Temperature modulates the interaction between fungicide pollution and disease: evidence from a *Daphnia*-microparasitic yeast model

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Abstract

Temperature is expected to modulate the responses of organisms to stress. Here, we aimed to assess the influence of temperature on the interaction between parasitism and fungicide contamination. Specifically, using the cladoceran *Daphnia* as a model system, we explored the isolated and interactive effects of parasite challenge (yeast *Metschnikowia bicuspidata*) and exposure to fungicides (copper sulphate and tebuconazole) at two temperatures (17 and 20 °C), in a fully factorial design. Confirming a previous study, *M. bicuspidata* infection and copper exposure caused independent effects on *Daphnia* life history, whereas infection was permanently suppressed with tebuconazole exposure. Here, we show that higher temperature generally increased the virulence of the parasite, with the hosts developing signs of infection earlier, reproducing less and dying at an earlier age. These effects were consistent across copper concentrations, whereas the joint effects of temperature (which enhanced the difference between non-infected and infected hosts) and the anti-parasitic action of tebuconazole resulted in a more pronounced parasite × tebuconazole interaction at the higher temperature. Thus, besides independently influencing parasite and contaminant effects, the temperature can act as a modulator of interactions between pollution and disease.

Introduction

Agents of disease (parasites and pathogens) are important and ubiquitous biotic factors, playing an important role in natural populations by regulating host population dynamics, maintaining host genetic diversity and modulating other trophic interactions (Jokela *et al.* 2009; Hall *et al.* 2011; Agha *et al.* 2016). Parasites can affect their host directly – for example through reduced fecundity, increased mortality and/or altered behaviour (Hall *et al.* 2011; Kekäläinen *et al.* 2014) – and indirectly – by inducing changes in the host's response to other stress factors, such as contaminants (Kelly *et al.* 2010; Buser *et al.* 2012*a*; Hanlon *et al.* 2015). Research on the interactive effects of parasitism and contamination (reviewed by Marcogliese and Pietrock, 2011) has produced contrasting evidence, with the outcome of the interaction being favourable either to the host (Blanar *et al.* 2010; Hanlon *et al.* 2012) or to the parasite (Johnson *et al.* 2007; Coors *et al.* 2008). A previous experimental study by our team (Cuco *et al.* 2017) demonstrated that simultaneous exposure of *Daphnia* to a microparasitic yeast (*Metschnikowia bicuspidata*) and fungicides resulted in independent effects from contaminant and parasite (for copper sulphate), whereas contaminant-driven parasite suppression was found for another fungicide, tebuconazole. Such contrasting results emphasize the need for more research on this subject.

Increasing temperature is expected to change contaminant properties (Noyes *et al.* 2009; degradation rates, environmental mobility, bioavailability; Delcour *et al.* 2014) as well as the susceptibility of organisms to contaminants (Seeland *et al.* 2012; Cuco *et al.* 2016). Simultaneously, temperature plays a pivotal role in the outcome of host–parasite relationships (Wolinska and King, 2009; Schoebel *et al.* 2011; Gallana *et al.* 2013), by influencing parasite infectivity and spore production (Mitchell *et al.* 2005; Vale *et al.* 2008), and by modulating the host's susceptibility to infection (Garbutt *et al.* 2014). Based on this rationale, research on interaction scenarios between parasitism and contamination should consider temperature as an important modulator of both phenomena. This is particularly important in the light of climate change predictions (namely warming), as temperature effects on pollution × disease interactions are still unresolved (Marcogliese, 2008; Noyes *et al.* 2009; Delcour *et al.* 2014).

The crustacean zooplankter *Daphnia* sp. plays a central role in aquatic food webs by consuming primary producers and serving as food for predators, and is also frequently parasitized by numerous microorganisms (Lass and Ebert, 2006; leading to epidemics in the field; Duffy and Sivars-Becker, 2007; Duncan and Little, 2007). These parasites play a pivotal role in *Daphnia* population dynamics (Hall *et al.* 2011), diversity (Duncan and Little, 2007; Wolinska and Spaak, 2009) and community structure (Hall *et al.* 2009; Buser *et al.* 2012b). The microparasitic yeast *M. bicuspidata* grows inside the host's body cavity and produces needle-like ascospores that are horizontally transmitted (Ebert, 2005). This virulent parasite reduces host fecundity in the late stage of the infection and kills its host within 2–3 weeks (Ebert, 2005; Hall *et al.* 2006; Duffy and Hall, 2008). Lake epidemics of *M. bicuspidata* can reach a prevalence up to 40% (Caceres *et al.* 2006; Duffy *et al.* 2009; Hall *et al.* 2011; Wolinska *et al.* 2011), and they are important in the ecological dynamics of *Daphnia* natural populations (Duffy and Sivars-Becker, 2007; Duffy *et al.* 2009, 2011). This host–parasite model allows experimental manipulation of spore load and parasite transmission (Lohr *et al.* 2010; Yin *et al.* 2011), as well as the discrimination of the interactive effects of parasite and other stressors (Duffy *et al.* 2011; Civitello *et al.* 2012; Cuco *et al.* 2017).

The aim of this study was to assess the interactive effects of fungicide contamination and disease, considering the influence of temperature. Copper sulphate and tebuconazole were selected based on their contrasting effects on the host–parasite system described (i.e. *Daphnia* infected with *M. bicuspidata*, Cuco *et al.* 2017). We hypothesize that temperature will affect the outcome of the contaminant × disease interaction in the case of tebuconazole (which causes a suppression of infection), but this may be less important in the case of copper sulphate because of the lack of interaction between parasite and toxicant effects (Cuco *et al.* 2017).

Materials and methods

Host–parasite system: origin and maintenance

Two host genotypes were tested, belonging to the *Daphnia longispina* species complex (*D. galeata × longispina* clone AMM_12 and *D. galeata* clone AMM_47, from now on designated as Clone 12 and Clone 47, respectively). The studied parasite was a single isolate of the endoparasitic yeast *M. bicuspidata*. Host genotypes and parasite strain were isolated from Lake Ammersee (Germany) and have been used in other studies (Yin *et al.* 2011; Buser *et al.* 2012b; Cuco *et al.* 2017).

Daphnia genotypes (Clone 12 and Clone 47) were reared as parthenogenetic lineages in moderately hard reconstituted water (123 mg L^{-1} MgSO$_4$·7H$_2$O, 96 mg L^{-1} NaHCO$_3$, 60 mg L^{-1} CaSO$_4$·2H$_2$O, 4 mg L^{-1} KCl) supplemented with a standard organic additive (algal extract) and vitamins (Cuco *et al.* 2016, 2017). Cultures were maintained with a 16h^L:8h^D photoperiod and the medium was renewed three times a week. Along with medium renewal, daphnids were fed with a *Raphidocelis subcapitata* suspension (1·5 × 10^5 cells mL^{-1}). *Daphnia* were reared in a controlled temperature room (at 20 ± 1 °C) and in a water bath equipped with a water chiller (17 ± 1 °C). The temperature was regularly checked with a temperature probe. Before the experiment, *Daphnia* were acclimated for at least five generations at the two tested temperatures, in order to remove potentially confounding effects of transient thermal stress (Cuco *et al.* 2016).

The parasite strain was maintained in a *Daphnia magna* clone, reared under the conditions described above, at 20 °C. Uninfected *Daphnia* juveniles were added to infected cultures every other week, guaranteeing a continuous supply of hosts and production of spores (Hesse *et al.* 2012; Cuco *et al.* 2017).

Test chemicals

Tebuconazole and copper sulphate concentrations were defined according to Cuco *et al.* (2016), which estimated reproductive EC$_x$ values that were then used to define a low dose (roughly corresponding to EC$_{10}$ for both contaminants) and a high dose (corresponding to EC$_{50}$ for tebuconazole and EC$_{20}$ for copper). A lower EC$_x$ value was used for copper because it causes substantial mortality along with reproductive impairment that could result in a highly unbalanced design (loss of experimental units due to high mortality). Concentrated stock solutions were prepared with ethanol (for dissolving tebuconazole) or distilled water (copper sulphate) and then diluted in reconstituted water (see *Host–parasite system: origin and maintenance*). Final test solutions were: 0·00, 100 and 250 µg L^{-1} for tebuconazole, and 0·00, 30·0 and 40·0 µg L^{-1} for copper sulphate (expressed as free Cu^{2+} ion). For tebuconazole exposure, ethanol was also added in the negative control (0·00 µg L^{-1}) at an equal volume to the one used in the tebuconazole concentrations (0·1 mL L^{-1}).

Analytical quantification of tested substances previously confirmed the validity of nominal concentrations within the range of 25·0–43·7 µg L^{-1} (for copper) and 100–240 µg L^{-1} (for tebuconazole), although measured concentrations slightly overestimated nominal concentrations: 6–16% for copper and 1–10% for tebuconazole. Detailed analyses of measured copper and tebuconazole concentrations are presented in Supplementary Table S1. These analyses were performed by an independent analytical laboratory (certified according to ISO/IEC 17025:2005), which analysed random samples by ICP-MS (for copper, according to ISO 17294-2:2003) and LC-MS/MS (for tebuconazole, according to an internal method adapted from ISO 11369:1997). We assumed that good correspondence between nominal and measured concentrations was valid for the experiments shown here (the same procedures were used and the range of concentrations is comparable).

Experimental design

Life history assays were initiated with neonates (<24 h old, born between the 3rd and the 5th broods) from the two *Daphnia* genotypes reared at each acclimation temperature (17 °C and 20 °C). *Daphnia* were individually exposed to a range of contaminant concentrations (see *Test chemicals*), at the two selected temperatures (17 °C and 20 °C), in the presence ('parasite') or absence ('no-parasite') of the parasite *M. bicuspidata*, in a fully crossed design. These temperatures were chosen based on previous work (Cuco *et al.* 2016), which showed substantial differences in the life history of these *Daphnia* clones at 17 °C when compared with warmer conditions (20 °C and 23 °C). For logistical reasons, experiments with tebuconazole and copper were carried out separately. Therefore, the experimental design for each contaminant consisted of 2 *Daphnia* clones × 3 contaminant concentrations (see *Test chemicals*) × 2 parasite treatments × 2 exposure temperatures × 13 replicates = 312 experimental units. Because infection is not always 100% successful, the parasite treatment was initiated with 20 replicates, and individual *Daphnia* that did not become infected were then excluded. In the case of tebuconazole, only 13 replicates were set up in the parasite treatment, as we anticipated a suppression of infection (Cuco *et al.* 2017). Each replicate corresponded to one *Daphnia* individually placed in a glass vessel containing 25 mL of the respective test solution, prepared by adding the proper amount of contaminant stock solution to the culture medium (see *Test chemicals*). Assays were carried out under the same conditions as described for maintenance, except that *Daphnia* were fed daily with an algal ration of 1·5 × 10^5 cells mL^{-1} and test medium was renewed every 3 days. In the parasite treatment, individual *Daphnia* were infected on day 5 with an *M. bicuspidata* spore suspension, following the
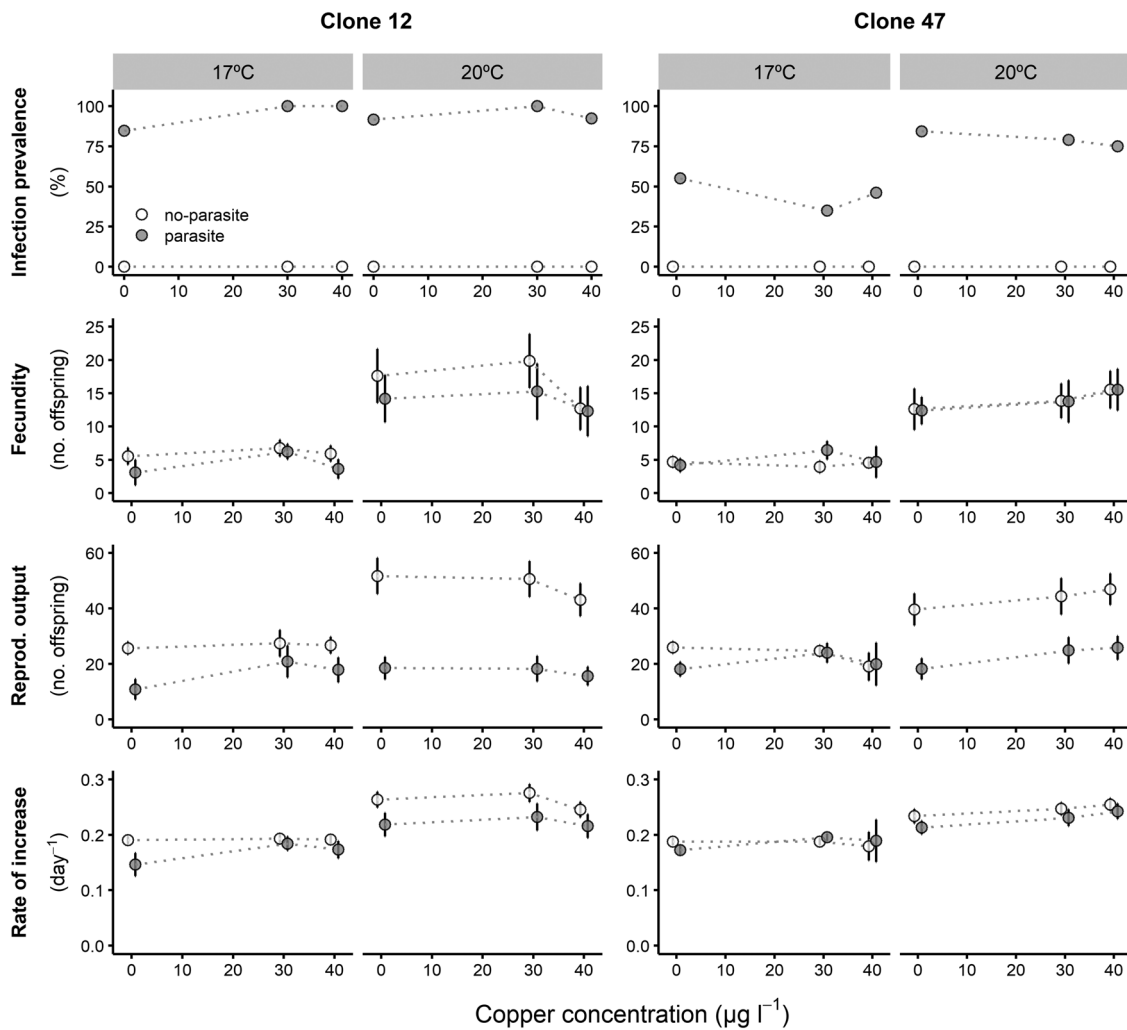
Fig. 1. Life history parameters (mean and respective 95% confidence intervals, except for infection) of two *Daphnia* genotypes (Clone 12 and 47) when exposed to increasing copper concentrations in the presence ('parasite') or absence ('no-parasite') of spores from the microparasitic yeast *M. bicuspidata*, at two temperatures (17 °C and 20 °C). For the calculation of the reproductive and demographic endpoints in the parasite treatment, only infected hosts were considered (see *Methods*).

methodology described below (see *Infection procedure*). Test procedures followed OECD (2012) recommendations.

Infection procedure

At experimental day 5 (first day of infection), test solution volume from all experimental units was reduced from 25 to 10 mL and *Daphnia* were not fed; this ensured higher spore encounter and filtration rates (Yin *et al.* 2011). Infected *D. magna* from the host-parasite culture were crushed in distilled water to obtain a homogenized spore suspension, and spore concentration was determined using a Neubauer improved counting chamber. A placebo suspension was also obtained by crushing the same number of uninfected *D. magna* in distilled water. Experimental units of the parasite treatment were inoculated with an *M. bicuspidata* spore suspension aliquot (700 spores mL^{-1}) while experimental units of the no-parasite treatment were given an equivalent placebo aliquot. The use of a placebo was important to rule out potential confounding effects of bacteria or alarm cue effects from the *Daphnia* homogenates. On day 6, test solution volume was increased to 15 mL and, on day 7, to 25 mL. Daily food ration (1.5×10^5 cells mL^{-1}) was restored on day 6. Between day 5 and day 7, vessels were slightly shaken once per day, to promote spore resuspension and subsequent filtration by the host. From day 8 onwards, initial conditions (25 mL of test solution with renewal every 3 days) were restored, as described above.

Data collection

Survival and reproduction data were recorded daily for each *Daphnia*. Reproductive parameters included fecundity (cumulative number of living offspring released per surviving female) and reproductive output (total number of living offspring released per initial female, including females, which died during the course of the experiment). Fecundity was estimated at day 15 before females started dying due to parasite action. For the calculation of reproductive output (measured at day 21), we excluded replicates in which *Daphnia* died before day 4, as this could be the result of accidental or natural early-age mortality. The experiment ended on day 21 for copper, later for tebuconazole (see below). At the time of death or at the end of the assay, daphnids were visually inspected for signs of infection (presence of spores inside the host's body cavity) under an Olympus CKX41 inverted microscope, and prevalence of infection was calculated per treatment. For the calculation of all parameters, we excluded experimental units where *Daphnia* died before the infection could be confirmed. Survival and reproduction data were used to determine the *per capita* intrinsic rate of population increase (r) from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

where *r* is the rate of population increase (day^{-1}), *x* is the age class in days, l_x is the probability of surviving to age *x*, and m_x is the
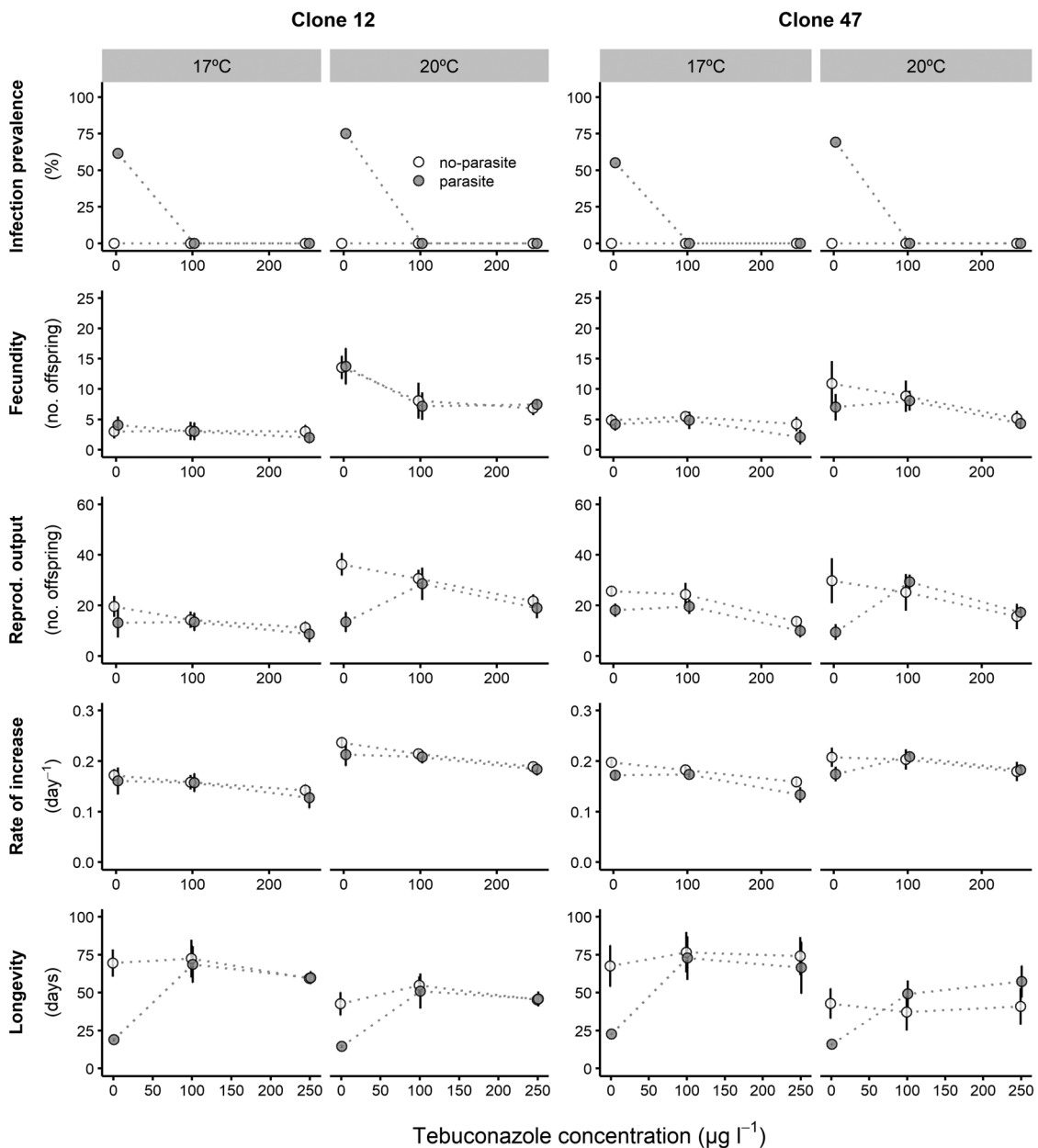
Fig. 2. Life history parameters (mean and respective 95% confidence intervals, except for infection) of two *Daphnia* genotypes (Clone 12 and 47) when exposed to increasing tebuconazole concentrations in the presence ('parasite') or absence ('no-parasite') of spores from the microparasitic yeast *M. bicuspidata*, at two temperatures (17 °C and 20 °C). Because of the antiparasitic nature of tebuconazole, no infected hosts were present in the parasite treatment, except for the negative control; in the latter case, only infected hosts were considered for the calculation of the reproductive and demographic endpoints (see *Methods*).

fecundity at age *x* (Meyer *et al.* 1986; McCallum, 2000). Replicate pseudo values for *r* were generated using the jack-knifing technique described by Meyer *et al.* (1986), thus enabling the use of statistical tests for analyzing differences in the rate of increase between experimental treatments.

Previous data (Cuco *et al.* 2017) showed that tebuconazole suppresses *M. bicuspidata* infection, unlike copper. Therefore, with the purpose of assessing if parasite infection is delayed or definitely suppressed by this fungicide, we continued to monitor *Daphnia* exposed to tebuconazole (and respective negative controls) throughout their lifespan (i.e. beyond the 21-d period). During this period, we monitored survival and the presence of infection.

Statistical analysis

Three-way analyses of variance (ANOVA) were performed separately for each experiment (copper and tebuconazole) to assess the

effects of temperature, parasite challenge and contaminant concentration (as fixed factors) on *Daphnia* life history parameters. Because we expected differences in the sensitivity of the tested clones to the stress factors (Cuco *et al.* 2016), analyses were conducted separately per clone, with a necessary adjustment in the significance level ($\alpha = 0.025$). Plots of residuals were analysed to assess departures from normality and homogeneity of variances, which were found on a few occasions; however, we assumed ANOVA to be sufficiently robust to deal with such deviations. ANOVAs were carried out with the General Linear Model procedure in Minitab 16 (Minitab Inc., USA).

Results

Infection data

The infection procedure was successful in both experiments and for both clones; 55–100% of exposed *Daphnia* became

Table 1. Three-way ANOVA summary table of the effects of temperature, parasite challenge, concentration and their interactions on different life history parameters of two *Daphnia* genotypes (Clone 12 and 47) exposed to copper

	Source	Clone 12	Clone 47
Fecundity	Temperature	$F_{(1, 136)} = 184 \cdot 71$; $P < 0 \cdot 001$	$F_{(1, 127)} = 203 \cdot 72$; $P < 0 \cdot 001$
	Parasite	$F_{(1, 136)} = 9 \cdot 43$; $P = 0 \cdot 003$	$F_{(1, 127)} = 0 \cdot 22$; $P = 0 \cdot 641$
	Concentration	$F_{(2, 136)} = 7 \cdot 07$; $P = 0 \cdot 001$	$F_{(2, 127)} = 2 \cdot 18$; $P = 0 \cdot 117$
	Temperature × Parasite	$F_{(1, 136)} = 0 \cdot 49$; $P = 0 \cdot 485$	$F_{(1, 127)} = 0 \cdot 39$; $P = 0 \cdot 532$
	Temperature × Concentration	$F_{(2, 136)} = 2 \cdot 60$; $P = 0 \cdot 078$	$F_{(2, 127)} = 1 \cdot 79$; $P = 0 \cdot 172$
	Parasite × Concentration	$F_{(2, 136)} = 0 \cdot 41$; $P = 0 \cdot 664$	$F_{(2, 127)} = 0 \cdot 56$; $P = 0 \cdot 575$
	Temperature × Parasite × Concentration	$F_{(2, 136)} = 1 \cdot 36$; $P = 0 \cdot 260$	$F_{(2, 127)} = 0 \cdot 49$; $P = 0 \cdot 614$
Reproductive output	Temperature	$F_{(1, 139)} = 79 \cdot 94$; $P < 0 \cdot 001$	$F_{(1, 128)} = 78 \cdot 06$; $P < 0 \cdot 001$
	Parasite	$F_{(1, 139)} = 259 \cdot 38$; $P < 0 \cdot 001$	$F_{(1, 128)} = 81 \cdot 99$; $P < 0 \cdot 001$
	Concentration	$F_{(2, 139)} = 2 \cdot 75$; $P = 0 \cdot 067$	$F_{(2, 128)} = 3 \cdot 52$; $P = 0 \cdot 032$
	Temperature × Parasite	$F_{(1, 139)} = 67 \cdot 58$; $P < 0 \cdot 001$	$F_{(1, 128)} = 49 \cdot 82$; $P < 0 \cdot 001$
	Temperature × Concentration	$F_{(2, 139)} = 4 \cdot 98$; $P = 0 \cdot 008$	$F_{(2, 128)} = 5 \cdot 24$; $P = 0 \cdot 007$
	Parasite × Concentration	$F_{(2, 139)} = 1 \cdot 88$; $P = 0 \cdot 156$	$F_{(2, 128)} = 1 \cdot 47$; $P = 0 \cdot 233$
	Temperature × Parasite × Concentration	$F_{(2, 139)} = 0 \cdot 93$; $P = 0 \cdot 398$	$F_{(2, 128)} = 0 \cdot 93$; $P = 0 \cdot 399$
Rate of increase	Temperature	$F_{(1, 139)} = 216 \cdot 46$; $P < 0 \cdot 001$	$F_{(1, 128)} = 179 \cdot 29$; $P < 0 \cdot 001$
	Parasite	$F_{(1, 139)} = 55 \cdot 69$; $P < 0 \cdot 001$	$F_{(1, 128)} = 4 \cdot 04$; $P = 0 \cdot 047$
	Concentration	$F_{(2, 139)} = 6 \cdot 32$; $P = 0 \cdot 002$	$F_{(2, 128)} = 6 \cdot 37$; $P = 0 \cdot 002$
	Temperature × Parasite	$F_{(1, 139)} = 3 \cdot 39$; $P = 0 \cdot 068$	$F_{(1, 128)} = 4 \cdot 89$; $P = 0 \cdot 029$
	Temperature × Concentration	$F_{(2, 139)} = 3 \cdot 00$; $P = 0 \cdot 053$	$F_{(2, 128)} = 2 \cdot 66$; $P = 0 \cdot 073$
	Parasite × Concentration	$F_{(2, 139)} = 2 \cdot 31$; $P = 0 \cdot 103$	$F_{(2, 128)} = 1 \cdot 92$; $P = 0 \cdot 151$
	Temperature × Parasite × Concentration	$F_{(2, 139)} = 1 \cdot 38$; $P = 0 \cdot 256$	$F_{(2, 128)} = 0 \cdot 73$; $P = 0 \cdot 485$

Analyses were conducted separately per *Daphnia* clone. Significant effects are highlighted in bold ($P \leqslant 0 \cdot 025$).

infected. No infections were detected in the no-parasite treatment. Almost all infected hosts died within the 21-d period, confirming that *M. bicuspidata* considerably reduces *Daphnia* life span. Specifically, longevity was reduced from 6 to 2 weeks at 20 °C and from 9 to 3 weeks at 17 °C.

Prevalence of infection was fairly consistent for both *Daphnia* clones across all copper concentrations, despite an overall lower proportion of infection for Clone 47 at 17 °C (Fig. 1). By contrast, infection was suppressed in the presence of tebuconazole (only control animals became infected), which was consistent for both clones and under both temperatures (Fig. 2).

Temperature × parasite × copper interaction

The tested copper concentrations did not elicit a pronounced effect on *Daphnia* reproductive parameters, although statistically significant effects (including a slight stimulation of reproduction) were observed under some circumstances (Table 1). These subtle effects were sometimes temperature-dependent (significant temperature × concentration interaction – Table 1). Temperature and the parasite caused major effects on *Daphnia* reproductive performance. The parasite-induced reduction in reproductive output was stronger at 20 °C (significant temperature × parasite interaction – Table 1 and Fig. 1). However, fecundity (calculated prior to the death of the hosts) was only reduced by the parasite in Clone 12, independently of temperature. The same was true for the rate of increase (Table 1 and Fig. 1). This means that the reproductive underperformance of infected *Daphnia* was mostly due to parasite-induced mortality rather than reproductive impairment, especially in Clone 47 (no differences in fecundity between parasite and no-parasite treatments). No significant interactions were detected for parasite × concentration or temperature × parasite × concentration (Table 1), demonstrating independent effects of parasite and contaminant action for both temperatures.

Temperature × parasite × tebuconazole interaction

Tebuconazole significantly decreased the reproductive performance of both *Daphnia* clones (Fig. 2 and Table 2). In some cases, these effects were temperature-dependent (significant temperature × concentration interactions, Table 2). Lower temperature significantly decreased the measured life history parameters of *Daphnia*, and exposure to the parasite also compromised host fecundity (only in clone 47), reproductive output and *per capita* rate of increase (both clones, Fig. 2 and Table 2). However, because infection only occurred in the absence of tebuconazole (see above), parasite effects were generally dependent on tebuconazole concentration (see significant interactions between parasite × concentration or temperature × parasite × concentration in Table 2). For example, the parasite caused a significant reduction in reproductive output and rate of increase, but only without tebuconazole (which suppressed infection), this effect is more pronounced at 20 °C (Fig. 2). Thus, unlike for copper, there is interdependence between tebuconazole contamination and parasitism, which is further modulated by temperature (Fig. 3).

The extended observations (>21 days) confirmed that infection was definitely suppressed in the presence of tebuconazole, at all tested concentrations. Consequently, longevity was significantly reduced by the parasite, but only in the absence of tebuconazole (see significant parasite × concentration interaction: Table 2,

Table 2. Three-way ANOVA summary table of the effects of temperature, parasite challenge, concentration and their interactions on different life history parameters of two *Daphnia* genotypes (Clone 12 and 47) exposed to tebuconazole

Parameter	Source	Clone 12	Clone 47
Fecundity	Temperature	$F_{(1, 138)} = 206.70$; $P < 0.001$	$F_{(1, 131)} = 50.10$; $P < 0.001$
	Parasite	$F_{(1, 138)} = 0.00$; $P = 0.975$	$F_{(1, 131)} = 11.55$; $P = 0.001$
	Concentration	$F_{(2, 138)} = 26.55$; $P < 0.001$	$F_{(2, 131)} = 18.69$; $P < 0.001$
	Temperature × Parasite	$F_{(1, 138)} = 0.00$; $P = 0.975$	$F_{(1, 131)} = 0.63$; $P = 0.427$
	Temperature × Concentration	$F_{(2, 138)} = 15.90$; $P < 0.001$	$F_{(2, 131)} = 3.51$; $P = 0.033$
	Parasite × Concentration	$F_{(2, 138)} = 0.55$; $P = 0.577$	$F_{(2, 131)} = 1.10$; $P = 0.335$
	Temperature × Parasite × Concentration	$F_{(2, 138)} = 0.86$; $P = 0.424$	$F_{(2, 131)} = 2.23$; $P = 0.112$
Reproductive output	Temperature	$F_{(1, 135)} = 116.84$; $P < 0.001$	$F_{(1, 138)} = 4.59$; $P = 0.034$
	Parasite	$F_{(1, 135)} = 34.36$; $P < 0.001$	$F_{(1, 138)} = 18.25$; $P < 0.001$
	Concentration	$F_{(2, 135)} = 15.66$; $P < 0.001$	$F_{(2, 138)} = 27.67$; $P < 0.001$
	Temperature × Parasite	$F_{(1, 135)} = 7.70$; $P = 0.006$	$F_{(1, 138)} = 0.04$; $P = 0.834$
	Temperature × Concentration	$F_{(2, 135)} = 4.10$; $P = 0.019$	$F_{(2, 138)} = 3.88$; $P = 0.023$
	Parasite × Concentration	$F_{(2, 135)} = 14.28$; $P < 0.001$	$F_{(2, 138)} = 13.02$; $P < 0.001$
	Temperature × Parasite × Concentration	$F_{(2, 135)} = 5.57$; $P = 0.005$	$F_{(2, 138)} = 7.59$; $P = 0.001$
Rate of increase	Temperature	$F_{(1, 135)} = 196.39$; $P < 0.001$	$F_{(1, 138)} = 42.08$; $P < 0.001$
	Parasite	$F_{(1, 135)} = 7.17$; $P = 0.008$	$F_{(1, 138)} = 15.52$; $P < 0.001$
	Concentration	$F_{(2, 135)} = 28.41$; $P < 0.001$	$F_{(2, 138)} = 25.96$; $P < 0.001$
	Temperature × Parasite	$F_{(1, 135)} = 0.12$; $P = 0.726$	$F_{(1, 138)} = 2.71$; $P = 0.102$
	Temperature × Concentration	$F_{(2, 135)} = 0.29$; $P = 0.749$	$F_{(2, 138)} = 5.76$; $P = 0.004$
	Parasite × Concentration	$F_{(2, 135)} = 1.06$; $P = 0.350$	$F_{(2, 138)} = 4.97$; $P = 0.008$
	Temperature × Parasite × Concentration	$F_{(2, 135)} = 0.68$; $P = 0.510$	$F_{(2, 138)} = 2.15$; $P = 0.120$
Longevity	Temperature	$F_{(1, 135)} = 54.19$; $P < 0.001$	$F_{(1, 138)} = 50.79$; $P < 0.001$
	Parasite	$F_{(1, 135)} = 44.06$; $P < 0.001$	$F_{(1, 138)} = 8.12$; $P = 0.001$
	Concentration	$F_{(2, 135)} = 45.28$; $P < 0.001$	$F_{(2, 138)} = 20.15$; $P < 0.001$
	Temperature × Parasite	$F_{(1, 135)} = 3.12$; $P = 0.080$	$F_{(1, 138)} = 9.09$; $P = 0.018$
	Temperature × Concentration	$F_{(2, 135)} = 0.26$; $P = 0.771$	$F_{(2, 138)} = 2.08$; $P = 0.117$
	Parasite × Concentration	$F_{(2, 135)} = 32.17$; $P < 0.001$	$F_{(2, 138)} = 16.62$; $P < 0.001$
	Temperature × Parasite × Concentration	$F_{(2, 135)} = 2.82$; $P = 0.063$	$F_{(2, 138)} = 0.15$; $P = 0.766$

Analyses were conducted separately for each *Daphnia* clone. Significant effects are highlighted in bold ($P \leqslant 0.025$).

Fig. 2). This pattern was similar for both clones, irrespective of exposure temperature.

Discussion

We explored how temperature modifies the outcome of the interaction between pollution and parasitism. We tested environmentally relevant scenarios, both in terms of applied pollutant concentrations (Kahle *et al.* 2008; Bereswill *et al.* 2012) and experienced temperatures. Confirming our expectations, elevated temperature exacerbated overall parasite effects and their interaction with tebuconazole (but not with copper sulphate) – see the conceptual model in Fig. 3. These results highlight the context-dependent nature of interactions between stress and parasitism, which might bring into question the pollution × disease debate, as pointed out by Hall *et al.* (2013).

The most striking influence of temperature was the increased effect of the parasite on the host reproductive output, indicating that *M. bicuspidata* is more virulent at higher temperatures. At 17 °C, *Daphnia* still produced several broods before dying from infection, in contrast to females at 20 °C. For one of the two tested clones, higher infection was observed at 20 °C (clone 47 in the

copper experiment). This may have resulted from increased filtration rates at 20 °C (Burns, 1969), leading to increased consumption of parasite ascospores (Vale *et al.* 2008). Increased infection at higher temperatures has been observed in other host–microparasite systems (Vale *et al.* 2008; Dietrich *et al.* 2014; Frenken *et al.* 2016). Thus, climate change scenarios involving rising temperatures might exacerbate the negative effects of parasites in some systems (Marcogliese, 2008). However, the relationship between temperature and disease may not always be straightforward, because it depends on host physiology and interactions with other factors, such as predation (Hall *et al.* 2006). Furthermore, the degree of temperature variability has also been shown to be important in host–parasite interactions (Dallas and Drake, 2016).

Tested copper concentrations were mostly non-toxic to *Daphnia*, but they also did not alter parasite effects on the host, confirming the independent effect of copper and parasitism, as observed by Cuco *et al.* (2017). Although temperature interacted with copper concentration and parasite challenge (see the previous paragraph), it did not interfere with the inexistent copper × disease interaction. With respect to tebuconazole, tested concentrations caused a significant reduction in the reproduction of *Daphnia*, but the most notable effect of the toxicant was the
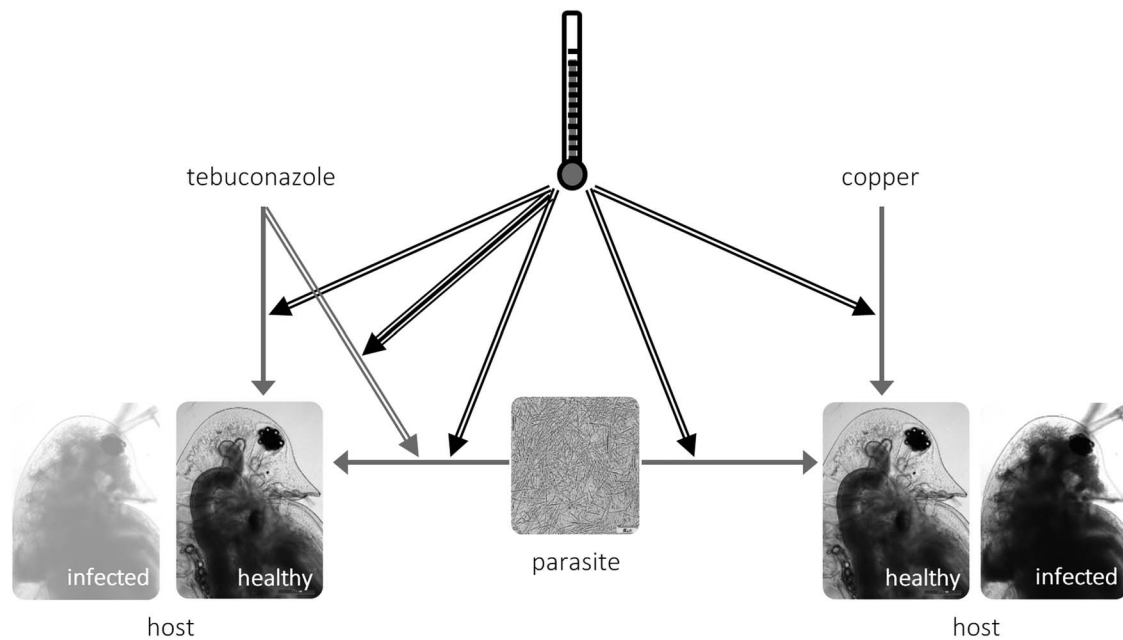
Fig. 3. Schematic representation of the interactions between temperature (black arrows), toxicant (tebuconazole, left; copper, right) and parasite (*M. bicuspidata*) in *Daphnia* life history. Simple arrows represent direct effects of a stressor (e.g. tebuconazole decreases reproduction in *Daphnia*), double arrows represent two-way interactions between stressors (e.g. higher temperature increases the virulence of the parasite); and the triple arrow depicts the three-way interaction between temperature × tebuconazole × parasite (see text).

suppression of infection. This confirms our previous results (Cuco *et al.* 2017) carried out in a distinct set of experiments. Unlike for copper, temperature clearly interfered in the interaction between tebuconazole and the parasite. The largest difference between infected and non-infected *Daphnia* was observed in the reproductive output in the control treatment (no tebuconazole) at 20 °C when compared with the controls at 17 °C. The temperature-dependent effect *via* parasite action was no longer perceptible in the presence of ecologically-relevant tebuconazole concentrations.

Few studies have addressed interactions between temperature, pollution and disease. For example, Dietrich *et al.* (2014) demonstrated increased susceptibility of chinook salmon to the microparasite *Aeromonas salmonicida* when exposed to a common organophosphate insecticide (malathion) at elevated temperatures. In that study, however, the applied elevated temperature (19 °C) was above the fish optimal temperature (11 °C), which probably resulted in thermal stress. This was not the case for our *Daphnia* clones (Cuco *et al.* 2016), and prior acclimation to each exposure temperature allowed potential effects of transient thermal shock or maladjustment to be discarded. Interactions between temperature, pollutants and biotic factors other than parasites have also been demonstrated. For example, Scherer *et al.* (2013) found that suboptimal temperature influences the toxicity of the fungicide pyrimethanil in *Daphnia*, but this effect is masked by the presence of predator chemical cues. Despite the numerous appeals to consider such multi stressor scenarios (Heugens *et al.* 2001; Noyes *et al.* 2009; Holmstrup *et al.* 2010; Fischer *et al.* 2013), a consolidated body of knowledge on pollutant effects in environmentally-realistic scenarios is still missing.

According to IPCC (2014) predictions for climate change, temperatures are expected to rise ca. 4 °C by the end of the 21st century. In the tested scenarios, an increase of 3 °C was enough to intensify the effects of the parasite and its interaction with contaminant challenge (in the case of tebuconazole). This result is relevant not only because of the projected increase in mean temperatures but also in the face of projected increases in maximum temperatures, number of hot days and heat waves (IPCC, 2014),

which may lead to transient periods of higher temperatures that could coincide with parasite epidemics. Thus, this is a clear indication that such an increase in temperature could lead to aggravated effects of parasitism and its interaction with anti-parasitic agents (such as tebuconazole) in natural populations.

Our extended observations (>21 days) confirmed that tebuconazole completely suppressed *M. biscuspidata* infection, which may have important ecological consequences. It is still not clear which stages of development (spore germination, growth, sporulation) are affected by tebuconazole exposure. Azole fungicides are considered fungistatic agents, preventing growth and sporulation of fungi (Kahle *et al.* 2008), including yeasts (Manavathu *et al.* 1998; Álvarez-Pérez *et al.* 2015). The anti-parasitic effect of tebuconazole here observed may compromise the extension and importance of parasite epidemics in the field. Such an event could affect host–parasite coevolution and endanger the ecological role of the parasite (Duffy and Sivars-Becker, 2007; Hall *et al.* 2011). The widespread use of azole fungicides (as agrochemicals, but also as pharmaceuticals) and their environmental persistence in aquatic systems (Kahle *et al.* 2008) could ultimately result in a weakening of the reciprocal selection of hosts and azole-sensitive microparasites (such as *M. bicuspidata*). Indeed, fungi are ubiquitous in aquatic systems (Marcogliese, 2008; Dighton, 2016; Grossart *et al.* 2016), being common parasites to a variety of organisms, such as cyanobacteria (Gleason *et al.* 2015; Agha *et al.* 2016), protists (Gleason *et al.* 2015; Jephcott *et al.* 2016), plants (Dean *et al.* 2012), invertebrates (Hall *et al.* 2011), fish (Gozlan *et al.* 2014) and amphibians (Hanlon *et al.* 2012, 2015). Besides playing important roles in energy transfer and in the regulation of host diversity, they also act as biological controllers of economically- and ecologically-important aquatic hosts, such as toxin-producing dinoflagellates or cyanobacteria (Gleason *et al.* 2015; Jephcott *et al.* 2016). Given the common occurrence and importance of fungi at the ecosystem level (Dighton, 2016), studies such as this one are necessary to understand the potential effects of climate change and aquatic pollution on fungi-mediated processes (e.g. disease) and their consequences on ecosystem functioning.

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