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

Mélanie Thierry[*1,2], Nicholas A. Pardikes[2], Benjamin Rosenbaum[3,4], Miguel G. Ximénez-Embún[2], & Jan Hrček[1, 2]

[1] University of South Bohemia, Faculty of Science, Branisovska 31, 37005, Czech Republic

[2] Biology Centre of the Czech Academy of Sciences, Institute of Entomology, Branisovska 31, 37005, Czech Republic

[3] Theory in Biodiversity Science, German Centre for Integrative Biodiversity Research (iDiv), Halle-Jena-Leipzig, Puschstr. 4, 04103 Leipzig, Germany

[4] Institute of Biodiversity, Friedrich Schiller University Jena, Dornburger Str. 159, 07743 Jena, Germany

[*] Corresponding author: melanie.thierry34@gmail.com

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 both 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, which could reduce top-down control in the long-term. 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 are altering the abiotic context, which can change the outcome of species interactions [1,2]. Global warming can weaken 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 does also alter 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 ecosystems integrity.

The effects of multiple predators on prey suppression are often not additive. Additivity would occur if predators have independent effects on prey, in which case predator density should enhance top-down control because of a 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 referred to as multiple predator effects (MPEs [17]). Emergent MPEs are particularly important in biological control where introduction of 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 for 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 parasitized, three outcomes are possible: the parasitoid successfully
63 develops, the host successfully eliminates its parasitoid through immune response (i.e., encapsulation and
64 melanization) and survives [24], or both parties die. When multiple parasitoids are present, they can
65 compete extrinsically as adults for space and oviposition (i.e., interference), and intrinsically within a host
66 [25]. Intrinsic competition is the result of a super- and/or multiparasitism events when two parasitoids -
67 conspecifics or heterospecifics respectively - parasitize the same host individual. In solitary parasitoids,
68 such as the species used in the present study, only one individual completes its development in each host,
69 suppressing the other(s) physically or physiologically. However, both parasitoid species can be observed
70 as eggs or larvae inside the host by dissecting the host larva. Parasitoids represent an excellent system to
71 study how warming directly changes the effects of multiple predators on top-down control because the
72 outcome of the interactions is directly observed by rearing the host, and intrinsic competitive interactions
73 between parasitoids can be observed by dissecting the host larva. In this study, we empirically measured
74 trophic interaction strength across temperatures and parasitoid assemblages. We recorded emergent effects
75 of multiple parasitoids on host suppression by comparing empirical data with estimates in which multiple
76 parasitoids would not interact (i.e., would have additive effect) using a mathematical model for multiple
77 co-occurring parasitoids with a functional response approach [26,27]. With this framework, we addressed
78 three specific questions: (1) Do multiple parasitoids have additive, synergistic, or antagonistic effects on
79 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 prevalent 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 are already living close to their upper thermal limits [29], and *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 prior the start of the experiments. Single parasitoid isofemale lines were used and maintained for approximately 25 to 40 non-overlapping generations prior to

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

107 *Experiments*

108 To investigate the effects of warming on the strength of trophic and non-trophic interactions, we used a
109 functional response approach following Mccoy's framework [26]. We first obtained the parameters of each
110 parasitoid functional response at ambient and warmed temperatures with single-parasitoid treatments
111 (Experiment 1). Then, we used these functional response parameter estimates to predict trophic interaction
112 strength for each temperature and parasitoid combination with the null hypothesis that parasitoids were not
113 interacting, and thus had additive effects on host suppression. In Experiment 2 we empirically measured
114 the effects of temperature and parasitoid combinations on trophic interaction strength, and compared the
115 predicted and observed values to identify emergent effects of multiple parasitoids on host suppression and
116 their dependence on the temperature regime. The two first blocks of Experiment 1 and entire Experiment 2
117 were performed in parallel, and controls and single-parasitoid treatments were common to both
118 experiments. In Experiment 3, we investigated the mechanisms of multiple parasitoid effects by dissecting
119 hosts rather than rearing them. This allowed us to measure rates of super- and multiparasitism and
120 encapsulation depending 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 [of
122 which 12,990 (73%) successfully emerged as adults (8,409 hosts and 4,581 parasitoids)], and 5,000 for
123 experiment 3 from which 1,000 larvae were dissected.

124 *Experiment 1: Single-parasitoid experiment*

125 Eggs of *D. simulans* were placed in a single 90 mm high and 28 mm diameter glass vial with 10mL of
126 *Drosophila* media at six different densities (5, 10, 15, 25, 50 or 100 eggs per 10mL of food media in vial;
127 Figure 1a). To collect *D. simulans* eggs, an egg-washing protocol was adapted from [33]. The day before
128 the egg-washing protocol was conducted, two batches of egg-laying medium (petri dishes with agar gel
129 topped with yeast paste) were introduced in each population cage for flies to laying eggs overnight. Eggs

were transferred in the experimental vials. Half of the vials were placed at ambient temperature (22.7°C ± 0.4 s.d. - current mean yearly temperature at the two study sites [28]), and the other half at warmed 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 other *Drosophila* species, the thermal performance curve of *Drosophila simulans* demonstrates a decrease in performance from temperatures above 25°C [35].

After 48 hours, one single naïve mated three to five-day-old female parasitoid was placed in each vial with *D. simulans* larvae. Twenty-four hours later, parasitoids were removed. This was repeated for all three parasitoid species, temperatures, and host densities. Controls without parasitoids were run at the same time to obtain the baseline for host survival without parasitism. Vials were checked 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, thus avoiding 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 1b and c). 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 1b) or different species (Figure 1c). Each treatment was replicated eight times across two blocks.

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 parasitoids conspecific or the two parasitoids heterospecific, resulting in five different parasitoid assemblages. Instead of rearing the insects to adults, we dissected ten 3rd instar larvae or pupae per vial (Figure 1e). Each host larva was individually transferred

into a glass petri dish containing PBS and dissected under stereomicroscope. We recorded the number of parasitoid larvae and eggs of each species to assess super- and multiparasitism events, and, when possible, the number and identity of encapsulated parasitoids. Pictures of the eggs, larvae, and encapsulated parasitoids for each species observed during the experiment are presented in Supplemental Material S1. Each treatment was replicated ten times across two blocks. At the elevated temperature, six replicates were dissected two days after infection (early dissection time) and four three days after infection (late dissection time), and at the ambient temperature, four replicates were dissected three days after infection (early dissection time) and six four days after infection (late dissection time). Different times for dissection were chosen for 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. At the late dissection time, *Ganaspis* larvae were also observed, and sometimes at the same time than *Asobara sp*. as larva within a 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, Markov chain Monte Carlo was used 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, numerical solutions of the equation were computed with the built-in *Runge-Kutta* ODE solver, to predict densities $\widehat{H}_1$ after 1 day for each given initial host density, H_0. 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. Vague priors were used 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 S1 and Figure S2).

Experiment 2: Multiple parasitoids experiment

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

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

where H is the number of adult hosts emerging from the experiment vial, H_C the mean number of adult hosts emerging from the controls without parasitoids, and P the number of parasitoid adults emerging from the experimental vial [40,41]. DI_{obs} was set to zero if the number of hosts emerging from the treatment was greater than the controls. If no parasitoid emerged or if the number of hosts suppressed was estimated to be zero, SP was set to zero. If the number of parasitoids that emerged was greater than to the estimated number of hosts suppressed, SP was set to one. For treatments with single parasitoid species, we assumed that each

of the two parasitoid individuals were attacking the hosts equally, therefore the number of parasitoid adults emerging was divided by two to calculate individual successful parasitism rate.

Data were analyzed with generalized linear models (GLMs). Model assumptions were verified with the *DHARMa* package [42]. To correct for overdispersion of the residuals and zero inflation, data were modeled using zero-inflation models with a beta binomial error distribution and a logit function using the *glmmTMB* function from the *TMB* package [43]. Two categories of predictor variables were used in separate models with temperature treatment (two levels: ambient and warming): (i) parasitoid treatment (three levels; single parasitoid, two parasitoids conspecific, and two parasitoids heterospecific), and (ii) parasitoid species assemblage (nine levels). For DI, two-way interactions between temperature and either parasitoid treatment or parasitoid assemblage were always kept in our models for better comparison with predicted DI values (see section below). For SP, these two-way interactions were tested and kept in our models if judged to be significant based on backward model selection using Likelihood-ratio tests. Significance of the effects was tested using Wald type III analysis of deviance with Likelihood-ratio tests. Factor levels were compared using Tukey's HSD *post hoc* comparisons of all means, and the *emmeans* package [44]. Results for developmental rate are presented in Supplement Material S3 (Figure S3).

Estimation of multiple parasitoid effects

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

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

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

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

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

Predictions from the population dynamic model were then compared with the observed values (DI_{obs}). Estimated DI values greater than observed DI translate to risk reduction while estimates that were lower than observed DI reflects 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 a quasibinomial GLM 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 event 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 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. 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 ($F_{1,93}$ = 9.89, P = 0.002), but parasitoid diversity did not ($F_{1,93}$ = 0.08, P = 0.772), 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 were no significant difference between predicted and observed DI at ambient temperature, implying neutral effects with multiple parasitoids (when looking at the intercept of the quasibinomial GLM with $DI_{0\ as}$ an offset; value ± SE: 0.18 ± 0.27, t value = 0.692, df = 942, P = 0.493), whereas under warming the predicted DI_0 significantly underestimated the observed DI_{ob}, implying risk enhancement for the host (value ± SE: 0.44 ± 0.20, t value = 2.139, df = 798, P = 0.038; Figure 2).

Effects of warming and parasitoid assemblages on the observed degree of infestation

Contrary to the effects of multiple parasitoids on host suppression, the observed degree of infestation DI_{obs} was not significantly affected by temperature ($\chi2_{(1)}$ = 1.17, P = 0.279), or parasitoid treatment (single, two conspecific or two heterospecific parasitoid assemblages: $\chi2_{(2)}$ = 4.34, P = 0.114) due to species-specific effects. DI only varied with parasitoid species assemblages ($\chi2_{(8)}$ = 258.92, P < 0.0001). DI was the highest in assemblages with *Ganaspis sp*., either alone, with a conspecific, or another parasitoid species (Figure S4). The interaction between temperature and parasitoid assemblages had no significant effect on DI_{obs} ($\chi2_{(1)}$ = 3.42, P = 0.166), despite some observed variation (Figure S4).

Effect of warming and parasitoid assemblages on parasitoid performance

Despite having no effect on DI, parasitoid treatment (single, two conspecific or two heterospecific parasitoid assemblages) significantly affected successful parasitism rate, and the effect varied among parasitoid species (two-way interaction: $\chi2_{(4)} = 16.88$, P = 0.002; Table 1). SP of *Ganaspis sp.* decreased by 95.7% (95% CI: 93.6 - 97.8%) with the presence of a parasitoid conspecific [*Post hoc* Odds Ratio (OR) = 0.043, P < 0.0001], and by 83.4% (CI: 75.4 - 91.3%) with the presence of a parasitoid heterospecific compared to when alone (OR = 0.166, P < 0.001). However, it increased by 287.6% (CI: 178.8 - 396.4%) when the parasitoid competitor was from another species compared to a conspecific (OR = 3.876, P < 0.0001). SP of *Asobara sp*. decreased by 55.2% (CI: 41.5 - 69.7%) when a parasitoid conspecific was present compared to when alone (OR = 0.448, P = 0.036), but was not significantly affected by the presence of a parasitoid heterospecific (OR = 0.712, P = 0.484). There were no significant effects of parasitoid treatments for SP of *Leptopilina sp*. Effects of parasitoid assemblages on SP also varied between parasitoid species and are presented in Supplementary Material S5 (Table S2 and Figure S5).

Effects of temperature on SP also depended on the species (two-way interaction: $\chi2_{(2)} = 7.31$, P = 0.026). 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). 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

294 increased frequency of super- and multiparasitism events by 173% (CI: 130-216%; OR = 2.73, P < 0.0001),
295 indicating an increase in intrinsic competition among parasitoids with warming.

296 The frequency of encapsulated parasitoids differed between parasitoid species, but not between
297 treatments (results presented in Supplement Material S6), indicating that host immune response did not
298 change depending on the treatments.

299 **Discussion**

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

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

305 Our results showed that warming led to a higher top-down control than expected with multiple predators.
306 Indeed, our mathematical model underestimated trophic interaction strength measured in multiple-
307 predators' treatments at elevated temperature. Our results are in concordance with previous studies on
308 diverse systems on the importance of considering non-trophic interactions to predict the effect of multiple
309 predators on top-down control under global changes. Drieu et al. [47] found that predator diversity enhanced
310 the biological control of insect pests in vineyards under warming due to functional complementarity among
311 predator species, while effects were substitutive at ambient temperature. Cuthbert et al. [11] also found an
312 effect of temperature on intraspecific multiple predator effects on an invasive Gammaridae species; but
313 effects contrasted ours: risk enhancement at low temperature and risk reduction with warming. Sentis et al.
314 [10] found a general trend of predation risk reduction for the prey with multiple predators in an aquatic food
315 web, but without any effect of temperature on those emergent MPEs. Our study goes further by showing
316 the important impact of warming on the effects of multiple predators on prey suppression across multiple
317 assemblages of conspecifics and heterospecifics. In addition to an increase in prey suppression with
318 multiple predators under warming in terrestrial ecosystems, a diverse predator community also increases

the chances of complementarity in face of environmental variation and disturbance [48]. Indeed, presence of multiple predator species could mitigate negative effects of warming on top-down control due to resource partitioning and/or functional redundancy [47,49,50]. Preserving predator biodiversity should therefore be generally beneficial for top-down control under climate change.

Mechanisms behind emergent multiple predator effects on the prey

Because of the synergistic effects of multiple parasitoids on host suppression under warming found in our study, we could have hypothesized that warming weaken interference between parasitoids, similarly to predator-prey systems [51]. However, our host-parasitoid system allowed us to investigate further the potential mechanisms behind our results, especially the strength of intrinsic competitive interactions between parasitoids (i.e., frequency of super- and multiparasitism event). We found generally higher intrinsic competition in multiple parasitoid treatments compared to single parasitoid treatments, and higher intrinsic competition under warming when the two species were present compared to ambient temperature. When super- or multi-parasitized, 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.

Here, the experiments were conducted in simplified laboratory conditions where parasitoids were forced 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 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 not affected by temperature, but by parasitoid assemblage

Despite multiple parasitoids enhancing host suppression under warming, 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 observed degree of infestation [55]. 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 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]. It is therefore important to look at 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 longer timeframe that include potential differences in phenology, increase in predator diversity should intensify prey suppression because of functional diversity rather than because of diversity *per se* [58–60]. Here, two predators of a same species rather than a single predator intensified prey suppression at warmer temperature despite the small scale of the experiment. Allowing for differentiation in habitat domain between predator species might have yielded higher prey suppression in treatments with heterospecifics, and lower rate of multiparasitism. Given the likely ubiquity of resource partitioning in nature [61], preserving predator biodiversity would be the best strategy to maintain top-down control.

No effects of treatments on observed degree of infestation

Prey suppression was generally higher when predator assemblages included the best-performing species, *Ganaspis sp.*, no matter the predator treatment, nor the temperature. A meta-analysis on the effects of

predator diversity on prey suppression found a similar trend across the 46 studies taken into account [62], but also found a general positive effect of multiple predators on top-down control. Contrastingly, a meta-analysis of 108 biological control projects found no relationship between the number of agents released and biological control success for insect pests [63]. However, increasing predator diversity should be generally beneficial for top-down control by increasing the chances to have a more effective natural enemy species in the community, as it was the case in our study (i.e., sampling effect model [64]). Moreover, presence of multiple species in the community could buffer any mismatch between predator and prey species induced by warming [65]. *Ganaspis sp*. was the best performing species for suppression of *D. simulans* across treatments, but its performance decreased with warming, suggesting that parasitism rate, and therefore host suppression, could also decreased in the longer-term due to a decrease in parasitoid population.

Conclusion

Overall, pairwise interaction strength generally failed to accurately estimate the trophic interaction strength observed, indicating that non-trophic interactions must be considered to predict the effects of multiple predators on prey suppression, and in food web studies in general [66]. Previous studies show altered MPEs with warming due to changes in resource partitioning [8,11], but our study is the first, to our knowledge, to show sign of direct effects of warming on predator interactions across predator assemblages, resulting in a higher top-down control with multiple predators at elevated temperature.

Acknowledgements

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

References

1. Song C, Von Ahn S, Rohr RP, Saavedra S. 2020 Towards a probabilistic understanding about the context-dependency of species interactions. *Trends Ecol. Evol.* **35**, 384–396. (doi:10.1016/j.tree.2019.12.011)
2. Davis AJ, Lawton JH, Shorrocks B, Jenkinson LS. 1998 Individualistic species responses invalidate simple physiological models of community dynamics under global environmental change. *J. Anim. Ecol.* **67**, 600–612. (doi:10.1046/j.1365-2656.1998.00223.x)
3. Rall BÖC, Vucic-Pestic O, Ehnes RB, Emmerson M, Brose U. 2010 Temperature, predator-prey interaction strength and population stability. *Glob. Chang. Biol.* **16**, 2145–2157. (doi:10.1111/j.1365-2486.2009.02124.x)
4. Parmesan C. 2006 Ecological and evolutionary responses to recent climate change. *Annu. Rev. Ecol. Evol. Syst.* **37**, 637–669. (doi:10.1146/annurev.ecolsys.37.091305.110100)
5. Thierry M, Hrček J, Lewis OT. 2019 Mechanisms structuring host–parasitoid networks in a global warming context: a review. *Ecol. Entomol.* , een.12750. (doi:10.1111/een.12750)
6. Hance T, Baaren J van, Vernon P, Boivin G. 2007 Impact of extreme temperatures on parasitoids in a climate change perspective. *Annu. Rev. Entomol.* **52**, 107–126. (doi:10.1146/annurev.ento.52.110405.091333)
7. Uszko W, Diehl S, Englund G, Amarasekare P. 2017 Effects of warming on predator–prey interactions – a resource-based approach and a theoretical synthesis. *Ecol. Lett.* **20**, 513–523. (doi:10.1111/ele.12755)
8. Barton BT, Schmitz OJ. 2009 Experimental warming transforms multiple predator effects in a grassland food web. *Ecol. Lett.* **12**, 1317–1325. (doi:10.1111/j.1461-0248.2009.01386.x)
9. Schmitz OJ, Barton BT. 2014 Climate change effects on behavioral and physiological ecology of predator-prey interactions: Implications for conservation biological control. *Biol. Control* **75**, 87–96. (doi:10.1016/j.biocontrol.2013.10.001)
10. Sentis A, Gémard C, Jaugeon B, Boukal DS. 2017 Predator diversity and environmental change

416 modify the strengths of trophic and nontrophic interactions. *Glob. Chang. Biol.* **23**, 2629–2640.
417 (doi:10.1111/gcb.13560)
418 11. Cuthbert RN, Wasserman RJ, Dalu T, Briski E. 2021 Warming mediates intraspecific multiple
419 predator effects from an invasive crustacean. *Mar. Biol.* **168**, 35. (doi:10.1007/s00227-021-03840-
420 z)
421 12. Schmitz OJ. 2007 Predator diversity and trophic interactions. *Ecology* **88**, 2415–2426.
422 (doi:10.1890/06-0937.1)
423 13. Sih A, Englund G, Wooster D. 1998 Emergent impacts of multiple predators on prey. *Trends Ecol.*
424 *Evol.* **13**, 350–355. (doi:10.1016/S0169-5347(98)01437-2)
425 14. Resetarits WJ, Bohenek JR, Pintar MR. 2021 Predator-specific responses and emergent multi-
426 predator effects on oviposition site choice in grey treefrogs, Hyla chrysoscelis. *Proc. R. Soc. B Biol.*
427 *Sci.* **288**. (doi:10.1098/rspb.2021.0558)
428 15. Schmitz OJ. 2009 Effects of predator functional diversity on grassland ecosystem function. *Ecology*
429 **90**, 2339–2345. (doi:10.1890/08-1919.1)
430 16. Laubmeier AN, Rebarber R, Tenhumberg B. 2020 Towards understanding factors influencing the
431 benefit of diversity in predator communities for prey suppression. *Ecosphere* **11**, e03271.
432 (doi:10.1002/ecs2.3271)
433 17. Soluk DA. 1993 Multiple predator effects: predicting combined functional response of stream fish
434 and invertebrate predators. *Ecology* **74**, 219–225. (doi:10.2307/1939516)
435 18. Tylianakis JM, Romo CM. 2010 Natural enemy diversity and biological control: making sense of
436 the context-dependency. *Basic Appl. Ecol.* **11**, 657–668. (doi:10.1016/j.baae.2010.08.005)
437 19. Thierry M, Pardikes NA, Lue CH, Lewis OT, Hrček J. 2021 Experimental warming influences
438 species abundances in a Drosophila host community through direct effects on species performance
439 rather than altered competition and parasitism. *PLoS One* **16**, e0245029.
440 (doi:10.1371/journal.pone.0245029)
441 20. Kéfi S, Berlow EL, Wieters EA, Joppa LN, Wood SA, Brose U, Navarrete SA. 2015 Network

structure beyond food webs: Mapping non-trophic and trophic interactions on Chilean rocky shores. *Ecology* **96**, 291–303. (doi:10.1890/13-1424.1)

21. Wootton JT. 1997 Estimates and tests of per capita interaction strength: Diet, abundance, and impact of intertidally foraging birds. *Ecol. Monogr.* **67**, 45–64. (doi:10.1890/0012-9615(1997)067[0045:EATOPC]2.0.CO;2)

22. Thierry M, Pardikes NA, Ximénez-Embún MG, Proudhom G, Hrček J. 2021 Multiple parasitoid species enhance top-down control, but parasitoid performance is context-dependent. *bioRxiv* , 2021.07.16.452484. (doi:10.1101/2021.07.16.452484)

23. Godfray HCJ. 2013 Parasitoids. In *Encyclopedia of Biodiversity: Second Edition*, pp. 674–682. (doi:10.1016/B978-0-12-384719-5.00152-0)

24. Carton Y, Poirié M, Nappi AJ. 2008 Insect immune resistance to parasitoids. *Insect Sci.* **15**, 67–87. (doi:10.1111/j.1744-7917.2008.00188.x)

25. Harvey JA, Poelman EH, Tanaka T. 2013 Intrinsic inter- and intraspecific competition in parasitoid wasps. *Annu. Rev. Entomol.* **58**, 333–351. (doi:10.1146/annurev-ento-120811-153622)

26. Mccoy MW, Stier AC, Osenberg CW. 2012 Emergent effects of multiple predators on prey survival: The importance of depletion and the functional response. *Ecol. Lett.* **15**, 1449–1456. (doi:10.1111/ele.12005)

27. Sentis A, Boukal DS. 2018 On the use of functional responses to quantify emergent multiple predator effects. *Sci. Rep.* **8**, 1–12. (doi:10.1038/s41598-018-30244-9)

28. Jeffs CT *et al.* 2021 Molecular analyses reveal consistent food web structure with elevation in rainforest *Drosophila* - parasitoid communities. *Ecography (Cop.).* **44**, 403–413. (doi:10.1111/ecog.05390)

29. Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC, Martin PR. 2008 Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 6668–6672. (doi:10.1073/pnas.0709472105)

30. Kellermann V, Overgaard J, Hoffmann AA, Fløjgaard C, Svenning J-C, Loeschcke V. 2012 Upper

thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci.* **109**, 16228–16233. (doi:10.1073/pnas.1207553109)

31. Overgaard J, Kearney MR, Hoffmann AA. 2014 Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. *Glob. Chang. Biol.* **20**, 1738–1750. (doi:10.1111/gcb.12521)

32. Lue C-H *et al.* 2021 DROP: Molecular voucher database for identification of *Drosophila* parasitoids. *Mol. Ecol. Resour.* (doi:10.1111/1755-0998.13435)

33. Nouhaud P, Mallard F, Poupardin R, Barghi N, Schlötterer C. 2018 High-throughput fecundity measurements in *Drosophila*. *Sci. Rep.* **8**, 4469. (doi:10.1038/s41598-018-22777-w)

34. Pachauri RK, Meyer L, Hallegatte France S, Bank W, Hegerl G, Brinkman S, van Kesteren L, Leprince-Ringuet N, van Boxmeer F. 2014 *IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change e [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]*. IPCC, Geneva, Switzerland. See http://www.ipcc.ch.

35. MacLean HJ, Sørensen JG, Kristensen TN, Loeschcke V, Beedholm K, Kellermann V, Overgaard J. 2019 Evolution and plasticity of thermal performance: An analysis of variation in thermal tolerance and fitness in 22 *Drosophila* species. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20180548. (doi:10.1098/rstb.2018.0548)

36. Rosenbaum B, Rall BC. 2018 Fitting functional responses: Direct parameter estimation by simulating differential equations. *Methods Ecol. Evol.* **9**, 2076–2090. (doi:10.1111/2041-210X.13039)

37. Sohlström EH, Archer LC, Gallo B, Jochum M, Kordas RL, Rall BC, Rosenbaum B, O'Gorman EJ. 2021 Thermal acclimation increases the stability of a predator-prey interaction in warmer environments. *Glob. Chang. Biol.* , gcb.15715. (doi:10.1111/gcb.15715)

38. Holling CS. 1959 Some characteristics of simple types of predation and parasitism. *Can. Entomol.* **91**, 385–398.

494 39. Fernández-Arhex V, Corley JC. 2003 The functional response of parasitoids and its implications for
495 biological control. *Biocontrol Sci. Technol.* **13**, 403–413. (doi:10.1080/0958315031000104523)
496 40. Carton Y, Kitano H. 1981 Evolutionary relationships to parasitism by seven species of the
497 *Drosophila melanogaster* subgroup. *Biol. J. Linn. Soc.* **16**, 227–241. (doi:10.1111/j.1095-
498 8312.1981.tb01849.x)
499 41. Boulétreau M, Wajnberg E. 1986 Comparative responses of two sympatric parasitoid cynipids to
500 the genetic and epigenetic variations of the larvae of their host, *Drosophila melanogaster*. *Entomol.*
501 *Exp. Appl.* **41**, 107–114. (doi:10.1111/j.1570-7458.1986.tb00516.x)
502 42. Hartig F. 2019 DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression
503 Models.
504 43. Lüdecke D, Makowski D, Waggoner P. 2019 *Performance: Assessment of regression models*
505 *performance*. See https://cran.r-project.org/package=performance.
506 44. Lenth R V. 2018 Emmeans: Estimated marginal means, aka least-squares means. *R Packag. version*
507 45. Soetaert K, Petzoldt T. 2010 Inverse modelling, sensitivity and monte carlo analysis in R using
508 package FME. *J. Stat. Softw.* **33**, 1–28. (doi:10.18637/jss.v033.i03)
509 46. Team RC. 2017 R: The R Project for statistical computing.
510 47. Drieu R, Rusch A. 2017 Conserving species-rich predator assemblages strengthens natural pest
511 control in a climate warming context. *Agric. For. Entomol.* **19**, 52–59. (doi:10.1111/afe.12180)
512 48. Macfadyen S, Craze PG, Polaszek A, van Achterberg K, Memmott J. 2011 Parasitoid diversity
513 reduces the variability in pest control services across time on farms. *Proc. R. Soc. B Biol. Sci.* **278**,
514 3387–3394. (doi:10.1098/rspb.2010.2673)
515 49. Cebolla R, Urbaneja A, Baaren J van, Tena A. 2018 Negative effect of global warming on biological
516 control is mitigated by direct competition between sympatric parasitoids. *Biol. Control* **122**, 60–66.
517 (doi:10.1016/j.biocontrol.2018.04.006)
518 50. Pepi A, McMunn M. 2021 Predator diversity and thermal niche complementarity attenuate indirect
519 effects of warming on prey survival. *Am. Nat.* (doi:10.1086/714590)

51. Lang B, Rall BC, Brose U. 2012 Warming effects on consumption and intraspecific interference competition depend on predator metabolism. *J. Anim. Ecol.* **81**, 516–523. (doi:10.1111/j.1365-2656.2011.01931.x)

52. Ives AR, Cardinale BJ, Snyder WE. 2005 A synthesis of subdisciplines: Predator-prey interactions, and biodiversity and ecosystem functioning. *Ecol. Lett.* **8**, 102–116. (doi:10.1111/j.1461-0248.2004.00698.x)

53. Abarca M, Spahn R. 2021 Direct and indirect effects of altered temperature regimes and phenological mismatches on insect populations. *Curr. Opin. Insect Sci.* (doi:10.1016/j.cois.2021.04.008)

54. Renner SS, Zohner CM. 2018 Climate change and phenological mismatch in trophic interactions among plants, insects, and vertebrates. *Annu. Rev. Ecol. Evol. Syst.* **49**, 165–182. (doi:10.1146/annurev-ecolsys-110617-062535)

55. Delava E, Fleury F, Gibert P. 2016 Effects of daily fluctuating temperatures on the *Drosophila–Leptopilina boulardi* parasitoid association. *J. Therm. Biol.* **60**, 95–102. (doi:10.1016/j.jtherbio.2016.06.012)

56. Lampropoulos PD, Perdikis DC, Fantinou AA. 2013 Are multiple predator effects directed by prey availability? *Basic Appl. Ecol.* **14**, 605–613. (doi:10.1016/j.baae.2013.08.004)

57. Griffin JN, Toscano BJ, Griffen BD, Silliman BR. 2015 Does relative abundance modify multiple predator effects? *Basic Appl. Ecol.* **16**, 641–651. (doi:10.1016/j.baae.2015.05.003)

58. Finke DL, Snyder WE. 2008 Niche partitioning increases resource exploitation by diverse communities. *Science (80-.).* **321**, 1488–1490. (doi:10.1126/science.1160854)

59. Krey KL *et al.* 2021 Prey and predator biodiversity mediate aphid consumption by generalists. *Biol. Control* **160**, 104650. (doi:10.1016/j.biocontrol.2021.104650)

60. Greenop A, Woodcock BA, Wilby A, Cook SM, Pywell RF. 2018 Functional diversity positively affects prey suppression by invertebrate predators: a meta-analysis. *Ecology* **99**, 1771–1782. (doi:10.1002/ecy.2378)

546 61. Chesson P. 1991 A need for niches? *Trends Ecol. Evol.* **6**, 26–28. (doi:10.1016/0169-
547 5347(91)90144-M)
548 62. Griffin JN, Byrnes JEK, Cardinale BJ. 2013 Effects of predator richness on prey suppression: A
549 meta-analysis. *Ecology* **94**, 2180–2187. (doi:10.1890/13-0179.1)
550 63. Denoth M, Frid L, Myers JH. 2002 Multiple agents in biological control: Improving the odds? *Biol.*
551 *Control* **24**, 20–30. (doi:10.1016/S1049-9644(02)00002-6)
552 64. Myers JH, Higgins C, Kovacs E. 1989 How many insect species are necessary for the biological
553 control of insects? *Environ. Entomol.* **18**, 541–547. (doi:10.1093/ee/18.4.541)
554 65. Pardikes N, Revilla T, Lue C-H, Thierry Mélanie, Soutos-Villaros D, Hrcek J. 2021 Community
555 context modifies response of host-parasitoid interactions to phenological mismatch under warming.
556 *Authorea* (doi:10.22541/au.162454818.82806593/v1)
557 66. Kéfi S *et al.* 2012 More than a meal… integrating non-feeding interactions into food webs. *Ecol.*
558 *Lett.* **15**, 291–300. (doi:10.1111/j.1461-0248.2011.01732.x)
559

560 **Table 1.** Odds ratios of a successful parasitism event between parasitoid treatments (single parasitoid, two
561 parasitoids conspecific, and two parasitoids heterospecific) for each parasitoid species. Results are averaged
562 over both temperatures because there was no significant interaction between temperature and parasitoid
563 treatments. Values less than or greater than one denote a decrease or an increase in the odds of successful
564 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

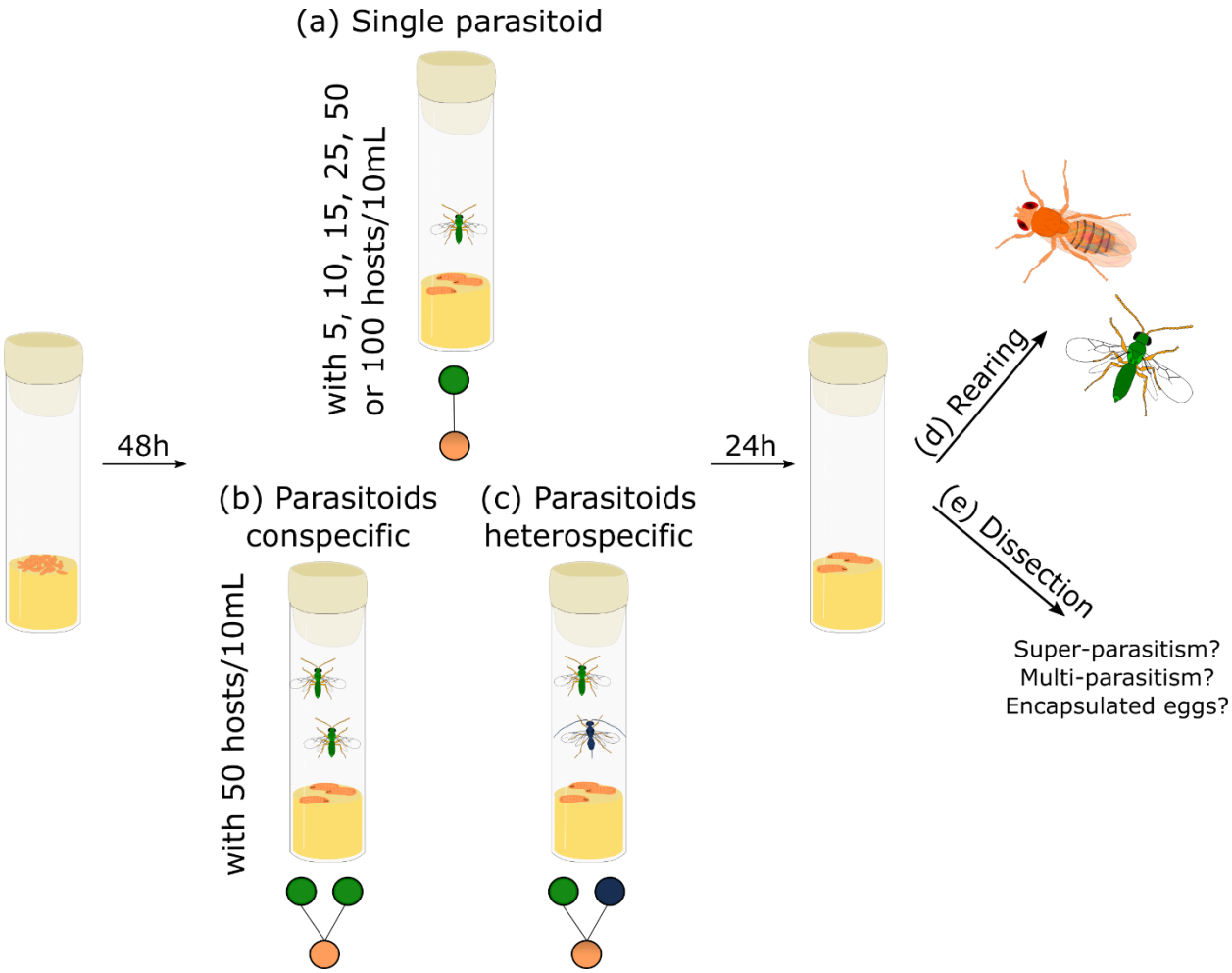
565

Figure legends

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

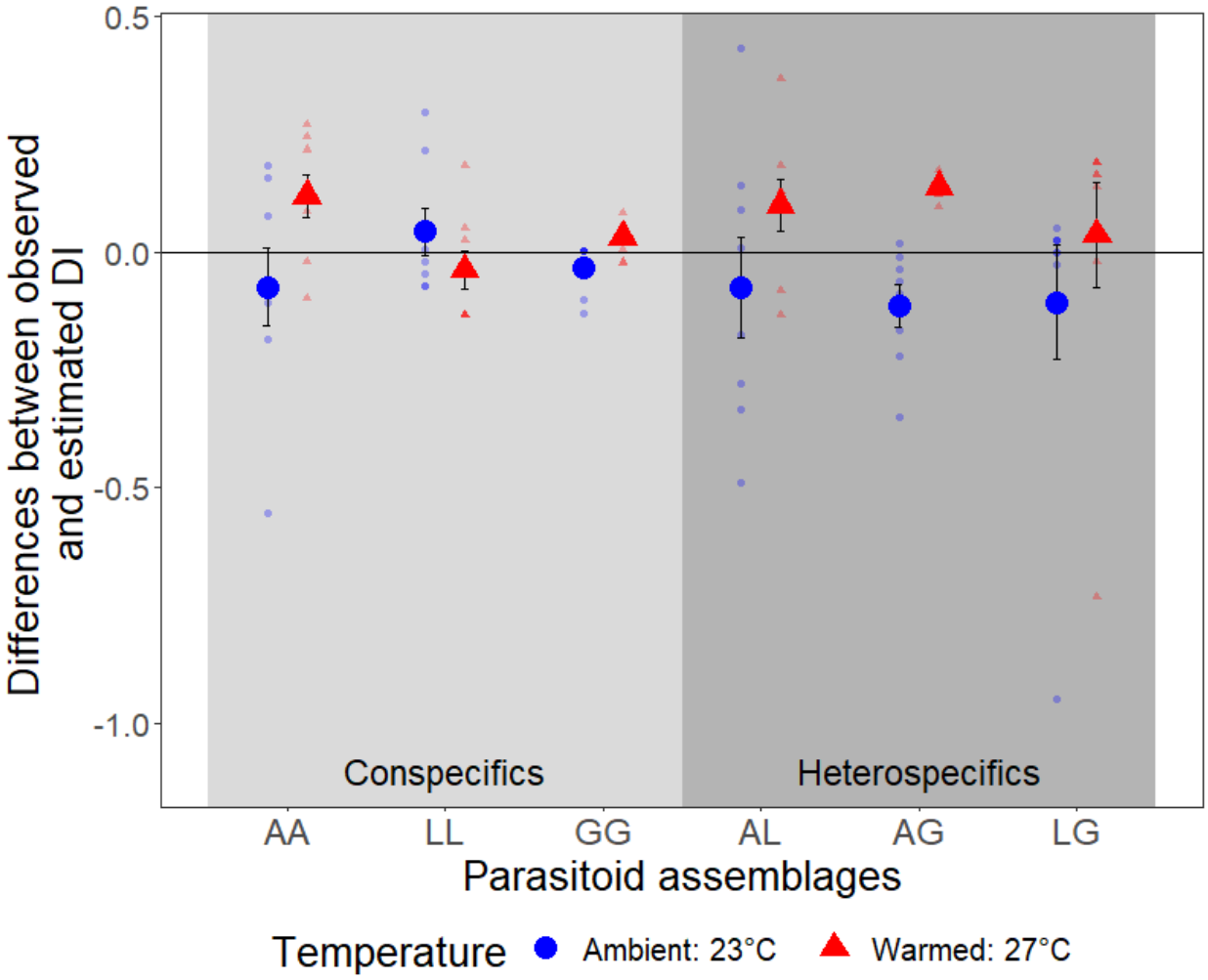
Figure 2. Differences between observed and estimated degree of infestation (DI) for each parasitoid assemblage and temperature. Negative values translate to risk reduction while positive values reflect risk enhancement for the host with multiple parasitoids. Light grey panel: two parasitoids conspecific, 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 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.



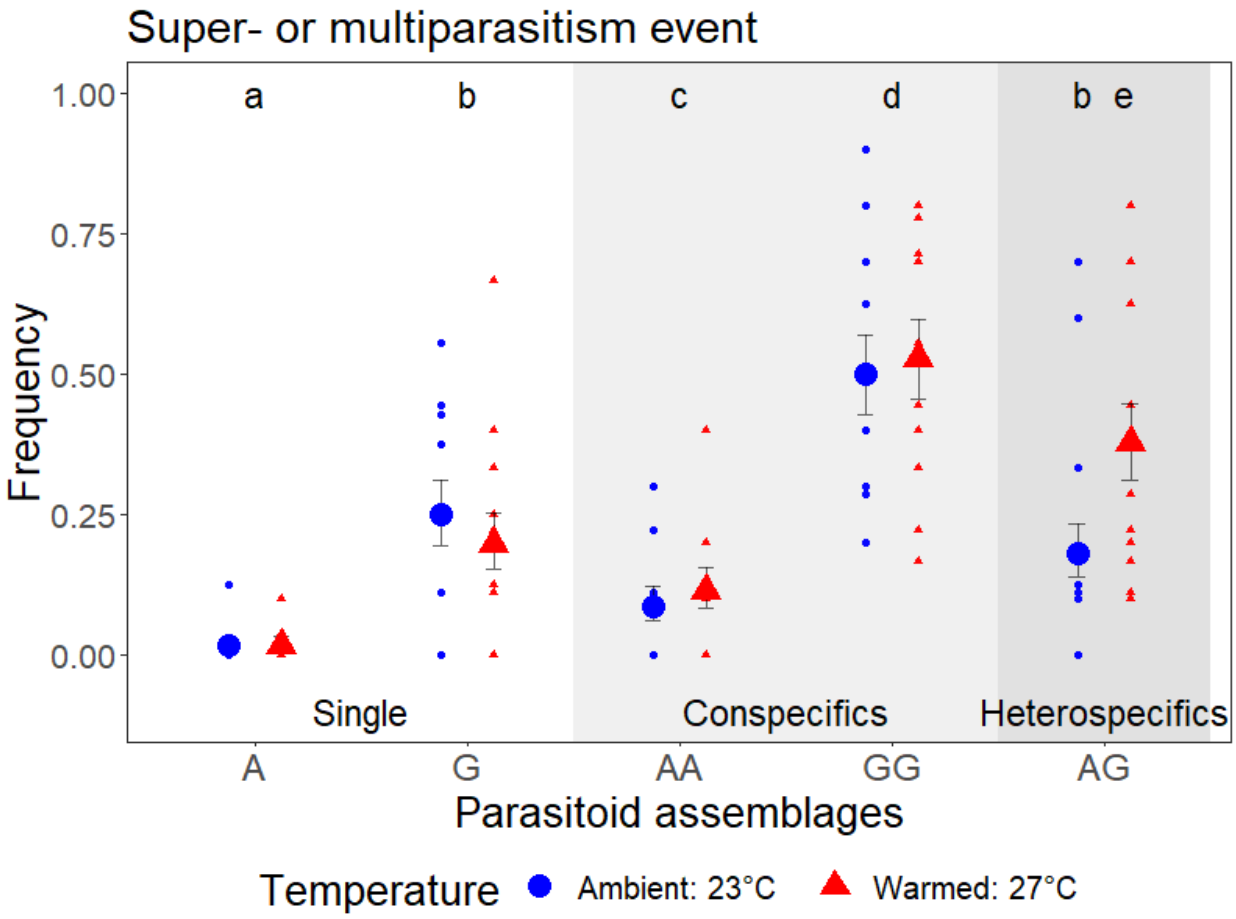
587

588 **Figure 1.**



589

590 **Figure 2**.



591

592 **Figure 3.**