Presence of multiple parasitoids decreases host survival under warming, but parasitoid performance also decreases

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Running title: Warming alters multiple predator effects

Abstract. Current global changes are reshaping ecological communities and modifying environmental conditions. We need to recognize the combined impact of these biotic and abiotic factors on species interactions, community dynamics, and ecosystem functioning. Specifically, the strength of predator-prey interactions often depends on the presence of other natural enemies: it weakens with competition and interference or strengthens with facilitation. Such effects of multiple predators on prey are likely to be affected by changes in the abiotic environment, altering top-down control, a key structuring force in natural and agricultural ecosystems. Here, we investigated how warming alters the effects of multiple predators on prey suppression using a dynamic model coupled with empirical laboratory experiments with *Drosophila*-parasitoid communities. While multiple parasitoids enhanced top-down control under warming, parasitoid performance generally declined when another parasitoid was present due to competitive interactions. This could reduce top-down control over multiple generations. Our study highlights the importance of accounting for interactive effects between abiotic and biotic factors to better predict community dynamics in a rapidly changing world and thus better preserve ecosystem functioning and services such as biological control.

Keywords: biodiversity-ecosystem functioning, global change, temperature, functional response, host-parasitoid networks, multiple predator effects

Introduction

Ongoing global anthropogenic changes alter the abiotic context, changing the outcome of species interactions [1,2]. Global warming can modify the strength of trophic interactions due to changes in metabolic rates [3], shifts in spatial distributions and seasonal phenology [4], lethal effects on predators, or altered attack rates [5–7]. But warming also alters the strength of non-trophic interactions among predators [8,9]. Altered non-trophic interactions among predators would change the effects of multiple predators on top-down control [10,11], yet to what extent is unclear. Effects of warming on non-trophic interactions among predators are often overlooked but essential to accurately forecast ecological consequences of warming for biological control and ecosystem integrity.

The effects of multiple predators on prey suppression are often not additive. Additivity would occur if predators had independent effects on prey, in which case increased predator density should enhance top-down control because of higher predatory pressure on the prey. However, direct and indirect interactions among predators may cause effects to deviate from additivity [12–14]. The effects of multiple predators on prey can be synergistic (i.e., the effects are greater than what would be expected if they were additive) due to niche complementarity or facilitation (i.e., risk enhancement for the prey) [15]. By contrast, the effects of multiple predators on prey can be antagonistic due to intraguild predation, competition, or interference when the degree of overlap between predator's foraging areas or phenologies is too high (i.e., risk reduction) [16]. All such potential effects are called multiple predator effects (MPEs [17]). Emergent MPEs are particularly important in biological control where introducing one or several predator species might result in risk reduction for the prey because of competition among predators instead of planned risk enhancement [18].

Warming can alter both trophic and non-trophic interactions. Changes in the strength of these interactions could modify emergent MPEs, either enhancing or decreasing top-down control. Climate change also disrupts species composition of communities [4,19], which would change the outcome of pairwise interactions that are influenced by other species in the community [20–22]. Changes in species

55 composition of communities are thus also likely to alter MPEs, affecting biological control. However,
56 interactive effects between warming and community composition on top-down control remain poorly
57 studied, and little is known about how warming alters the effects of multiple predators on top-down control.

58 Here, we used mathematical models in combination with a series of three laboratory experiments on
59 *Drosophila simulans* and three of its co-occurring larval parasitoids to investigate the effects of warming
60 on multiple predator effects on top-down control. Host-parasitoid interactions are a particular type of
61 predator-prey interaction in which parasitoid larvae feed and develop inside or on an arthropod host while
62 adults are free-living [23]. When host is parasitized, three outcomes are possible: the parasitoid successfully
63 develops and kills the host, the host survives and successfully eliminates its parasitoid through immune
64 response (i.e., encapsulation and melanization) [24], or both parties die. The presence of multiple
65 parasitoids can result in extrinsic competition between adults for space and oviposition (i.e., interference)
66 and intrinsic competition within a host [25]. Intrinsic competition results from superparasitism or
67 multiparasitism events when two parasitoids (conspecific or heterospecific) parasitize the same host
68 individual. A single parasitoid can also lay multiple eggs in a single host as part of a strategy to overwhelm
69 the host immune system. In solitary parasitoids, such as the species used in the present study, only one
70 individual completes development in each host, suppressing the other(s) physically or physiologically.
71 Parasitoids represent an excellent system to study how warming directly changes the effects of multiple
72 predators on top-down control because the outcome of the interactions is easily observed by rearing the
73 host, and intrinsic competitive interactions between parasitoids can be observed by dissecting the host larva.
74 In this study, we empirically measured trophic interaction strength across temperatures and parasitoid
75 assemblages. We recorded emergent effects of multiple parasitoids on host suppression by comparing
76 empirical data with estimates in which multiple parasitoids would not interact (i.e., would have an additive
77 effect) using a mathematical model for multiple co-occurring parasitoids with a functional response
78 approach [26,27]. With this framework, we addressed three specific questions: (1) Do multiple parasitoids
79 have additive, synergistic, or antagonistic effects on host suppression? (2) To what extent does temperature

modify the outcomes of MPEs? (3) Are changes in host immune response or competitive interaction strength causing emergent MPEs? Our results demonstrate the principal role of temperature for non-trophic interactions among parasitoids, with cascading effects on host suppression.

Materials and Methods

Biological system

Cultures of *Drosophila simulans* and their associated parasitoids collected from two tropical rainforest locations in North Queensland Australia: Paluma (S18° 59.031' E146° 14.096') and Kirrama Range (S18° 12.134' E145° 53.102'; both <100 m above sea level; [28]) were used for the experiments. Tropical species already live close to their upper thermal limits [29]. *Drosophila* species are limited in their evolutionary potential for thermal adaptation [30,31], making our tropical *Drosophila*-parasitoid community a relevant system to study effects of future warming conditions on communities. *D. simulans* and parasitoid cultures were established between 2017 and 2018, identified using both morphology and DNA barcoding, and shipped to the Czech Republic under permit no. PWS2016-AU-002018 from Australian Government, Department of the Environment. All cultures were maintained at 23°C and 12:12 hour light and dark cycle at Biology Centre, Czech Academy of Sciences. The three larval parasitoid species *Asobara sp*. (Braconidae: Alysiinae; strain KHB, reference voucher no. USNMENT01557097, reference sequence BOLD process ID: DROP043-21), *Leptopilina sp*. (Figitidae: Eucolinae; strain 111F, reference voucher no. USNMENT01557117, reference sequence BOLD process ID: DROP053-21), and *Ganaspis sp*. (Figitidae: Eucolinae; strain 84BC, reference voucher no. USNMENT01557102 and USNMENT01557297, reference sequence BOLD process ID: DROP164-21) were used (for more details on the parasitoid strains see [32]). *Drosophila simulans* isofemale lines were kept on standard *Drosophila* medium (corn flour, yeast, sugar, agar, and methyl-4-hydroxybenzoate) for approximately 45 to 70 non-overlapping generations before the experiments. To revive genetic variation, five host lines were combined to establish two population cages of mass-bred lines before the start of the experiments. Single parasitoid isofemale lines were used and maintained for approximately 25 to 40 non-overlapping generations before the start of the experiment by

105 providing them every week with two-day-old larvae of a different *Drosophila* species – *Drosophila*
106 *melanogaster*.

107 *Experiments*

108 We used a functional response approach following Mccoy's framework to investigate the effects of warming
109 on the strength of trophic and non-trophic interactions [26]. We first obtained each parasitoid functional
110 response parameter at ambient and warmed temperatures with single-parasitoid treatments (Experiment 1).
111 Then, we used these functional response parameter estimates to predict trophic interaction strength for each
112 temperature and parasitoid combination with the null hypothesis that parasitoids were not interacting and
113 thus had additive effects on host suppression. In Experiment 2 we empirically measured the effects of
114 temperature and parasitoid combinations on trophic interaction strength and compared the predicted and
115 observed values to identify emergent effects of multiple parasitoids on host suppression and their
116 dependence on the temperature regime. We performed the two first blocks of Experiment 1 and entire
117 Experiment 2 in parallel, and controls and single-parasitoid treatments were common to both experiments.
118 In Experiment 3, we investigated the mechanisms of multiple parasitoid effects by dissecting hosts rather
119 than rearing them. This allowed us to measure super- and multiparasitism rates and encapsulation depending
120 on the temperature regime and parasitoid combinations.

121 A total of 22,920 *D. simulans* eggs were collected. 13,120 for experiment 1, 4,800 for experiment 2, and
122 5,000 for experiment 3 (from which 1,000 larvae were dissected). In experiments 1 and 2, 12,990 eggs
123 (73%) successfully developed into adults (8,409 hosts and 4,581 parasitoids). The remaining 23% either
124 died naturally or the host was suppressed without successful development of the parasitoid.

125 *Experiment 1: Single-parasitoid experiment*

126 Eggs of *D. simulans* were placed in a single 90 mm high, and 28 mm diameter glass vial with 10mL of
127 *Drosophila* media at six different densities replicated eight times each (5, 10, 15, 25, 50, or 100 eggs per
128 10mL of food media in a vial, total number of eggs = 13,120, N = 384 vials; Figure 1b). To collect *D.*
129 *simulans* eggs, an egg-washing protocol was adapted from [33]. The day before the egg-washing protocol

was conducted, two batches of egg-laying medium (Petri dishes with agar gel topped with yeast paste) were introduced in each population cage for flies to lay eggs overnight. Eggs were transferred to the experimental vials. We placed half of the vials at ambient temperature (22.7°C ± 0.4 s.d. - the current mean yearly temperature at the two study sites [28]), and the other half under elevated temperature (27.4°C ± 0.5 s.d. - projected change in global mean surface temperature for the late 21st century is 3.7˚C for the IPCC RCP8.5 baseline scenario [34]). Like in other *Drosophila* species, the thermal performance curve of *Drosophila simulans* demonstrates a decrease in performance from temperatures above 25°C [35].

After 48 hours, we placed one single naïve mated three to five-day-old female parasitoid in each vial with *D. simulans* larvae. We removed the parasitoids twenty-four hours later. We repeated this for all three parasitoid species, temperatures, and host densities. We simultaneously performed controls without parasitoids to obtain the baseline for host survival without parasitism and potential variation during egg collection (Figure 1a). We checked vials were daily for adult emergences until the last emergence (up to 41 days for the species with the longest developmental time). We waited five consecutive days without any emergence to stop collecting and thus avoided confounding counts with a second generation. All emerged insects were collected, identified, sexed, and stored in 95% ethanol. Each treatment was replicated eight times across eight experimental blocks.

Experiment 2: Multiple parasitoids experiment

To investigate the effect of warming on MPEs, we manipulated parasitoid assemblages and temperature in a fully factorial design (Figure 1c and d). We followed the same protocol described above for Experiment 1, using 50 *D. simulans* eggs per vial with two female parasitoids either from the same (Figure 1c) or different species (Figure 1d). Each treatment was replicated eight times across two blocks (N = 96). Controls and single-parasitoid treatments were standardized to experiment 1 with the 50 eggs per vial density. 50 eggs per standard *Drosophila* vial corresponds to low competition between hosts, a suitable host/parasitoid ratio when using one or two parasitoids, and enough host per vial for meaningful statistics.

Experiment 3: Mechanisms of MPEs

In a follow-up experiment, we conducted a subset of the treatments described for Experiments 1 and 2 with *Asobara sp*. and *Ganaspis sp*. We put 50 *D. simulans* eggs per vial with 10 mL of food media under ambient and warming temperatures and introduced one parasitoid, two conspecific parasitoids, or the two heterospecific parasitoids, resulting in five different parasitoid assemblages. Each treatment was replicated ten times across two blocks (N = 100). Instead of rearing the insects to adults, we dissected ten 3rd instar larvae or pupae per vial (Figure 1f). We individually transferred each host larva into a glass petri dish containing PBS and dissected it under a stereomicroscope. We recorded the number of parasitoid larvae and eggs of each species to assess superparasitism and multiparasitism events. When possible, we also identified the number and species of encapsulated parasitoids. Pictures of the eggs, larvae and encapsulated parasitoids for each species observed during the experiment are presented in Supplemental Material S1 Figure S1. At the elevated temperature, six replicates were dissected two days after infection (early dissection time) and four three days after infection (late dissection time). At the ambient temperature, four replicates were dissected three days after infection (early dissection time) and six four days after infection (late dissection time). We selected different times for dissection at each temperature to standardize parasitoid developmental stage while still being able to identify all the parasitoids that have parasitized the host. At the early dissection time, *Asobara sp*. were already at the larval stage, whereas *Ganaspis sp*. were still eggs. *Ganaspis* larvae were also observed at the late dissection time, sometimes simultaneously with a larva of *Asobara sp*. within the same host.

Data analysis and modelling

Experiment 1: Single-parasitoid experiment

We combined numerical simulations of host density dynamics, accounting for host depletion [36]:

$$\frac{dH}{dt} = -F(H)P,$$

with Bayesian parameter estimation using the *rstan* package (e.g. [37]). $P = 1$ is the parasitoid density, and $F(H)$ denotes the host density-dependent functional response. In the model fitting, we used Markov chain Monte Carlo to sample from the functional response's model parameters' posterior probability distribution $p(\theta|H_{sup})$ given the observations H_{sup}, based on the likelihood function $p(H_{sup}|\theta)$ and prior distributions $p(\theta)$, with θ the free parameters. H_{sup} is the number of *D. simulans* suppressed (the difference between adult hosts emerging from the controls without parasitoids and from the experiment). In each iteration, we computed numerical solutions of the equation with the built-in *Runge-Kutta* ODE solver to predict densities $\hat{H}_1$ after one day for each given initial host density, H_0. The initial host densities were taken from the average number of hosts that emerged from the controls for each density and temperature to account for potential deviations between the aimed and actual densities (Table S1). The likelihood was evaluated assuming a binomial distribution for observed numbers of suppressed hosts H_{sup} with $n = H_0$ trials and $p = \frac{H_0 - \hat{H}_1}{H_0}$ success probability. We used vague priors for all model parameters.

We fitted three different functional response models (Type II, Type III and generalized Type III) and retained the Type II functional response [38] after model comparison (see Supplement Material S2). The equation for the instantaneous attack rate of a parasitoid is as follows:

$$F(H) = \frac{aH}{1 + ahH}$$

where a is the attack rate, and h is the handling time. Type II functional responses are thought to characterize the attack rate of many types of predators and parasitoids [39]. Parameter estimates and the functional responses for each species at each temperature are presented in Supplement Material S2 (Table S2 and Figure S2).

197 *Experiment 2: Multiple parasitoids experiment*

198 Host-parasitoid interaction strength was described with the combination of Degree of Infestation (DI; i.e.,
199 host suppression) and Successful Parasitism rate (SP; i.e., parasitoid performance). The observed degree of
200 infestation (DI_{obs}) and Successful parasitism rate (SP) were measured as:

201 $$DI_{obs} = 1 - \frac{H}{H_C} \; ; \; SP = \frac{P}{H_c - H}$$

202 where H is the number of adult hosts emerging from the experiment vial, H_C is the mean number of adult
203 hosts emerging from the controls without parasitoids, and P is the number of parasitoid adults emerging
204 from the experimental vial [40,41]. DI_{obs} was set to zero if the number of hosts emerging from the treatment
205 was greater than the controls. If no parasitoid emerged or the number of hosts suppressed was estimated to
206 be zero, SP was set to zero. If the number of parasitoids that emerged was greater than the estimated number
207 of hosts suppressed, SP was assigned to one. For treatments with parasitoid conspecifics, we assumed that
208 each of the two parasitoid individuals was attacking the hosts equally; therefore, the number of parasitoid
209 adults emerging was divided by two to calculate individual successful parasitism rate.

210 We analyzed these data with generalized linear models (GLMs) and verified model assumptions with
211 the *DHARMa* package [42]. To correct for overdispersion of the residuals and zero inflation, data were
212 modeled using zero-inflation models with a beta-binomial error distribution and a logit function using the
213 *glmmTMB* function from the *TMB* package [43]. Two categories of predictor variables were used in separate
214 models with temperature treatment (two levels: ambient and warming): (i) parasitoid treatment (three levels;
215 single parasitoid, two parasitoids conspecific, and two parasitoids heterospecific), and (ii) parasitoid species
216 assemblage (nine levels). The two-way interactions between temperature and either parasitoid treatment or
217 parasitoid assemblage were tested and kept in our models if judged to be significant based on backward
218 model selection using Likelihood-ratio tests. The significance of the effects was tested using Wald type III
219 analysis of deviance with Likelihood-ratio tests. Factor levels were compared using Tukey's HSD *post hoc*

220 comparisons of all means and the *emmeans* package [44]. Results for developmental rate are presented in
221 Supplement Material S3 Figure S3.

222 *Estimation of multiple parasitoid effects*

223 To predict the degree of infestation if parasitoids have independent effects on host suppression, we used
224 the method developed by Mccoy *et al*. [26], which considers host depletion. This method uses the functional
225 responses obtained from Experiment 1 in a population-dynamic model to predict how host density changes
226 in time as a function of initial density and parasitoid combination for each temperature. We thus calculated
227 the estimated Degree of Infestation (DI_0) by integrating the aggregate attack rates over the duration of the
228 experiment as host density declines. We first solved the equation

229 $$\frac{dH}{dt} = -\sum_{i=1}^{n} \frac{a_i H_t P_i}{1 + a_i h_i H_t}$$

230 similar to the equation described for Experiment 1 but adapted to *n* parasitoids. Then we calculated the
231 estimated Degree of Infestation as

232 $$DI_0 = 1 - \frac{H_T}{H_0}$$

233 where H_0 is the initial host density, and H_T is the estimated host population at the end of the experiment
234 (time T = 1 day). This method allows a reasonable estimate of DI_0 for the null hypothesis that predators do
235 not interact [27]. The lower and upper confidence intervals (CI) around the predicted values were estimated
236 with a global sensitivity analysis based on the functional response parameters estimates to generate 100
237 random parameter sets using a Latin hypercube sampling algorithm [45]. The expected degree of infestation
238 was calculated for each parameter set using the *sensRange* function in the R package *FME*. The 2.5% and
239 the 97.5% quantiles of the values obtained from these simulations were used as 95% CIs around the
240 predictions.

241 Predictions from the population dynamic model were then compared with the observed values (DI_{obs}).
242 Estimated DI values greater than observed DI translate to risk reduction, while lower estimates reflect risk

enhancement for the host with multiple parasitoids. We calculated the difference between DI_{obs} and mean DI_0 for each treatment and investigated the effects of temperature (ambient versus warmed), parasitoid diversity (one or two species), and their interaction if significant, using an analysis of variance (ANOVA) with the *aov* function. We statistically compared the observed and estimated DI for each temperature regime using GLMs with a beta-binomial error distribution and a logit function with DI_0 as an offset (i.e., predictor variable) following Sentis et al. (2017). A positive or negative significant intercept indicates that DI_0 values underestimate or overestimate DI_{obs}, respectively.

Experiment 3: MPEs mechanisms

The frequency of super- and multiparasitism events was calculated out of the larvae parasitized per vial (total of 1,000 larvae dissected across 100 vials, out of which 868 were parasitized: presence of either one or both parasitoid species and/or trace of melanization). The frequency of encapsulated parasitoids was calculated out of the total number of parasitoids per larva. Effects of temperature and parasitoid assemblages on these frequencies were analyzed with generalized linear mixed models (GLMMs) with the method described for Experiment 2 and the time of dissection (early or late) as a random effect. All analyses were performed using R 4.0.2 [46].

Results

Effects of multiple parasitoids on host suppression under warming

The degree of infestation observed in the experiment varied from the model estimations (Figure 2). Temperature significantly affected these differences ($F_{1,93} = 13.9$, $P < 0.0001$), but parasitoid diversity did not ($F_{1,93} = 0.09$, $P = 0.766$) (Table S3, Table S4), implying that parasitoid density rather than their diversity is important for host suppression. The comparison of the estimated and observed DI revealed that, in most cases, there was no significant difference between predicted and observed DI at ambient temperature, implying neutral effects with multiple parasitoids (when looking at the intercept of the GLM with $DI_{0\ as}$ an offset; value ± SE: 0.12 ± 0.32, z value = 0.381, df = 40, P = 0.703), whereas under warming the predicted

267 DI_0 significantly underestimated the observed DI_{ob}, implying risk enhancement for the host (value ± SE:
268 0.44 ± 0.18, z value = 2.431, df = 40, P = 0.015; Figure 2).

269 *Effects of warming and parasitoid assemblages on the observed degree of infestation*
270 Contrary to the effects of multiple parasitoids on host suppression, the observed degree of infestation DI_{obs}
271 was not significantly affected by temperature ($\chi2_{(1)}$ = 1.05, P = 0.306), or parasitoid treatment (single, two
272 conspecific or two heterospecific parasitoid assemblages: $\chi2_{(2)}$ = 4.26, P = 0.119; Table S5) due to species-
273 specific effects. DI only varied with parasitoid species assemblages ($\chi2_{(8)}$ = 251.92, P < 0.0001, Table S6).
274 DI was the highest in assemblages with *Ganaspis sp*., either alone, with a conspecific, or another parasitoid
275 species (Figure S4).

276 *Effect of warming and parasitoid assemblages on parasitoid performance*
277 Despite having no effect on DI, parasitoid treatment (single, two conspecific, or two heterospecific
278 parasitoid assemblages) significantly affected successful parasitism rate, and the effect varied among
279 parasitoid species (two-way interaction: $\chi2_{(4)}$ = 16.88, P = 0.002; Table 1; Table S7). SP of *Ganaspis sp.*
280 decreased by 95.7% (95% CI: 93.6 - 97.8%) with the presence of a parasitoid conspecific [*Post hoc* Odds
281 Ratio (OR) = 0.043, P < 0.0001 for contrast 2Pc/1P; Table S8], and by 83.4% (CI: 75.4 - 91.3%) with the
282 presence of a parasitoid heterospecific compared to when alone (OR = 0.166, P < 0.001 for contrast 2Ph/1P;
283 Table S8). However, it increased by 287.6% (CI: 178.8 - 396.4%) when the parasitoid competitor was from
284 another species compared to a conspecific (OR = 3.876, P < 0.0001, for contrast 2Pc/2Ph; Table S8). SP of
285 *Asobara sp*. decreased by 55.2% (CI: 41.5 - 69.7%) when a parasitoid conspecific was present compared
286 to when alone (OR = 0.448, P = 0.036), but was not significantly affected by the presence of a parasitoid
287 heterospecific (OR = 0.712, P = 0.484). There were no significant effects of parasitoid treatments for SP of
288 *Leptopilina sp*. Effects of parasitoid assemblages on SP also varied between parasitoid species and are
289 presented in Supplementary Material S6 (Table S9, Table S10, and Figure S5).

290 Effects of temperature on SP also depended on the species (two-way interaction: $\chi2_{(2)}$ = 7.31, P = 0.026;
291 Table S7). Only *Ganaspis sp.* was significantly affected by temperature, and its SP decreased by 58.8%

(CI: 69.8 - 47.8%) with warming (OR = 0.412, $\chi2_{(1)}$ = 10.17, P = 0.001). However, all species developed faster under warming (Figure S3).

Mechanisms of MPEs

The frequency of either super- or multiparasitism events, reflecting strength of intrinsic competition among parasitoids, was significantly affected by parasitoid assemblages ($\chi2_{(4)}$ = 572.40, P < 0.0001), temperature ($\chi2_{(1)}$ = 4.49, P = 0.034), and the interaction between parasitoid assemblages and temperature $\chi2_{(4)}$ = 36.04, P < 0.0001; Figure 3, Table S11). Superparasitism rate increased by 239% (CI: 230-308%) when *Ganaspis sp*. was with a conspecific (OR = 3.69, P < 0.0001), and by 581% (CI: 411-751%) when *Asobara sp*. was with a conspecific (OR = 6.81, P < 0.0001) compared to when they were alone, but without significant differences between temperature treatments. In the parasitoids heterospecific treatments, warming significantly increased the frequency of super- and multiparasitism events by 173% (CI: 130-216%; OR = 2.73, P < 0.0001), indicating an increase in intrinsic competition among parasitoids with warming.

The frequency of encapsulated parasitoids differed between parasitoid species, but not between treatments (results presented in Supplement Material S8: Table S12 and Figure S6), indicating that host immune response did not change depending on the treatments.

Discussion

The key result from our study is the synergistic effects of multiple predators for top-down control at elevated temperature across predator assemblages. However, parasitoid performance often decreased when multiple parasitoids were present due to intrinsic competition among parasitoids, potentially limiting the long-term benefits for ecosystem functioning.

Warming increases the effects of multiple predators on the risk of predation

Our results showed that warming led to a higher top-down control than expected with multiple predators. Indeed, our mathematical model underestimated trophic interaction strength measured in multiple predators treatments at elevated temperatures. Our results are in concordance with previous studies on diverse systems

316 on the importance of considering non-trophic interactions to predict the effect of multiple predators on top-
317 down control under global changes. Drieu et al. [47] found that predator diversity enhanced the biological
318 control of insect pests in vineyards under warming due to functional complementarity among predator
319 species, while effects were substitutive at ambient temperature. Cuthbert et al. [11] also found an impact of
320 temperature on intraspecific multiple predator effects on an invasive Gammaridae species (Amphipoda).
321 Yet, the direction of the effects contrasted ours as they observed risk enhancement at low temperature and
322 risk reduction with warming. Sentis et al. [10] found a general trend of predation risk reduction for the prey
323 with multiple predators in an aquatic food web but without any effect of temperature on those emergent
324 MPEs. Our study goes further by showing the important impact of warming on the impacts of multiple
325 predators on prey suppression across multiple assemblages of conspecifics and heterospecifics. In addition
326 to increasing prey suppression with multiple predators under warming in terrestrial ecosystems, a diverse
327 predator community also increases the chances of complementarity in the face of environmental variation
328 and disturbance [48]. Indeed, the presence of multiple predator species could mitigate the adverse effects
329 of warming on top-down control due to resource partitioning and/or functional redundancy [47,49,50].
330 Therefore, preserving predator biodiversity should be generally beneficial for top-down control under
331 climate change.

332 *Mechanisms behind emergent multiple predator effects on the prey*

333 Because of the synergistic effects of multiple parasitoids on host suppression under warming found in our
334 study, we could have hypothesized that warming weakens interference between parasitoids, similarly to
335 predator-prey systems [51]. However, our host-parasitoid system allowed us to investigate further the
336 potential mechanisms behind our results, especially the strength of intrinsic competitive interactions
337 between parasitoids (i.e., frequency of superparasitism and multiparasitism events). We found generally
338 higher intrinsic competition in multiple parasitoid treatments than single parasitoid treatments and higher
339 intrinsic competition under warming when the two species were present compared to ambient temperature.
340 When superparasitized or multiparasitized, the host was less likely to survive, possibly because its immune

response was less likely to overcome multiple parasitoids. Therefore, the higher top-down control observed under warming with multiple parasitoids was due to a higher parasitism pressure and not because of weaker interactions between parasitoids.

We conducted the experiments in simplified laboratory conditions and forced parasitoids to share the same habitat (a vial) and overlapped in time (24 hours), which does not allow for resource partitioning [52]. This might have enhanced the rate of super- and multi-parasitism events and thus top-down control. In nature, warming could also change predator habitat use [8,9], and phenology [53,54], leading to changes in MPEs. However, the impact of temperature on MPEs was consistent across parasitoid assemblages, suggesting a general pattern for synergistic effects with multiple natural enemies under warming in our system.

Parasitoid performance was mostly affected by parasitoid assemblage

Despite multiple parasitoids enhancing host suppression under warming, the successful parasitism rate was often lower at both temperatures when another parasitoid individual was present, probably due to the strong intrinsic competitive interactions observed through dissections. A decrease in parasitoid performance would potentially limit the synergistic effects of multiple parasitoids for host suppression in the long term. Similarly, another study on *Drosophila*-parasitoid interactions observed a significant impact of thermal regime on parasitoid success, but still without changes in the observed degree of infestation [55]. The long-term effects of warming on parasitoid populations are thus uncertain, and hosts from the next generation might benefit from lower parasitoid abundances due to a lower rate of successful parasitism.

Similar effects of intra- versus interspecific multiple parasitoids on top-down control

Similar to other studies, we did not find significant differences between treatments with multiple conspecifics or heterospecific predators for prey suppression [56–58]. Therefore, it is essential to look at the effects of both predator diversity and density on prey suppression, rather than only using a substitutive approach (i.e., keeping predator density constant [52]), which might confound the results. When niche differentiation is allowed, for example, with habitat heterogeneity or a more prolonged timeframe that

366 includes potential differences in phenology, an increase in predator diversity should intensify prey
367 suppression because of functional diversity rather than because of diversity *per se* [58–60]. Here, two
368 predators of the same species rather than a single predator intensified prey suppression at warmer
369 temperatures despite the small scale of the experiment. Allowing for differentiation in habitat domain
370 between predator species might have yielded higher prey suppression in treatments with heterospecifics
371 and a lower rate of multiparasitism. Given the likely ubiquity of resource partitioning in nature [61],
372 preserving predator biodiversity would be the best strategy to maintain top-down control.

373 *No effects of treatments on the observed degree of infestation*

374 Warming had a significant effect on the differences between observed and estimated degree of infestation.
375 However, temperature treatment had no significant effect on the observed degree of infestation. Moreover,
376 prey suppression was generally higher when predator assemblages included the best-performing species,
377 *Ganaspis sp.*, no matter the predator treatment or temperature. A meta-analysis on the effects of predator
378 diversity on prey suppression found a similar trend across the 46 studies taken into account [62], but also
379 found a general positive effect of multiple predators on top-down control. Contrastingly, a meta-analysis
380 of 108 biological control projects found no relationship between the number of agents released and
381 biological control success for insect pests [63]. However, increasing predator diversity should be generally
382 beneficial for top-down control by increasing the chances to have a more effective natural enemy species
383 in the community, as was the case in our study (i.e., sampling effect model [64]). Moreover, the presence
384 of multiple species in the community could buffer any mismatch between predator and prey species induced
385 by warming [65]. *Ganaspis sp*. was the best performing species in suppressing *D. simulans* across
386 treatments. Still, its performance decreased with warming, suggesting that parasitism rate, and therefore
387 host suppression, could also be reduced in the longer term due to a decrease in parasitoid population.

388 *Conclusion*

389 Overall, pairwise interaction strength generally failed to accurately estimate the trophic interaction strength
390 observed, indicating that non-trophic interactions must be considered to predict the effects of multiple

391 predators on prey suppression and in food web studies in general [66]. While previous studies show altered
392 MPEs with warming due to changes in resource partitioning [8,11], our study is the first, to our knowledge,
393 to show signs of direct effects of warming on predator interactions across predator assemblages, resulting
394 in a higher top-down control with multiple predators at elevated temperature.

395 **Acknowledgements**

396 We thank Anna Mácová, Andrea Weberova, Grégoire Proudhom, and Joel Brown for their help during the
397 setup of the experiments, and Tereza Holicová and Vincent Montbel for their help dissecting the larvae.
398 Tereza Holicová made the drawings used for Figure 1. We acknowledge funding support from the Czech
399 Science Foundation grant no. 20-30690S. BR acknowledges the support of iDiv funded by the German
400 Research Foundation (DFG–FZT 118, 202548816).

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570

571 **Table 1.** Odds ratios of a successful parasitism event between parasitoid treatments (single parasitoid, two
572 parasitoids conspecific, and two parasitoids heterospecific) for each parasitoid species. Results are averaged
573 over both temperatures because there was no significant interaction between temperature and parasitoid
574 treatments. Values less than or greater than one denote a decrease or an increase in the odds of successful
575 parasitism, respectively. Significant differences are highlighted in bold.

Parasitoid species	Contrast	Odds Ratio	P-value
Ganaspis sp.	2 conspecifics/single	**0.043**	**<0.0001**
	2 heterospecifics/single	**0.166**	**0.0007**
	heterospecifics/conspecifics	**3.876**	**<0.0001**
Asobara sp.	2 conspecifics/single	**0.448**	**0.036**
	2 heterospecifics/single	0.711	0.484
	heterospecifics/conspecifics	1.589	0.251
Leptopilina sp.	2 conspecifics/single	0.182	0.494
	2 heterospecifics/single	0.871	0.994
	heterospecifics/conspecifics	4.764	0.295

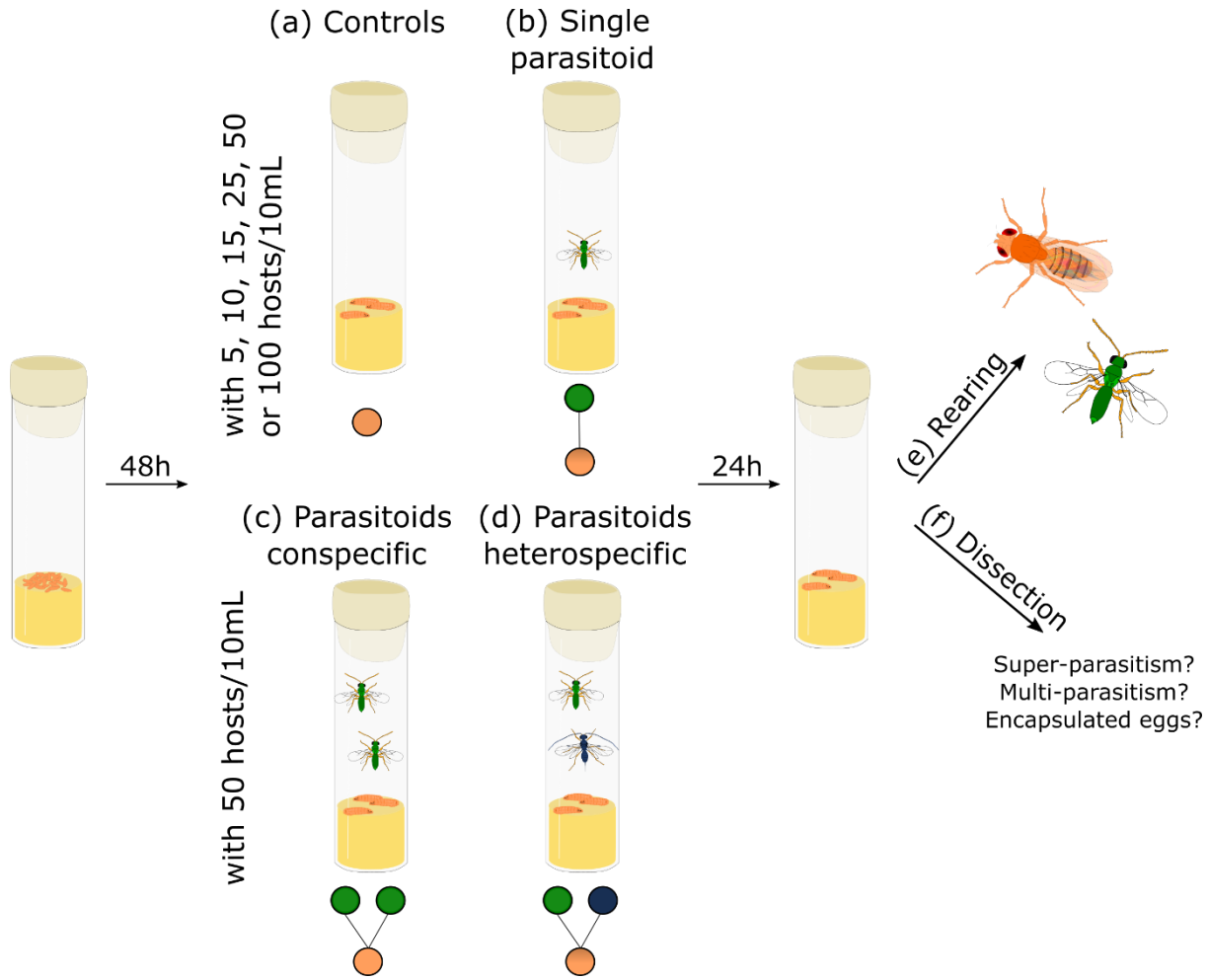
576

Figure legends

Figure 1. Schematic representation of the experimental design. (a) Controls or (b) one single parasitoid female with either 5, 10, 25, 50 or 100 *D. simulans* per 10 mL of media (N = 424 vials), (c) two parasitoids conspecific (N = 88 vials) or (d) two parasitoids heterospecific (N = 68 vials) with 50 *D. simulans* per 10 mL of media. (e) Rearing until adults emerge for Experiments 1 and 2 (up to 41 days), or (f) dissection of 10 3rd instar larvae or pupae per vial two, three, or four days after infection for Experiment 3.

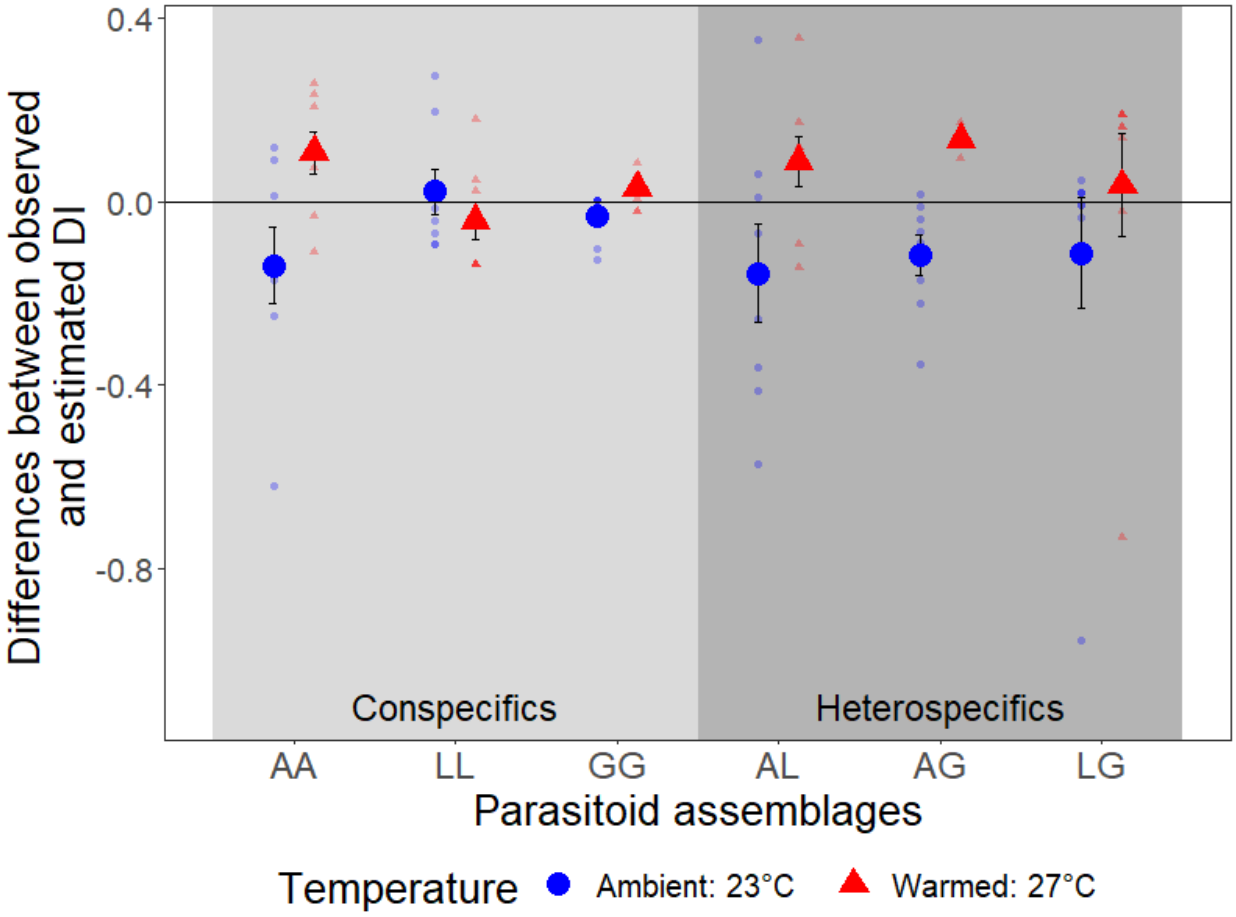
Figure 2. Differences between observed and estimated degree of infestation (DI) for each parasitoid assemblage and temperature (i.e., DI_{obs} - DI_{0}). Negative values translate to risk reduction, while positive values reflect risk enhancement for the host with multiple parasitoids. Light grey panel: two parasitoids conspecific, darker grey panel; two parasitoids heterospecific. Parasitoid abbreviations: A: *Asobara sp*., L: *Leptopilina sp*., and G: *Ganaspis sp*. Big dots represent the means (±SE), and small dots represent raw data.

Figure 3. Frequency of super- or multiparasitism events out of the total of parasitized hosts per vial (N = 100 vials) significantly changed depending on parasitoid assemblage and temperature regime, indicating changes in intrinsic competitive interaction strength among parasitoids. Within each plot, different small letters denote significant differences between parasitoid assemblages (and temperature regime if significant). White panel: single parasitoid, light grey panel: two parasitoids conspecific, darker grey panel; two parasitoids heterospecific. Parasitoid abbreviations: A: *Asobara sp*., and G: *Ganaspis sp*. Big dots represent the estimated means (±95% CIs), and small dots represent raw data.



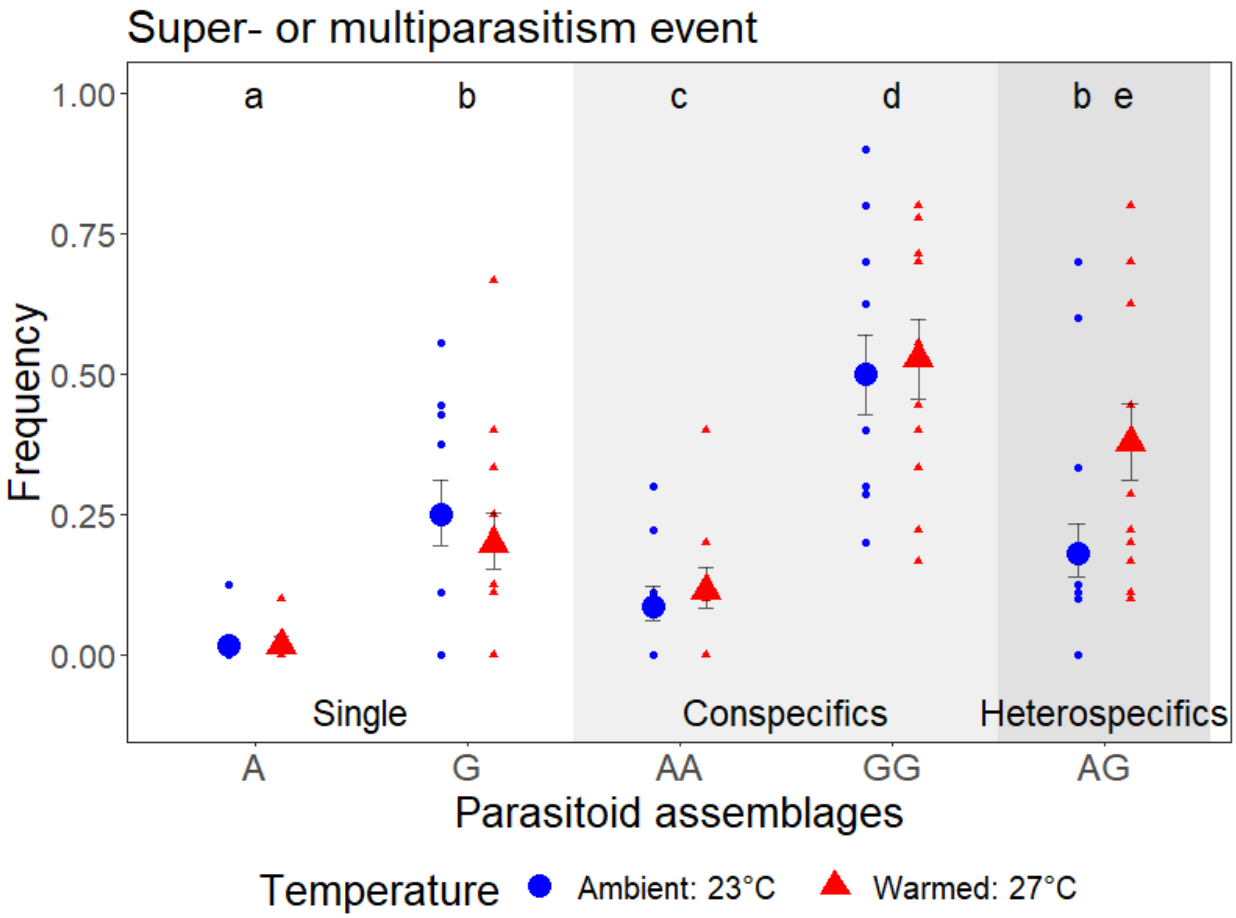
598

599 **Figure 1.**



600

601 **Figure 2**.



602

603 **Figure 3.**