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# Warming decreases host survival with multiple parasitoids, but parasitoid performance also decreases

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**Running title:** Warming alters multiple predator effects

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**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 the effects of multiple parasitoids on host suppression were the average of the effects of individual parasitoid at ambient temperature, host suppression with multiple parasitoids was higher than expected under warming. Multiple parasitoid species had equivalent effect to multiple individuals of a same species. 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 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

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## Introduction

Ongoing global changes are eroding global biodiversity (Wagner, Grames, Forister, Berenbaum, & Stopak, 2021). Predators are generally more at risk of extinction and loss of biomass than their prey because they are impacted by these environmental changes both directly and indirectly through the effects on lower trophic levels (Voigt et al., 2003). The loss of predatory biodiversity could result in a decline in top-down control, threatening biological control and further eroding biodiversity through cascading effects on lower trophic levels (Kehoe, Frago, & Sanders, 2020). Yet, little is known about how warming alters the effects of multiple predators on top-down control.

The abiotic context is key for the outcome of species interactions (Davis, Lawton, Shorrocks, & Jenkinson, 1998; Song, Von Ahn, Rohr, & Saavedra, 2020). Global warming can weaken the strength of trophic interactions due to changes in metabolic rates (Rall, Vucic-Pestic, Ehnes, Emmerson, & Brose, 2010), shifts in spatial distributions and in seasonal phenology (Parmesan, 2006), lethal effects on predators, or altered attack rates (Hance, Baaren, Vernon, & Boivin, 2007; Thierry, Hrček, & Lewis, 2019; Uszko, Diehl, Englund, & Amarasekare, 2017). But warming does also alter the strength of non-trophic interactions among predators. For instance, warming can induce changes in predator habitat use through phenotypic plasticity to adjust to the changing environment (Barton & Schmitz, 2009; Schmitz & Barton, 2014), which could lead to habitat overlap among predator species that were not previously interacting. Warming could also change predator foraging behavior, changing predator efficiency due to changes in encounter and attack rates (Uszko et al., 2017). Altered non-trophic interactions among predators would change the effects of multiple predators on top-down control (Cuthbert, Wasserman, Dalu, & Briski, 2021; Sentis, Gémard, Jaugeon, & Boukal, 2017), 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.

The effects of multiple predators on prey suppression are often assumed to be additive, which would be true if predators have independent effects on prey. Predator density should therefore enhance top-down

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control because of a higher predatory pressure on the prey. However, direct and indirect interactions among predators, or change in the prey demographic habitat selection with the presence of multiple predators, may cause effects to deviate from additivity (Resetarits, Bohenek, & Pintar, 2021; Schmitz, 2007; Sih, Englund, & Wooster, 1998). 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). Niche complementarity can be achieved when predators have complementary phenologies or habitat domains (Schmitz, 2009). In contrary, 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) (Laubmeier, Rebarber, & Tenhumberg, 2020). All such potential effects are referred to as multiple predator effects (MPEs; Soluk 1993). A meta-analysis from Griffin et al. (2013) revealed an overall positive effect of predator diversity on top-down control, suggesting a general pattern of niche complementarity (Northfield, Snyder, Ives, & Snyder, 2010). Contrastingly, a meta-analysis focused on biological control studies (59 projects against weeds and 108 projects against insect pests) revealed that, in more than half of the studies considered, the success of biological control efforts was due to a single species (Denoth, Frid, & Myers, 2002). This observation can be explained by the high specificity of the natural enemies used as biocontrol agents, resulting in strong competitive interactions among them. Efficiency of a single species versus diverse predator assemblages for biological control depends on potential for niche differentiation between predators, and the overall efficiency of each predator on the focal prey species (Pedersen & Mills, 2004). Functionally diverse predator communities, in term of habitat domain, diet breadth, and hunting strategy, are essential to preserve to maximize pest control ecosystem services, and should increase pest suppression above that of the most effective predator species (Greenop, Woodcock, Wilby, Cook, & Pywell, 2018). 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 predator instead of planned risk enhancement (Tylianakis & Romo, 2010).

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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 (Parmesan, 2006; Thierry, Pardikes, Lue, Lewis, & Hrček, 2021), which would shift the outcome of pairwise interactions that are influenced by other species in the community (Kéfi et al., 2015; Thierry, Pardikes, Ximénez-Embún, Proudhom, & Hrček, 2021; Wootton, 1997). Changes in species composition of communities are thus also likely to alter MPEs, affecting biological control. However, interactive effects between warming and community composition on top-down control remain poorly studied.

Here, we used mathematical models in combination with a series of three laboratory experiments on *Drosophila simulans* and three of its co-occurring larval parasitoids to investigate the effects of warming on multiple predator effects for top-down control. Host-parasitoid interactions are a particular type of predator-prey interaction in which parasitoid larvae feed and develop inside or on an arthropod host, while adults are free living (Godfray, 2013). When parasitized, three outcomes are possible: the parasitoid successfully develops, the host successfully eliminates its parasitoid through immune response (i.e., encapsulation and melanization) and survives (Yves Carton, Poirié, & Nappi, 2008), or both parties die. When multiple parasitoids are present, they can compete extrinsically as adults for space and oviposition (i.e., interference), and intrinsically within a host (reviewed in Harvey et al. 2013). Intrinsic competition is the result of a super- and/or multiparasitism event when two parasitoids - conspecifics or heterospecifics respectively - parasitize the same host individual. In solitary parasitoids, such as the species used in the present study, only one individual completes its development in each host, suppressing the other(s) physically or physiologically. Still, both parasitoid species can be observed as eggs or larvae inside the host by dissecting the host larva. Parasitoids thus represent an excellent system to study how warming directly changes the effects of multiple predators on top-down control because the outcome of the interactions is directly observed by rearing the host, and intrinsic competitive interactions between parasitoids can be observed by dissecting the host larva. In this study, we empirically measured trophic interaction strength

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across temperatures and parasitoid assemblages. We deducted emergent effects of multiple parasitoids on host suppression by comparing empirical data with estimates in which multiple parasitoids would not interact (i.e., would have additive effect) using a mathematical model for multiple co-occurring parasitoids with a functional response approach (Mccoy, Stier, & Osenberg, 2012; Sentis & Boukal, 2018). With this framework, we addressed three specific questions: (1) Do multiple parasitoids 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 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; Jeffs et al., 2021) were used for the experiments. *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 on a 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 Lue et al. 2021). *Drosophila simulans* isofemale lines were kept on standard *Drosophila* medium (corn flour, yeast, sugar, agar and methyl-4-

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

*Experiments*

To investigate the effects of warming on the strength of trophic and non-trophic interactions, we used a functional response approach following the framework of Mccoy et al. (2012). We first obtained the parameters of each parasitoid functional response at ambient and warmed temperatures with single-parasitoid treatments (Experiment 1). Then, we used these functional response parameter estimates to predict trophic interaction strength for each temperature and parasitoid combination with the null hypothesis that parasitoids were not interacting, and thus had additive effects on host suppression. In Experiment 2 we empirically measured the effects of temperature and parasitoid combinations on trophic interaction strength and compared the predicted and observed values to identify emergent effects of multiple parasitoids on host suppression and their dependence on the temperature regime. The two first blocks of Experiment 1 and entire Experiment 2 were performed in parallel, and controls and single-parasitoid treatments were common to both experiments. In Experiment 3, we investigated the mechanisms of multiple parasitoid effects (intrinsic competition, rate of encapsulation) by dissecting hosts rather than rearing them. This allowed us to measure rates of super- and multiparasitism and encapsulation depending on the temperature regime and parasitoid combinations.

A total of 22,920 *D. simulans* eggs were collected: 13,120 for experiment 1, 4,800 for experiment 2 [of which 12,990 (73%) successfully emerged as adults (8,409 hosts and 4,581 parasitoids)], and 5,000 for experiment 3 from which 1,000 larvae were dissected.

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*Experiment 1: Single-parasitoid experiment*

Eggs of *D. simulans* were placed in a single glass vial with 10mL of *Drosophila* media at six different densities (5, 10, 15, 25, 50 or 100 eggs per 10mL of food media in vial; Figure 1a). To collect *D. simulans* eggs, an egg-washing protocol was adapted from Nouhaud et al. (2018). The day before the egg-washing protocol was conducted, two egg-laying mediums (petri dishes with agar gel 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), and the other half at warmed temperature (27.4°C ± 0.5 - projected change in global mean surface temperature for the late 21$^{st}$ century is 3.7˚C for the IPCC RCP8.5 baseline scenario; Pachauri et al., 2014).

After 48 hours, one single naïve mated three to five-days-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 emerges until the last emergence (up to 41 days for the species with the longest developmental time). We waited five consecutive days without any emerges to stop collecting, thus avoiding collecting the second generation. All emerges 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 used 50 *D. simulans* eggs per 10 mL of food media in vial under ambient

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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 warmed temperature, six replicates were dissected two days after infection (early dissection time) and four three days after infection (late dissection time), and at 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.

*Data analysis and modelling*

*Experiment 1: Single-parasitoid experiment*

We combined numerical simulations of host density dynamics, accounting for host depletion (Rosenbaum & Rall, 2018):

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

with Bayesian parameter estimation using the *rstan* package (e.g. Sohlström et al. 2021). $P = 1$ is the parasitoid density, and $F(H)$ denotes the host density-dependent functional response. In the model fitting, MCMC was used to sample from the functional response's model parameters' posterior probability distribution $p(\theta|H_{att})$ given the observations $H_{att}$, based on the likelihood function $p(H_{att}|\theta)$ and prior distributions $p(\theta)$. $H_{att}$ is the number of *D. simulans* attacked (the difference between adult hosts emerging

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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 attacked hosts $H_{att}$ with $n = H_0$ trials and $p = \frac{H_0 - \widehat{H}_1}{H_0}$ 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 (Holling, 1959) 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 (Fernández-Arhex & Corley, 2003). 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 (Boulétreau & Wajnberg, 1986; Y. Carton & Kitano, 1981). $DI_{obs}$ was set to zero if the number of hosts emerging from the treatment was greater than the controls. If no parasitoid emerged or

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if the number of hosts attacked 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 attacked, *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 (Hartig, 2019). 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 (Lüdecke, Makowski, & Waggoner, 2019). 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 (Lenth, 2018). 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. (2012) 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.

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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 (Sentis & Boukal, 2018). 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 (Soetaert & Petzoldt, 2010). 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.

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*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). To correct for overdispersion of the residuals, data were modeled using zero-inflation models with a beta binomial error distribution and a logit function with the *glmmTMB* function. The two-way interaction between temperature and parasitoid assemblage was tested and kept in our models if judged to be significant based on backward model selection using Likelihood-ratio tests. We included dissection time (two levels: early and late) as a random factor in our models to account for potential effects of sampling design on our results. 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. All analyses were performed using R 4.0.2 (Team, 2017).

## 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 not parasitoid diversity ($F_{1,93}$ = 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).

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

Contrary to the emergent 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). Infestation rates were 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 between 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 = 0.0007). 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).

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*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. *Asobara sp*. almost never laid more than one egg per host when alone (only two occurrences with more than one egg out of the 200 larvae dissected for this treatment). In the parasitoids heterospecific treatments, warming significantly increased frequency of super- and multiparasitism events by 173% (CI: 130-216%; OR = 2.73, $P < 0.0001$). However, we were not able to identify the winner of the competitive interaction as several parasitoid larvae were often still alive at the time of the dissection.

52.4% of the parasitized larvae (n = 868) had evidence of melanization (traces, melanized egg, and/or melanized larvae), signaling a host immune response. The frequency of encapsulated parasitoids was significantly affected by parasitoid assemblages ($\chi2_{(4)} = 23.89$, $P < 0.0001$), and the interaction between temperature and parasitoid assemblages ($\chi2_{(4)} = 11.42$, $P = 0.0223$), but only because of the difference between *Asobara sp*. and *Ganaspis sp*., not because of parasitoid treatments (single, conspecifics, and heterospecifics; Figure S6). *Asobara sp*. escaped encapsulation due to its fast development time (Figure S3). Indeed, when larvae were parasitized by *Asobara sp*., most traces of melanization were observed on the empty eggshell, with the parasitoid larva still alive. Moreover, observations through dissections did not inform us on the outcome of the interactions. Indeed, when larvae were parasitized by *Ganaspis sp*., some eggs were only partially encapsulated, and parasitoid larvae were still able to hatch from these.

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## Discussion

The key result from our study is the synergistic effects of multiple predators for top-down control at elevated temperature across predator assemblages. Preventing further predator population decline is therefore crucial for top-down control of arthropods. However, parasitoid performance often decreased when multiple parasitoids were present due to a strong intrinsic competition among parasitoids, potentially limiting the long-term benefits for biological control.

*Warming increases the effects of multiple predators on 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 temperature. Our results are in concordance with previous studies on diverse systems on the importance of considering non-trophic interactions to predict the effect of multiple predators on top-down control under global changes. Drieu and Rusch (2017) found that predator diversity enhanced the biological control of insect pests in vineyard under warming due to functional complementarity among predator species, while effects were substitutive at ambient temperature. Cuthbert et al. (2021) also found an effect of temperature on intraspecific multiple predator effects on an invasive Gammaridae species; but effects contrasted ours: risk enhancement at low temperature and risk reduction with warming. Sentis et al., (2017) found a general trend of predation risk reduction for the prey with multiple predators in an aquatic food web, but without any effect of temperature on those emergent MPEs. Our study goes further by showing the important impact of warming on the effects of multiple predators on prey suppression across multiple assemblages of conspecifics and heterospecifics. In addition to an increase in prey suppression with multiple predators under warming in terrestrial ecosystems, a diverse predator community also increases the chances of complementarity in face of environmental variation and disturbance (Macfadyen, Craze, Polaszek, van Achterberg, & Memmott, 2011). Indeed, presence of multiple predator species could mitigate negative effects of warming on top-down control due to resource partitioning and/or functional redundancy (Cebolla, Urbaneja, Baaren, & Tena, 2018; Drieu & Rusch, 2017;

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Pepi & McMunn, 2021). Preserving predator biodiversity should therefore be generally beneficial for top-down control under climate change.

Here, emergent MPEs were explored with constant initial prey density. But the unimodal relationship between prey density and trophic interaction strength (i.e., type II functional response) suggests that varying prey densities might lead to different outcomes for prey suppression with multiple predators (Cuthbert et al., 2021; Sentis et al., 2017). With prey densities too low or too high, one would fail to detect non-trophic interactions among predators, while intermediate prey density would result in high resource limitation, and thus antagonistic interactions among predators. The synergistic effects of multiple predators found in our study under warming suggested that our experimental design with a single prey density was adequate to detect emergent MPEs. Because observed degree of infestation did not significantly vary with temperature, studying the effects of warming on top-down control without investigating MPEs would have failed to uncover any effects.

*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. Indeed, in predator-prey systems, predators have a higher search rate at warmer temperature, with thus less time for interference (Lang, Rall, & Brose, 2012). 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, maybe 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.

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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 (Ives, Cardinale, & Snyder, 2005). 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 (Barton & Schmitz, 2009; Schmitz & Barton, 2014), and phenology (Abarca & Spahn, 2021; Renner & Zohner, 2018), 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 (Delava, Fleury, & Gibert, 2016). 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 (Finke & Snyder, 2008; Griffin, Toscano, Griffen, & Silliman, 2015; Lampropoulos, Perdikis, & Fantinou, 2013). 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; Ives et al., 2005), which might bias the results. When niche differentiation is allowed, for example with habitat heterogeneity or longer timeframe that include

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potential differences in phenology, increase in predator diversity should intensify prey suppression because of functional diversity rather than because of diversity *per se* (Finke & Snyder, 2008; Greenop et al., 2018; Krey et al., 2021). 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 (Chesson, 1991), 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 (Griffin et al., 2013), 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 (Denoth et al., 2002). However, increasing predator diversity should be generally beneficial for top-down control by increasing the chances to have a performant natural enemy species in the mix, as it was the case in our study (i.e., sampling effect model; Myers et al. 1989). Moreover, presence of multiple species in the community could buffer any mismatch between predator and prey species induced by warming (Pardikes et al., 2021). *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

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predators on prey suppression, and in food web studies in general (Kéfi et al., 2012). Previous studies show altered MPEs with warming due to changes in resource partitioning (Barton & Schmitz, 2009; Cuthbert et al., 2021), 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.

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## Author contributions

MT conceived the project; NP and JH contributed to the experimental design; MT, NP, and MG collected the data; BR provided the methodology and R scripts to estimate functional responses. MT analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

## Competing interests

The author(s) declare no competing interests.

## Data availability

All data used for this study are available from the Zenodo database: 10.5281/zenodo.5237204.

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

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## 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 3$^{rd}$ 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. 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.

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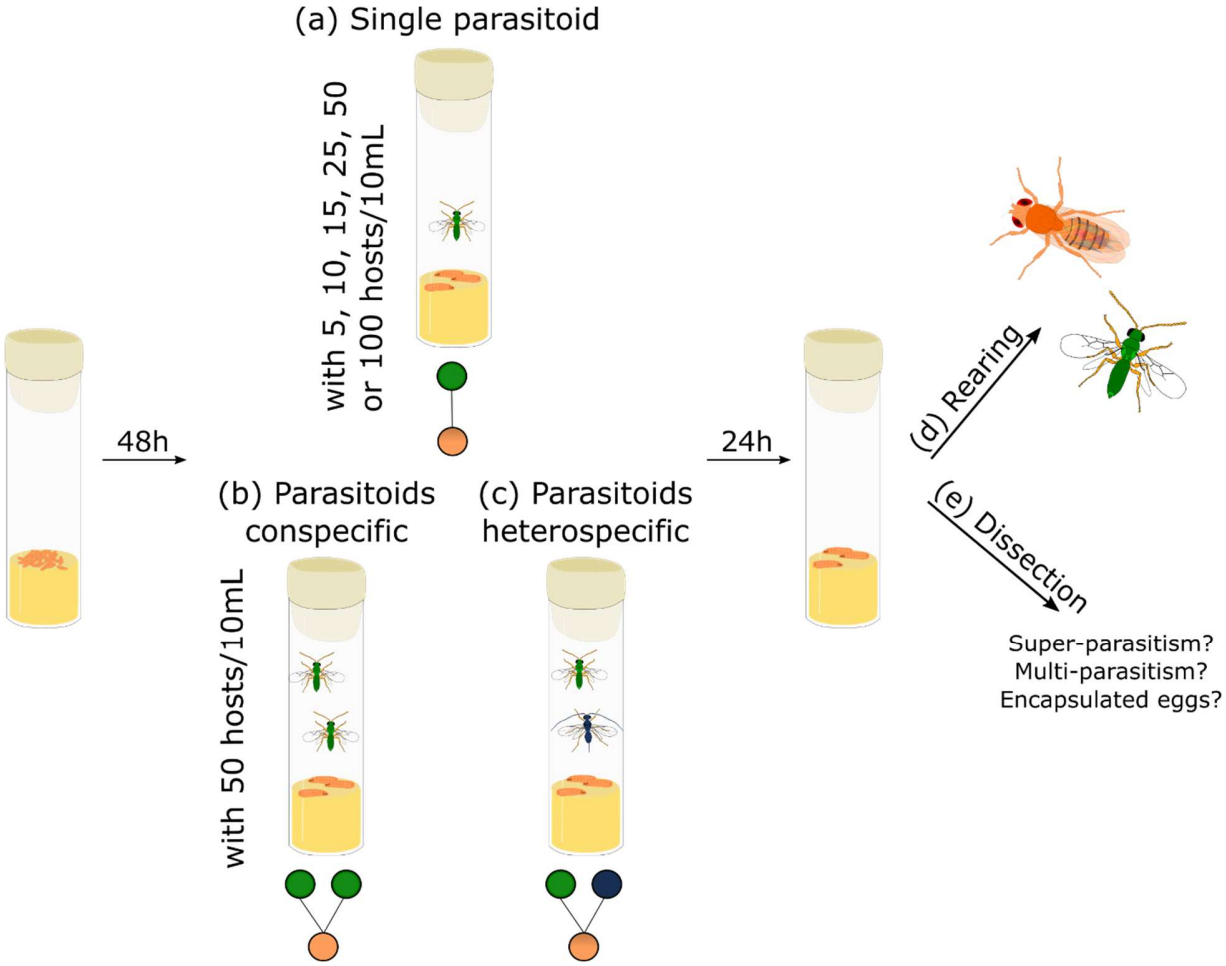


**Figure 1.**

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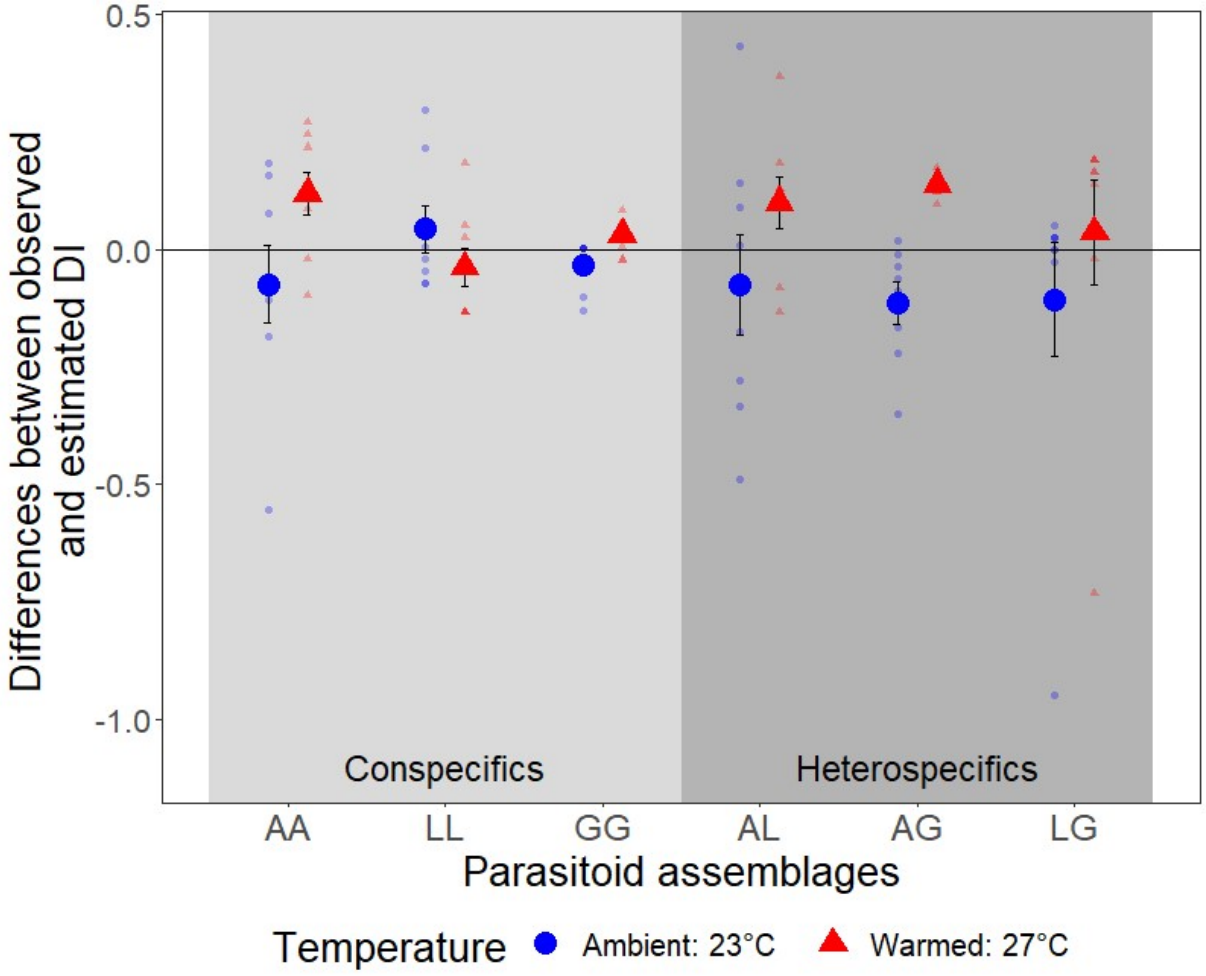


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681 **Figure 2**.

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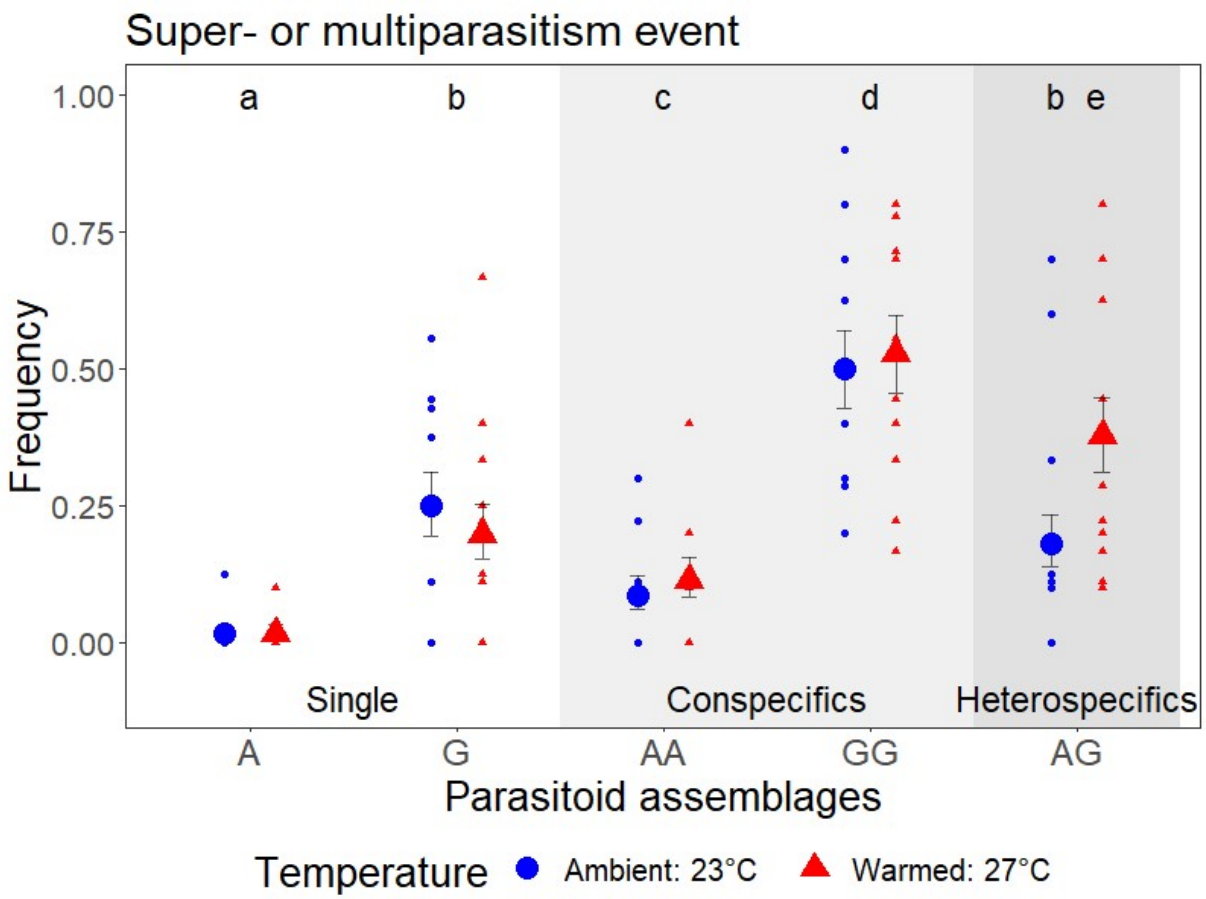


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683 **Figure 3.**