likely and may affect various aquatic fungal communities. In line with recent pleas for increased awareness to the environmental risk of fungicides (see Ittner et al., 2018; Zubrod et al., 2019, 2015), we need more information on fungicide resistance in non-target species and on negative long-term effects of fungicides in aquatic or terrestrial fungi communities. Confirming evidence from other studies (Álvarez-Pérez et al., 2015; Cuco et al., 2017a; Dimitrov et al., 2014; Ortiz-Cañavate et al., 2019; Zubrod et al., 2015), our findings should place azole fungicides on the spotlight for environmental researchers and regulators.

CRediT authorship contribution statement

Ana P. Cuco: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Justyna Wolinska:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Joana I. Santos:** Investigation, Writing - review & editing. **Nelson Abrantes:** Conceptualization, Resources, Funding acquisition, Writing - review & editing. **Fernando J.M. Gonçalves:** Conceptualization, Resources, Funding acquisition, Writing - review & editing. **Bruno B. Castro:** Conceptualization, Methodology, Formal analysis, Resources, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105584.

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