Synergistic effects of elevated temperature with pesticides on reproduction, development and survival of dung beetles

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Abstract

In times of global change, high temperatures can potentiate the negative effects of pesticides and other stressors. Here, we evaluated under controlled laboratory conditions the effect of a moderate increase in temperature in combination with ivermectin (an antiparasitic drug used in cattle that is excreted in dung), an herbicide, and parasitic pressure, on the reproductive success, development time and adult survival of dung beetles *Euoniticellus intermedius*, which are naturally exposed to these stressors. We found that heat increased the number and proportion of emerged offspring, but in combination with ivermectin, herbicide and parasitic treatments, it had synergistic negative effects. Moreover, heat in combination with ivermectin and with parasitism caused a synergistic increase of adult offspring mortality and, in combination with the herbicide, heat synergistically accelerated development. Our results indicate that heat can enhance the negative effect of other stressors and act synergistically with them, harming dung beetles, a group with important ecological and economic value in natural and productive ecosystems. Although adult sex ratio was not affected by experimental treatments, contrasting responses were found between males and females, supporting the idea that both sexes use different physiological mechanisms to cope with the same environmental challenges. The effects that combined stressors have on insects deepen our understanding of why we are losing beneficial species and their functions in times of drastic environmental change.

1. Introduction

Nowadays, increasing environmental temperature acts in combination with other factors and influences the reproductive success of insects. Factors linked to changes in land cover and biodiversity loss, such as the presence of chemical contaminants, higher rates of parasitism, or the decrease in the availability of resources, can have negative consequences on the survival and reproduction of insects (Folt et al., 1999; Siviter et al., 2021; Verheyen and Stoks, 2022). When two or more stressors occur simultaneously, their combined effect on organisms can be additive (the sum of their individual effects), antagonistic (smaller effect together than alone), or synergistic (greater effect in combination of stressors than the sum of their individual effects). The latter are of particular concern, due to their great threat to living organisms (Folt et al., 1999; Verheyen et al., 2019; Siviter et al., 2021; Verheyen and Stoks, 2022).

Insects are particularly vulnerable to temperature changes since they depend on environmental temperature to control their internal temperature, and because their small size makes them more susceptible (Atkinson, 1994; González-Tokman et al., 2020a; Mastore et al., 2023). Moreover, adaptation to a different temperature is unlikely to occur quickly, increasing the threat of fast changes (García-Robledo et al., 2016), particularly for species or populations already living at the edge of their critical thermal limits (Sunday et al., 2014).

Heat can potentiate the toxicity of polluting compounds found free in the environment (Noyes et al., 2009). For example, an increase in temperature combined with the insecticide chlorpyrifos increases mortality and decreases growth rate and thermal tolerance in larvae of the damselfly *Ischnura elegans*

(Odonata; Verheyen et al., 2019). Similarly, *Culex pipens* mosquitoes exposed to high temperature combined with chlorpyrifos had lower survival, developmental time, and size at emergence, indicating that some pesticides are more toxic at higher temperature (Tran et al., 2018).

Temperature also plays an important role in the activation of the immune system, since a thermal shock can be decisive in the survival of parasitized insects, or of their offspring (Wojda, 2017). For example, *Gryllus texensis* and *Tenebrio molitor* can modify their behavior when they are parasitized, inducing anorexia or causing behavioral fever, to face the immune challenge, and deal with the allocation of energy to their different metabolic pathways (Adamo et al., 2010; Catalan et al., 2012). Therefore, small changes in environmental temperature can induce physiological, morphological and behavioral changes, in addition to enhancing the effect of other biological and chemical stress factors (Sunday et al., 2014; Wojda, 2017).

Cattle pastures are habitats where different stressors occur, including excessive use of herbicides and veterinary medications that are excreted in dung, besides naturally occurring pathogens and parasites (Adamo et al., 2010; Cruz et al., 2012; Villada-Bedoya et al., 2019; Villada-Bedoya et al., 2020; Servín-Pastor et al., 2021). These stressors threaten coprophagous fauna, mainly flies and beetles. Worryingly, these harmful effects could be enhanced by the increasing environmental temperature, potentially resulting in negative effects on beetle fitness (Verdú et al., 2020; Ambrožová et al., 2021). For example, for the dung fly species *Scatophaga stercoraria*, the increase in temperature combined with the presence of ivermectin, one of the most widely used antiparasitic drugs in cattle, synergistically decreases the survival of developing larvae (González-Tokman et al. al., 2022).

Dung beetles (Coleoptera: Scarabaeinae) play a fundamental role in the functioning of cattle pastures (Doube, 1990; Nichols et al., 2008; Cruz et al., 2012; Doube, 2018). When they bury cattle dung to feed and reproduce, they fertilize the soil, reduce the exposure of cattle to feces, and reduces the emission of methane from dung into the atmosphere (Cruz et al., 2012; Miranda-Flores et al., 2020; Verdú et al. al., 2020). However, the presence of different stressors at high temperatures can reduce the survival and reproductive success of these organisms, affecting their function in the ecosystem (González-Tokman et al., 2017a; Ishikawa and Iwasa, 2019; Servín-Pastor et al., 2021). For example, an increase in temperature modifies the production of brood balls (each containing dung and an egg; Mamantov and Sheldon, 2020) and parasitic pressure reduces the size of such balls (Servín-Pastor et al., 2021). Medicines as, ivermectin delays development (Martínez et al., 2017; Ishikawa and Iwasa, 2019), reduces larval survival (Cruz et al., 2012; González-Tokman et al 2017b; Ishikawa and Iwasa, 2019), and the number of brood balls produced during reproduction (Pérez-Cogollo et al., 2016; Ishikawa and Iwasa, 2019), besides having other sublethal negative effects on their physiology and behavior (Verdú et al., 2018). Regarding pesticides as herbicides have been less studied, but some studies have shown them to be less harmful (González-Tokman et al., 2017a).

Even though some experiments have evaluated the effect of isolated stressors on some aspects of insect life history, it is still unknown whether an increase in temperature enhances the negative effects of other stressors that are applied to grassland, such as veterinary medicines and agrochemicals, or natural factors such as parasites that exert pressure on the coprophagous fauna. Pesticides are the second major threat for dung beetles in cattle pastures, only after habitat loss (Alvarado et al., 2017), but their effect in combination with high temperature has not been evaluated. The same is true for the effect of parasitism, which has not been evaluated in dung beetles but could also become more dangerous at high temperatures. Furthermore, since males and females may differ in their sensitivity to different environmental stressors, stressful conditions may cause changes in sex ratio, with potential implications for reproductive behavior and mate availability, and ultimately with consequences on population sizes and dynamics (Kappeler et al., 2023). But the combined effect of heat with other stressors on beetles remains untested.

Here, we evaluated the hypothesis that heat enhances the negative effects of three stressors: ivermectin, an herbicide, and parasitism, on the reproductive success, larval development time, sex ratio and survival of adult dung beetles *Euoniticellus intermedius*. We expected particularly synergic effects between heat and ivermectin, which is known to be highly toxic for these insects.

2. Materials and methods

2.1 Study species

Euoniticellus intermedius is an African species that was introduced into America more than 30 years ago to bury cattle dung in pastures (Montes de Oca and Halffter, 1998). It is a diurnal, multivoltine species that depends on bovine dung for its reproduction. Larval development lasts 28 days since the female makes brood balls, each consisting of an egg covered by dung. These brood balls can vary in terms of weight and size, and will provide the food resource for the developing larvae (Martínez et al., 2017; Pokhrel et al., 2020). Its reproductive strategies allow this species to establish in non-native sites (Pokhrel et al., 2020), and disperse despite the high levels of contamination with agrotoxic products registered in some regions (González- Gomez et al., 2023). This species is well known for its response to different sources of environmental stress, and it is known that its reproductive success, physiological condition, and stress tolerance are traits with plasticity in response to stressors such as contaminants in the dung and parasitism (Cruz et al., 2012; González-Tokman et al. 2017a, b; Servín-Pastor et al. 2021).

2.2 Study site

To carry out the experiments, 200 beetles were collected directly from the dung at San Román Ranch, Medellín de Bravo, Veracruz, Mexico (18° 58'19.37" N, 96° 04'5143" W), an artificial pasture of 17 ha. They were transported to the laboratory and placed in terraria (57 x 30 x 37 cm) covered with a mesh, with a 15 cm layer of moist soil. 30 couples were placed in each terrarium to allow reproduction in captivity. They were fed every third day with 500 g of medication-free cow dung, which was homogenized in a blender and sterilized by keeping at -20°C for 36 h. To eliminate environmental effects on experimental beetles, a second generation of *E. intermedius* was obtained before starting the treatments.

2.3 Experimental setup

We carried out an experiment under controlled laboratory conditions to determine the effect of different temperatures in combination with other stressors. Two temperatures, 29°C and 33°C (humidity at 73% and 70% respectively), were used in the experiment. Temperature was controlled in climatic chambers, where the beetles were placed in pairs, formed by one male and one female, and exposed to the respective treatments applied in dung (described below, Fig. 1). The pairs were placed in 1 L containers, filled 75% with sterile sieved soil and covered with a mesh, and kept at a photoperiod of 13 h light and 11 h dark (Villada-Bedoya et al., 2020). We used these temperatures because this species has successful breeding above 26°C (González-Tokman et al., 2017a; Villada-Bedoya et al., 2020). The soil moisture remained constant throughout the experiment. We placed fifteen replicates per treatment. At each experimental temperature, three stressors were used: ivermectin, herbicide, parasitism, and a control group without stressors, leaving a total of 8 experimental treatments. Ivermectin (10 µg per kg of fresh dung) was used because it is the most common antiparasitic drug used in the region and in other parts of the world (González-Gómez et al., 2018), and because it has negative effects on the reproductive success of *E. intermedius* (González-Tokman et al. 2017b). This concentration is considered sublethal for the study species and has effects on its metabolic rate and body size, in addition to triggering a physiological stress response (Villada-Bedoya et al., 2020) and at higher concentrations delays development (Krüger and Scholtz, 1997; Martínez et al., 2017). The herbicide used was the combination picloram + 2-4-dichlorophenoxyacetic acid 0.4 mg per kg of dung), which is a formula frequently used in the study area (González-Gómez et al. 2018; Villada-Bedoya et al., 2019) and is one of the most persistent herbicides in the environment (Volodymyr et al., 2018). Although it has been found that this herbicide, by itself, does not have an obvious negative effect on *E. intermedius* (González-Tokman et al., 2017a), its effect could be enhanced in combination with heat. Ivermectin and herbicide treatments were diluted in acetone (10 ml of acetone per kg of fresh dung) and applied in homogenized dung. Acetone was used as a control (see similar procedures in González-Tokman et al. 2017 a, b). In addition, the effect of parasitism was evaluated, since it can cause changes in the reproduction and thermal tolerance of our study species (Reaney and Knell, 2010; González-Tokman et al., 2020b), indicating a link between the response to heat and the immune system (Wojda, 2017). Parasitism was simulated with the implantation of a 2 mm long and 0.8 mm thick nylon monofilament between the pronotum and the elytra to both parents, since a wound generated by a sharp object activates the immune system and the melanization of foreign objects in insects (Rantala et al., 2000; Reaney and Knell, 2010; Catalán et al., 2012), and may have effects on the reproductive investment of our study species (Servín-Pastor et al. 2021). Each pair was exposed to the treatment for 15 days, while they could reproduce and form brood balls. After this period, both parents were removed from the breeding chamber. The brood balls were counted and left in the terraria for 30 days, after which we registered the number and sex of offspring that emerged. Three recently emerged adult beetles of each sex (5–7 days old) were randomly selected per terrarium and were isolated in 15 ml glass bottles (covered with moistened cotton, at 28°C and at a photoperiod of 12:12 h of light and darkness, deprived of food) to record adult survival. Mortality was registered every 6 h until the last individual died. A fitness index was calculated with the product of the standardized response variables (values between 0 and 1). Higher index fitness values indicate higher number of brood balls, emerged beetles, and proportion emerged, fast emergence time, and high survival of the offspring (Garrido et al., 2012).

2.4 Statistical analysis

All analyses were performed in R (version 4.0.2, R Core Team, 2020), using RStudio. Model fitting, evaluation, and selection were made following Crawley (2005) and Zuur (2009). To evaluate the effect of the combination of heat with other stressor (+ ivermectin or + herbicide or + parasitism), on the number of brood balls, the number of emerged adult offspring, the proportion of emerged beetles, the sex ratio of emerged beetles and the emergence time, we used Generalized Linear Models (GLM), using as predictor variables: temperature (with two levels; 29 and 33°C), treatment (with three levels; ivermectin, pesticide, parasitism) and the interaction between temperature and treatment. Linear Mixed Models (LMM) were used to evaluate the effect of the temperature and the treatment on the fitness index, considering the terrarium (family) as a random effect. The interaction between temperature and treatment was eliminated from the model when it was non-significant (Engqvist, 2005). We calculated the analysis of variance with type 2 sum of squares using the 'car' package for all the models (Fox and Weisberg, 2019).

We implemented GLMs to compare the number of brood balls and the number of emerged beetles, with negative binomial error distribution because since the Poisson models showed overdispersion (Resid. Dev. / Resid. D. f. >2). To analyze the proportion of emerged adults, a quasibinomial GLM with logit function was implemented, where the dependent variable contained information on the number of emerged and non-emerged adults. The quasibinomial model was used instead of binomial because the latter presented high overdispersion. To compare the sex ratio, a binomial GLM with logit function was performed, where the sex ratio variable contained the number of emerged males and females. To analyze differences in the emergence time between temperatures and treatments, a linear model (LM), was carried out. To analyze survival, a mixed-effects Cox model was performed with the Coxme package (Therneau, 2022), in which no case was censored, since all organisms died at the end of the experiment. In this survival analysis, the effect of the interaction temperature×treatment×sex, with the Terrarium as a random factor, was also tested. As the triple interaction was significant, Cox mixed effects models were performed separately by sex. The survival of each sex was modeled by temperature and treatment, with the Terrarium as a random factor. Survival curves were made for each treatment with the survminer package (Kassambara et al., 2021). The fitness index was modeled by temperature × treatment with an LMM, with Terrarium as random factor with the nlme package (Pinheiro and Bates, 2023). We tested the LMM of fitness index without an outlier detected, but the results did not change significantly, so we reported the results with all the data. Post hoc tests were performed with a false discovery rate correction from all the models (Benjamini and Hochberg, 1995) (Appendix 1). The results are presented as means and 95% confidence intervals, except for survival, where survival curves by treatment are shown.

To know the combination type that caused the combination between heat with each of the stressors, independently if the interaction in the models where significant or not, the independent action model was calculated to detect additive, antagonistic, or synergistic effects on the analyzed response variables and fitness index, following Coors and De Meester (2008) and Verheyen et al. (2019), with the following formula:

$$Ei = \frac{e_i - e_{control}}{e_{max} - e_{control}} \quad 1$$

Where e_i is the effect of the combination of heat with each stressor, $e_{control}$ is the effect of the control, e_{max} is the difference between the maximum value minus the minimum. To convert the effect into relative units, the following formula was used:

$$Ejoint = 1 - Prod\left(1 - E_1\right) * \left(1 - E_2\right) \quad 2$$

Where E1 and E2 are the effects of each stressor divided by e_{max}. Finally, to obtain the value in absolute units we used formula 3.

$$ejoint = E_{joint} * \left(e_{max} - e_{control}\right) + e_{control} \quad 3$$

Interactions are additive when the e_{joint} value falls within the calculated 95% confidence intervals; they are synergistic when the value falls above the 95% confidence intervals, and antagonistic when it falls below the 95% confidence intervals.

3. Results

The total number of brood balls laid by *E. intermedius* females did not differ at different temperature or stressor treatments (Table 1). However, the number and proportion of emerged beetles were marginally affected by temperature (p=0.051 for number and p=0.049 for proportion of emerged beetles; Table 1), and significantly by the stressor treatment (Table 1). In particular, the number and proportion of emerged adults were higher at 33 °C than at 29 °C, and the herbicide decreased the average number of emerged adults from 22 to 15, compared to the control group (Figure 2A, 2B; Table A1, A3, A4). There was no effect of ivermectin, parasitism, or of the temperature × stressor treatment interaction on the number of emerged adults. According to the independent action model, the combination of heat and the other stressors had synergistic negative effects on the number of emerged beetles (Fig. 2A, B). The sex ratio of emerged adults was not affected by temperature or the stressor treatments (Table 1; Table A5).

Heat shortened the emergence time of *E. intermedius* in all treatments (Fig. 2C), but this effect depended on the specific stressor (interaction between temperature × stressor treatment: F_3=4.42, p=0.005; Fig. 2C; Table A6). According to the independent action model, heat combined with ivermectin had additive effects on emergence time, while heat with parasitism had antagonistic effects (Fig. 2C). The effect of heat was enhanced in the presence of the herbicide, a combination that caused a synergistic decrease in the number of development days from 33 to 20 days.

Regarding the survival of emerged adults under starvation conditions, a significant effect of the interaction temperature × stressor treatment × sex was found (χ^2_3=9.73, p=0.021), so both sexes were analyzed separately. For females, the temperature × stressor treatment interaction was not significant (Table 2), these results were in the opposite direction of the prediction, since ivermectin reduced survival at 29 °C but not at 33 °C (Table A7). Heat combined with other stressors caused antagonistic effects on female survival (Table A2). On the contrary, in males, there was no effect of the temperature × stressor treatment interaction, and only the stressor treatment had an effect (Table 2). Compared to the control group, both ivermectin and parasitism decreased male survival (from 42 to 32 and 31 h, respectively; Table A1 and A8). According to the independent action model, heat combined with ivermectin and combined with parasitism had synergistic effects on male survival, while the effect of heat combined with herbicide was additive (Table A2).

The fitness index did not change with temperature nor with the stressor treatment (Table 1 and Table A9). However, according to the independent action model, heat had additive effects with ivermectin and the herbicide, whereas heat combined with an immune challenge acted antagonistically on the fitness index (Fig. 2D).

4. Discussion

In a changing and degrading world, organisms are increasingly being subjected to multiple, intense, and frequently novel, stressors. How do multiple stressors interact, and affect biologically and/or economically important insects, remains largely unknown. Importantly, the negative effects of stressors can be potentiated synergistically, meaning multiple stressors can affect organisms more strongly together than expected by the simple addition of their effects (Noyes et al., 2009; Verheyen et al., 2019; Verdú et al., 2020). Here, we found that under controlled laboratory conditions, heat increased the number of offspring produced by *E. intermedius* dung beetles breeding at 33 ° than at 29°C. However, heat also potentiated the negative effect of ivermectin, herbicide, and parasitism, reducing the number and proportion of emerged offspring, and increasing mortality of adult male offspring synergistically. Furthermore, heat combined with an herbicide synergistically accelerated larval development. Finally, heat in combination with parasitism antagonistically affected a global measure of fitness. These results confirm that a 4°C increase in temperature can enhance the effect of other stressors, affecting these insects with important functions in natural and productive ecosystems.

Heat synergistically potentiated the negative effects of ivermectin and of parasitism on the survival of the adult offspring. A similar effect was observed in the dung fly, *Scathophaga stercoraria*, exposed to ivermectin and high temperature, which suffered a synergistic reduction in survival (González-Tokman et al., 2022). Heat also acted synergistically with parasitism, possibly because parasitism causes a trade-off in the use of the energy between body maintenance and reproductive behavior (Rantala et al., 2000). However, negative effects on adult survival were only observed in males, and antagonistic results caused by high temperature and parasitism were seen in females. These could be caused by sex differences in response to parasites and contaminants (Villada-Bedoya et al. 2020; Córdoba-Aguilar and Munguía-Steyer, 2013). Transcriptomic analyses of stressed insects could shed light on the mechanisms explaining why this effect is not found in females, where even antagonistic responses are found.

As predicted, heat accelerated larval development, an effect that was enhanced synergistically by the presence of the herbicide. Faster emergence may increase the possibilities of having more reproductive events per year for the parents, which can be beneficial in some cases, for instance, in more variable and less predictable environments (i.e., a bet-hedging strategy). Nevertheless, earlier emergence might bring fitness costs caused by mismatches between animals and their resources (Trakimas et al. 2019; Buckley et al. 2017). Future experimental studies in natural dung beetle populations could evaluate the potential costs and benefits of emerging earlier, and whether these costs and benefits co-vary with specific ecological traits (like food resources or rainfall patterns).

Our results showed contrasting responses in survival between adult emerged females (antagonism) and males (synergies) exposed to different combinations of stressors. This is likely caused by genetic differences between the sexes (Xirocostas et al., 2020), which translate into sex-specific morphological and physiological responses in the face of environmental challenges. Specifically, in *E. intermedius*, ivermectin activates sex-specific damage repair systems under heat. While females activate heat shock proteins, males use their antioxidant capacity (Villada-Bedoya et al., 2020). Likewise, an immune challenge causes a differential use of energy reserves between males and females, where females are the most affected (Servín-Pastor et al., 2021). Activating the stress defense system can have consequences on fertility (González-Tokman et al., 2013; Clint et al., 2018), and could bring costs in terms of survival (Sokolova, 2023). These differential responses between females and males can also be explained by differences in the concentrations of juvenile hormone (Nijhout and Laub, 2018), or 20-hydroxyecdysone (Bhardwaj et al., 2017), which have different functions in adult insects of both sexes. Future studies in males and females exposed to different stressors could clarify the physiological mechanisms responsible for the differential responses between sexes. More broadly, different responses between the sexes to similar ecological and climatic pressures can potentially modify population dynamics and life history traits, such as operational sex ratios, sexual selection, and parental care evolution.

For other fitness-related variables in *E. intermedius*, we found no effects of heat in combination with other stressors. Females of this species produced the same number of brood balls in all treatments, even when stressors were combined with heat, reflecting a similar reproductive investment by females in this first reproductive event. Whether this reproductive effort under stressors has costs on future reproductive events remains to be tested. Maintaining the same investment in reproductive aspects, even in scenarios where multiple stressors are acting on organisms, may be the effect of one of the stressors causing a decrease in the negative impact of the others, an effect known as hormesis (Guedes and Cutler, 2013; Rix et al., 2022). Also, it has been found that young organisms can maintain the same reproductive effort in several events, even when parasitized (Polak and Starmer, 1998). For example, the activation of heat shock proteins can be mediators of the effects of multiple stressors (González-Tokman et al., 2020; Villada-Bedoya et al., 2020), where it is dampened, or the effect of heat or that of other stressors, thus preventing them from having a severe impact, in aspects of survival of the larvae for this species. Future studies could analyze the physiological aspects responsible for hormesis processes in insects facing multiple stressors. However, heat can also affect parasite populations, and influence their prevalence and intensity, which may increase or decrease a parasite's ability to infect its insect host (Tinsley et al., 2011;

Musgrave et al., 2019). Incorporating the effect of heat and other stressors on parasite populations, as well as a combination of multiple stressors joined with heat, are promising lines of research.

We did not observe differences on the sex ratio of emerged beetles exposed to any of the combinations of stressors, reflecting that the eggs and larvae of both sexes have similar sensitivity to the combination of stressors. Although it has been found that the sex ratio for *E. intermedius* does not change due to the effect of ivermectin (Cruz et al., 2011; Baena-Díaz et al., 2018), another study found that this substance at certain concentrations causes a decrease in the emergence of females, which would increase competition between males for reproductive partners (González-Tokman et al., 2017b). Future studies could evaluate combined effects of multiple stressors on sex ratios and measure aspects of competition between males or female choice, which may further increase the evidence of the ubiquity of the equal proportion of males and females (i.e., Fisher’s principle; Fisher, 1930).

5. Conclusions

In this warming and contaminated world, high temperatures potentiate the negative effect of other stressors, in this case of herbicides, ivermectin and parasitism, on life-history and fitness of dung beetles *E. intermedius*. Our results present a pessimistic scenario for dung beetles in cattle pastures, where the already negative effects of pesticides on biodiversity are becoming worse with only moderate increases in temperature. A transition towards reduced cattle production, and sustainable cattle production systems, such as sylvopastoral systems, where trees and natural enemies of pests reduce the need of pesticides while improving production is urgent to conserve biodiversity and ecosystem services in warming productive lands (De la Peña, 2022).

Declarations

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Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AER. The first draft of the manuscript was written by AER and DGT, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Model outputs analyzing the effects of different stressors on dung beetles *E. intermedius,* breeding at different temperatures. *df*= degrees of freedom. NS= non-significant interaction. The contrasts between treatments are described in the supplementary material. Significant values with false discovery rate (FDR) *p* correction method are shown in **bold**.

Response variables/ Explanatory variables	Temperature			Treatment			Temperature×Treatment		
	χ^2	*df*	*p*	χ^2	*df*	*p*	R.D.	*df*	*p*
Number of brood balls **glm negative binomial**	0.563	1	0.453	0.993	3	0.803	NS	NS	NS
Number of emerged beetles **glm negative binomial**	3.780	1	0.051	10.895	3	**0.012**	NS	NS	NS
Proportion of emerged beetles **glm quasibinomial**	3.874	1	**0.049**	20.843	3	**0.0001**	7.864	3	0.049
Sex ratio of emerged beetles **glm binomial**	0.001	1	0.980	3.023	3	0.388	NS	NS	NS
Fitness index **Linear mixed model**	0.006	1	0.940	3.773	3	0.287	5.158	3	0.161
Development time	*F*	*df*	*p*	*F*	*df*	*p*	*F*	*df*	*p*
Linear model	70.52	1	**<0.001**	0.614	3	0.608	4.427	3	**0.005**

Table 2. Effects of different stressors on the survival of emerged *E. intermedius* beetles under controlled laboratory conditions. *df*=degrees of freedom. NS=non-significant interaction. NA=factor not tested. Contrasts between treatments are described in the supplementary material. Significant values after false discovery rate correction are shown in bold.

	Survival			Female survival			Male survival		
	Coxme regression			**Coxme regression**			**Coxme regression**		
	χ^2	*df*	*p*	χ^2	*df*	*p*	χ^2	*df*	*p*
Temp	1.86	1	0.172	2.055	1	0.152	0.483	1	0.487
Treat	14.34	3	**0.003**	6.657	3	0.084	19.723	1	**0.0001**
Sex	0.75	1	0.388	NA	NA	NA	NA	NA	NA
Temp×Treat	2.86	3	0.413	7.518	3	0.057	NS	NS	NS
Temp×Sex	0.76	1	0.384	NA	NA	NA	NA	NA	NA
Treat×Sex	9.82	3	**0.020**	NA	NA	NA	NA	NA	NA
Temp×Treat×Sex	9.73	3	**0.021**	NA	NA	NA	NA	NA	NA

Figures

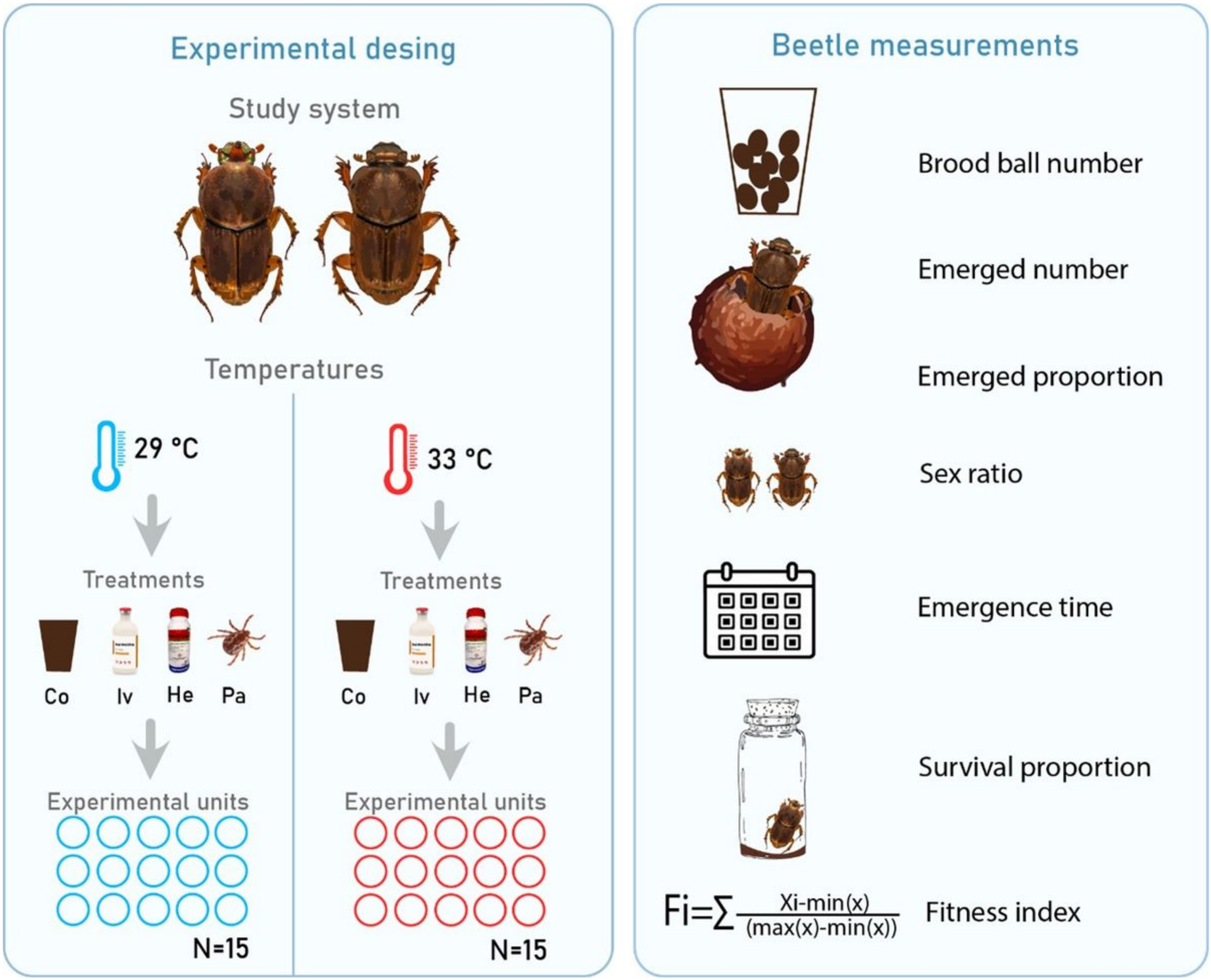

Figure 1

Experiment designed to evaluate the effect of the combination of heat with other stressors on the survival and reproductive success of *E. intermedius*, under controlled laboratory conditions. Co=Control; Iv=Ivermectin; He=Herbicide; Pa=Parasitism; N=number of replicates per treatment.

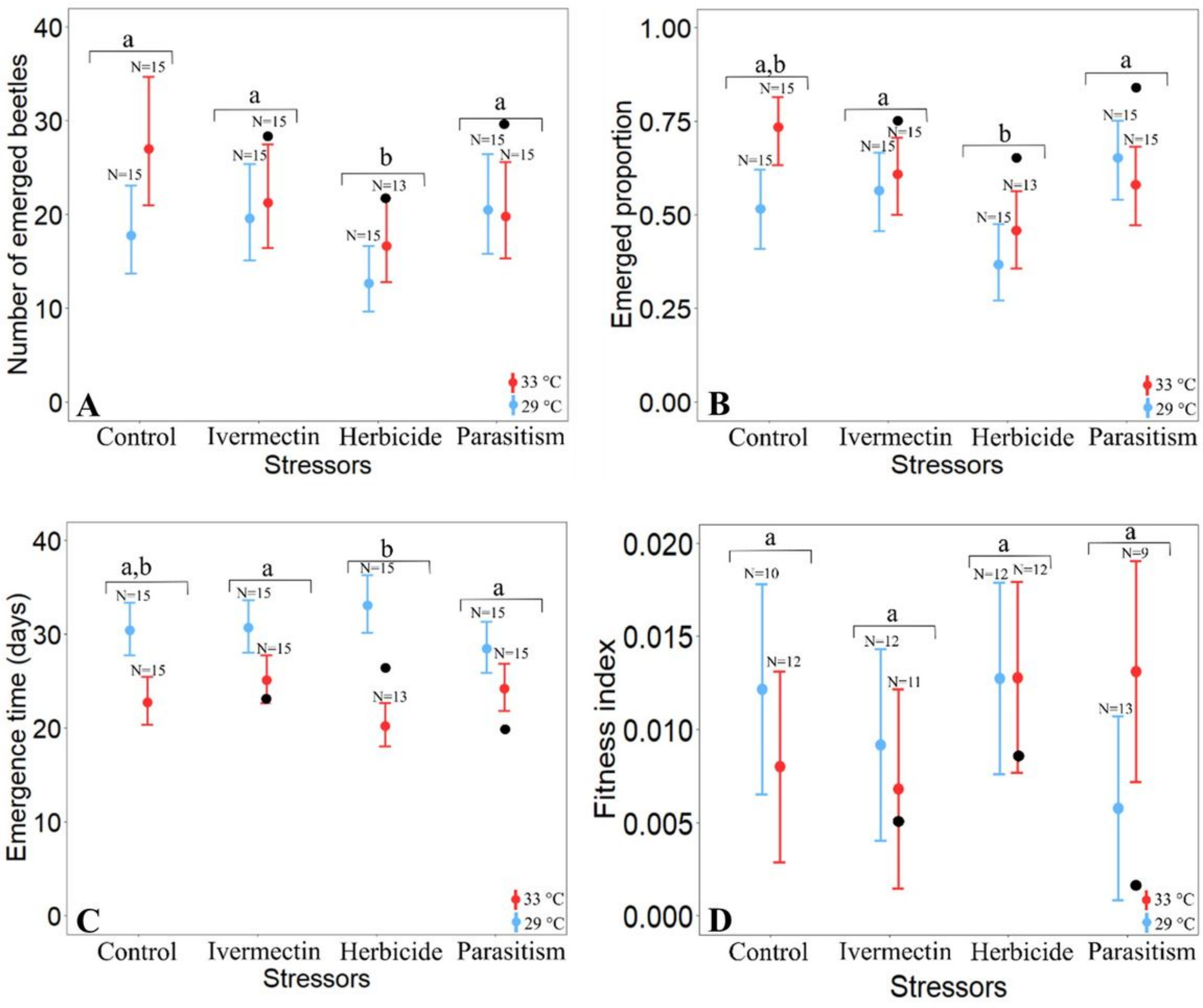

Figure 2

Effect of different stressors on the number of emerged beetles (A), the proportion of masses with emerged adults (B), developmental time (C) and fitness index (D), at different temperatures of beetles E. intermedius. N= number of replicates in each treatment. The points represent the means and the lines the 95 % confidence intervals. Letters indicate significant differences between treatments. Black dots are the expected additive effect for each stressor's combination according to the independent action model.

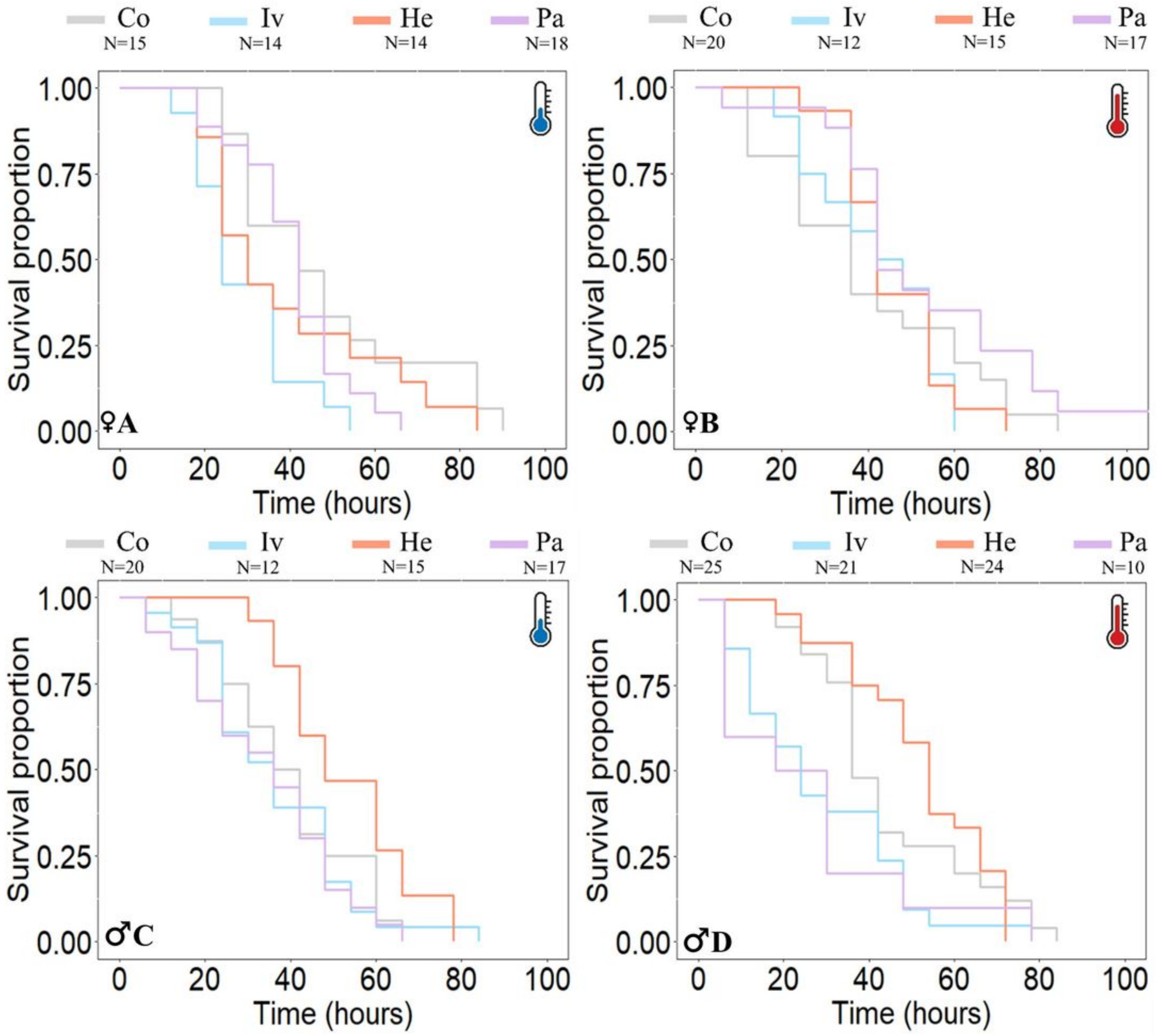

Figure 3

Effect of different stressors on the survival of emerged beetles of the species *E. intermedius* under starvation conditions in captivity. Top panels: females at 29 °C (A) and 33 °C (B); bottom panels: males at 29 °C (C) and 33 °C (D). N= number of beetles exposed to starvation in each treatment. The results of the independent action model are shown in Table A2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- BroodballsandEmerged.SynergisticEffects.AER.24.11.2023.csv
- EsquiveletalSupInfEcotoxicology.docx
- Survival.SynergisticEffects.AER.24.11.2023.csv