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Effects of exposures to repeated heat stress on the survival of the pea aphid *Acyrthosiphon pisum* and its endoparasitoid *Aphidius ervi*

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Abstract

Organisms could be exposed to several heat waves during their life, and their ability to survive a heat wave strongly depends on the effects of the previous one. Exposure to extreme temperatures can have important effects on the outcome of host-parasitoid interactions, as the ability of the parasitoid to survive depends on the ability of its host to cope successfully with these stresses. In the present study we address the impact of repeated exposure to heat stress on the survival of the pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera Aphididae) and its endoparasitoid *Aphidius ervi* Haliday (Hymenoptera Braconidae). The first treatment consisted of a heat stress of 35 °C for 30 minutes performed on 4 days old aphids, the second and third heat stresses of 39 °C were performed on 5 days old and on adult aphids, respectively. The three treatments were applied alone or in all their combinations. We found that aphid thermal tolerance is positively influenced by heat hardening if a severe stress occurs a few days after the first event. Adult parasitized aphids show significantly higher survival than unparasitized ones; however, the effects of parasitization and hardening on host survival after heat shock are not additive. We also found that *A. ervi* has a lower thermotolerance capacity than its host and does not show apparent hardening effects. In addition, parasitoid survival after mummification is not affected by the previously experienced heat shock. The possible explanations of the observed phenomena are discussed.

Key words: thermal stress, hardening, host-parasitoid interactions, thermal tolerance.

Introduction

Temperature is the most important environmental factor affecting the distribution, colonization, phenology, behaviour, and life history traits of insects (Cossins and Bowler, 1987; Hoffmann *et al.*, 2003; Peng *et al.*, 2020). In nature, temperatures fluctuate in repeated irregular waves. As a consequence, organisms are exposed to several heat waves rather than a single heat event (Zhang *et al.*, 2015; Bailey and van de Pol, 2016). When repeated thermal stresses occur, the ability to survive to a given stress strongly depends on the effects of the previous ones (Williams *et al.*, 2016), since the possibility to display some heat-induced resistance mechanisms could be enhanced (active acclimatization responses) or suppressed (accumulation of cellular damages and loss of performance). Insect responses to thermal stress may vary depending on the simultaneous occurrence of biotic and other abiotic stressors. The response to a combination of different stressors can increase, decrease, or leave unchanged the response to thermal stress (Kaunisto *et al.*, 2016; Trotta *et al.*, 2018).

Species exist within interconnected ecological communities and the biological aspects of organism vulnerability will also depend on how Heat Shock (HS) alters the interactions with competitors, predators, parasites, diseases, and mutualists (Lagos *et al.*, 2001; Pincebourde and Casas, 2006; Gilman *et al.*, 2010; Harley, 2011; Huey *et al.*, 2012). Exposure to extreme temperatures can have important effects on host plants, as well as on trophic levels that depend on the ability of lower trophic levels to cope successfully with these changes (Harrington *et al.*, 2001; Flores-Mejia *et al.*, 2017). For example, extreme temperature can have important effects on the outcome of host-parasitoid interactions (Hance *et al.*, 2007; Cayetano and Vorburger, 2013; Trotta *et al.*, 2018).

Aphids (Homoptera Aphididae) are sap-sucking insects with soft bodies, small sizes, a thin cuticle, and a limited ability to buffer thermal changes (Dixon and Kindlmann, 1998; Sandhi and Reddy, 2020). The pea aphid *Acyrthosiphon pisum* Harris is a common pest on peas, alfalfa, and other legume species (van Emden and Harrington, 2007; Sandhi and Reddy, 2020). *Aphidius ervi* Haliday (Hymenoptera Braconidae) is a koinobiotic parasitoid of the pea aphid that regulates the host physiology through the injection of venom and other factors of embryonic origin, so assessing suitable conditions for the developing parasitoid (Quicke, 1997; Digilio *et al.*, 1998; 2000; Pennacchio and Strand, 2005; Grossi *et al.*, 2016). The parasitoid larva develops inside the aphid through 3 larval stages until the formation of a mummy (the skeletonised aphid containing the parasitoid pupa). Parasitoids are considered effective natural enemies of insect pests. Parasitoid insects are also considered interesting models for exploring insect interactions because they can adapt their behaviour and development depending on the effects of host environmental conditions (Hance *et al.*, 2007; Benelli *et al.*, 2014). Parasitoids also have lower thermal tolerance than their hosts, suggesting that the interaction with the hosts could be negatively affected by heat stress (Furlong and Zalucki, 2017). Flores-Mejia *et al.* (2017) investigated the effects of increased temperature on the food web consisting of the potato plant, the aphid *Macrosiphum euphorbiae* Thomas, and the parasitoid wasp *A. ervi*. The authors showed that exposure to

high temperatures causes a direct reduction in plant and parasitoid biomass, while a crowding effect reduces aphid biomass.

Host-parasitoid interactions could be also negatively affected by heat waves. In a previous study (Trotta *et al.*, 2018), we observed that the parasitic wasp *A. ervi* can affect the thermal sensitivity of *A. pisum*. Since organisms in agricultural and natural ecosystems are exposed to repeated heat waves, here we address the impact of repeated exposure to heat stress rather than a single exposure on the survival of both aphids and parasitoids. Several methods have been developed to measure insect heat resistance in the laboratory (Hoffmann *et al.*, 2003) but the relevance of these measures in nature remains largely unknown. However, laboratory experiments constitute an important resource for the study of resistance and adaptation to thermal extremes. We have three main aims in comparing the effects of single and/or repeated exposures to stressful temperatures (one sublethal and two extremes) in a trophic model system consisting of the parasitoid *A. ervi* and its host *A. pisum*. First, we investigated the possible beneficial effects of heat hardening (and, in general, of heat-induced resistance mechanisms) on aphid thermal tolerance when a subsequent HS occurs during different stages of development. Second, we investigated the cumulative effects of parasitism and repeated HS on thermal tolerance in *A. pisum* parasitized by *A. ervi*. Finally, we considered the effects of repeated HS on parasitoid survival: we examined whether (and how) a parasitoid can be affected by repeated stresses suffered by its host. We discuss the thermal biology of complex host-parasitoid systems and their potential implications in the context of biological pest control under climate change.

Materials and methods

Plant and insects rearing

Broad bean plants (*Vicia faba* L.) of the cultivar "Aguadulce" were grown in pots (10 cm diameter) containing commercial soil (COMPO Naturasol®) in a greenhouse. To standardise the possible effects of the plant on the growth of aphids (Guldemond *et al.*, 1998), young vegetative plants, with two well-developed pairs of leaves (3 weeks after sowing seeds) were used.

A colony of the green pea aphid *A. pisum* was started in 1985 from a few hundred specimens collected in a field of alfalfa (*Medicago sativa* L.) near Salerno, Italy, and reared in the laboratory on broad bean plants. *A. ervi*, a solitary endoparasitoid, was obtained from Koppert Biological Systems and reared on *A. pisum*. The parasitoid provided by this company is commonly used in biological control programs. Aphid and parasitoid cultures were kept in separate climatic chambers (Binder KBF) at 22 ± 1 °C and 75 ± 5% relative humidity (mean values ± accuracy), under a photoperiod of 18L:6D. Even if two laboratory-adapted insect populations were used in the present experiment, we do not expect that the host-parasitoid relationships under heat stress changed.

Since all the experiments required same-aged aphids, approximately 100 adult virginoparae females were isolated from the mass rearing colony and put on a fresh potted broad bean plant kept in a plastic box (22 × 15 × 40 cm height) for 12 hours at the previously described conditions. The adult females were then removed and discarded. The new born nymphs were maintained as a synchronous colony on a broad bean plant for 4 days, roughly corresponding, at this rearing temperature, to the beginning of the third nymphal instar. However, before their use in the experimental trials, aphid morphological features (Digilio, 1995) were checked under a stereo microscope and the nymphs that were not in the required stage were discarded. Aphids were used at the beginning of the third nymphal instar, as they allow the parasitoid development with higher success (Trotta *et al.*, 2014; 2018). Four independent replicates, each consisting of 10 synchronous colonies (i.e., different plants) were generated with an interval of 14 days from each other.

The parasitoids were removed from the culture at the mummy stage and enclosed in a separate plastic 150 ml cylinders until adult emergence. After emergence and before the experiments, adult parasitoid females were left for 24 hours with two males and were provided with water and honey ad libitum. Water was provided by a soaked cotton ball, whereas a drop of honey was placed on a piece of paper at the base of the cylinders. All the parasitoid females used in the bioassays were two-three days post emergence old and were assumed to be mated.

Parasitization procedure

In each group, approximately 1100-1400 4-day-old aphids were removed from the plants with a soft paint brush and placed together in a plastic box (27 × 19 × 8 cm). Cohorts of twenty aphids were randomly transferred from the box into 60 plastic 150 ml cylinders (except for one group in which only 52 cylinders were generated) and then split into two groups.

Parasitoid females were given oviposition experience by exposing them to two aphids in a Petri dish before the experiment. Females with experience in oviposition (that is, observed to oviposit in an aphid within 2 minutes after introduction into the Petri dish) were randomly assigned to plastic cylinders belonging to the parasitized group (one parasitoid per cylinder) and removed after 3 hours. In *A. ervi*, the decision to accept or reject a host follows the insertion of the ovipositor (Larocca *et al.*, 2007), and there is no way to know for sure whether an aphid hosts a parasitoid egg without dissecting it. After the parasitization procedure, all aphids were merged to form a homogeneous group with the same percentage of parasitization. Then groups of 55-60 parasitized aphids formed the thermal experimental groups. This procedure was carried out four times. The same protocol was used for the unparasitized aphids. Preliminary experiments showed that these manipulations caused negligible aphid mortality (less than 3%).

For both the unparasitized and parasitized aphid groups not subjected to any HS treatment, aphid survival was checked on days 4, 5, 9 and 14, according to the experimental design (see figure 1 for details). The parasitization rate was measured as the mean number of mummies observed on the initial number of parasitized aphids.

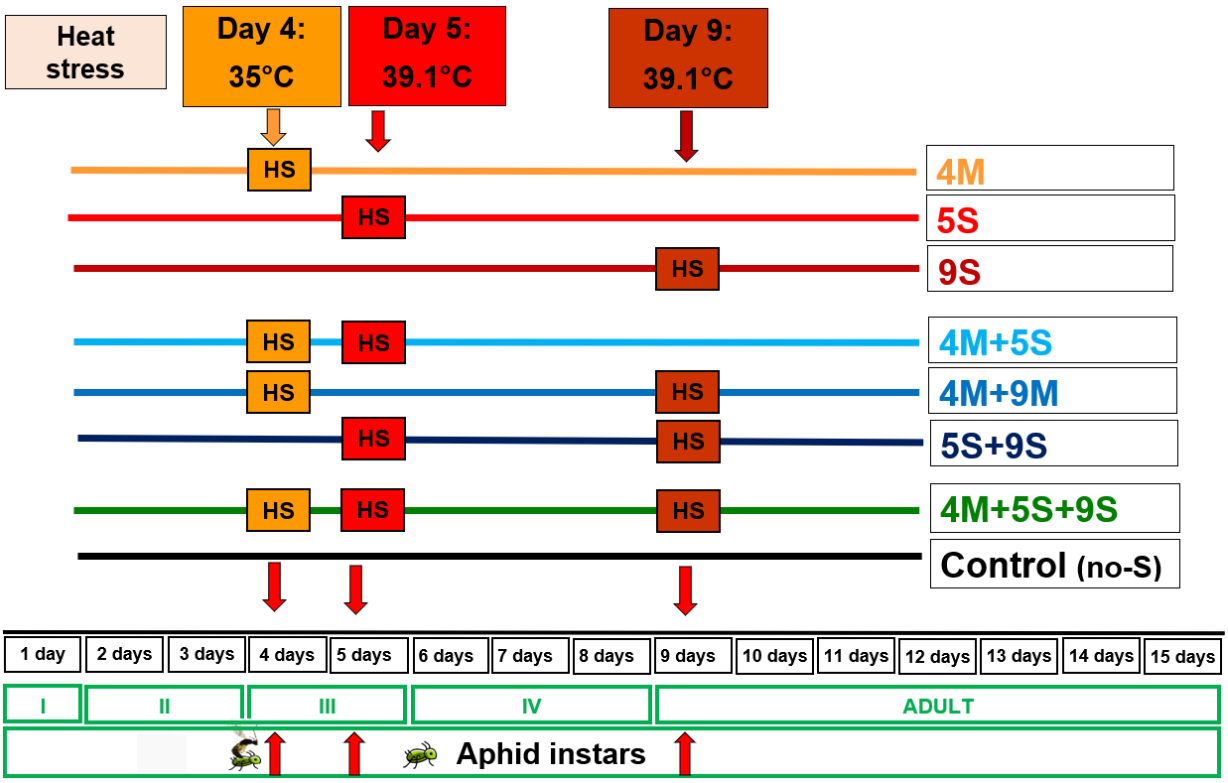

Figure 1. Timing of heat shock treatments in relation to the developmental stages of aphids. HS: heat shock; 4M: mild HS at 35 °C performed on 4-days-old aphids; 5S: HS at 39.1 °C performed on 5-days-old aphids; 9S: HS at 39.1 °C performed on 9-days-old aphids; I, II, III, and IV: first, second, third, and fourth nymph instar.

Heat shock treatments on parasitized and unparasitized aphids

Plastic cylinders (150 ml) with a mesh-covered ventilation hole at the top that contain aphids have been soaked in water. Lead weights were placed at the base of the cylinders to allow them to float and protrude 1 cm from the surface of the water. HS resistance was assessed by measuring the survival of unparasitized and parasitized aphids after exposure to heat treatment in a water bath (Argolab WB 12) for 30 minutes. A rapid exposure of 30 minutes was chosen because this procedure minimised the chance of any heat hardening response during heat shock and did not induce starvation or desiccation in aphids (Hoffmann *et al.*, 2003; Terblanche *et al.*, 2010). The first thermal treatment consisted of a HS at 35 ± 0.2 °C (mean values ± accuracy) performed on 4-days-old aphids (4M, applied 3 hours after parasitization). The second thermal treatment consisted of a HS at a temperature of 39.1 ± 0.2 °C performed on 5-day-old aphids (5S, applied 1 day after parasitization). The third thermal treatment consisted of a HS at the temperature of 39.1 ± 0.2 °C and was performed on 9-day-old aphids (9S, applied 5 days after parasitization).

The three HS treatments were applied alone or in all their combinations, generating seven experimental lines and one control. Three lines were exposed to a single thermal stress: 4M, 5S, and 9S; the other three lines were exposed to two thermal stresses: 4M + 5S, 4M + 9S, and 5S + 9S; one line was exposed to three thermal stresses: 4M + 5S + 9S. The unparasitized experimental groups were exposed to the same HS treatments as the parasitized ones. The control groups (No-S) were not exposed to HS (see figure 1 for details). Thermal stresses were applied during different developmental stages of aphids and parasitoids.

Survival of aphids and parasitoids

During the heat treatment, the experimental groups of parasitized and unparasitized aphids were placed in a plastic cylinder within a water bath. After HS, the aphids were transferred on a Petri dish lid and placed at the base of a new broad bean plant within a plastic box in an environmental chamber at 22 ± 1 °C. Survival was considered to occur if the aphids were able to walk away from the Petri dish lid and climb the plant. This response was checked 24 hours after heat exposure, as many insects are immobilised for some time after heat stress (Hazell *et al.*, 2010; Trotta *et al.*, 2018). For the survival check, the Petri dish lid was removed from the plant and the aphids that remained on the lid were counted. In the groups exposed to repeated HS, aphids were collected from the plants prior to the treatment, placed in plastic cylinders and subjected to the next thermal stress. Survival was measured as previously described.

Survival was also checked at the end of the experiment on 14-day-old aphids. For the parasitized groups, the number of mummies was also counted, since a parasitized aphid after 10-11 days turns into a mummy. The mummies were removed from the plant by incising the leaf blade around each mummy with a scalpel, enclosed in separate plastic cylinders, and the adult parasitoids were counted after their emergence.

Statistical analysis

Since the raw data of the present experiments have a discrete probability distribution (alive/dead individuals), binomial generalised linear models with a logit link function were considered for the data analyses. Linear mixed models for the analysis of survival data were also considered, with and without the percentage arcsine transformation. We finally chose linear models performed on the percentage of aphid survival after a square root arcsine transformation because they have the lowest Akaike Information Criterion (AIC), minimizing the lack of model fit to the observed data (Johnson and Omland, 2004). The homoscedasticity and normality assumptions were checked and met on these data. Aphid survival measured 24 hours after heat exposure were independently analysed for each of the 3 thermal experiments using mixed model ANOVAs with "Parasitization" (two levels, unparasitized and parasitized aphids) and "Treatment" (the applied heat treatments: two levels for 4M, three levels for 5S, and five levels for 9S) as fixed effects, and "Replicate" nested within "Parasitization" and "Treatments" (four levels/parasitization/treatment). The *P*-values for the differences between parasitization, treatments, their interactions, and replicates were obtained by mixed model ANOVA (Type II sum-of-square tests).

The effects of HS treatment on the survival of 14-day-old aphids, on the rate of parasitoid mummification, and on the rate of parasitoid emergence from aphid mummies were also analysed with mixed model ANOVAs with "Treatment" (eight levels) as fixed effect and "Replicate" nested in "Treatment".

To detect significant differences among groups, Tukey post-hoc tests for multiple comparisons of means were also performed. All statistical analyses were performed in R version 4.1.2 "Bird Hippie" (R Core Team, 2021).

Results

Aphid survival 24 hours after a HS treatment at 35 °C on 4-days-old aphids (4M)

The ANOVA performed on the data of aphid survival (after the arcsin transformation) recorded 24 hours after the 4M gave significant differences related to treatments (no-S *vs.* 4M: $F_{1,59} = 13.4$, $P < 0.001$) but not between parasitization ($F_{1,59} = 1.41$, $P = 0.24$) or for the "parasitization by treatment" interaction ($F_{1,59} = 0.4$, $P = 0.55$). No significant differences were found among replicates within parasitization and treatment ($F_{12,59} = 0.16$, $P = 0.33$). The 4M HS (figure 2) reduced the survival of aphids for both parasitized (−5.6%) and unparasitized (−4.4%) aphids.

Aphid survival 24 hours after HS treatment at 39.1 °C on 5-day-old aphids (5S)

The ANOVA performed on the survival values of the aphid recorded 24 hours after the 5S showed significant differences related to treatments ($F_{2,36} = 139$, $P < 0.001$) but not between parasitization ($F_{1,36} = 0.31$, $P = 0.58$) or for the interaction of "parasitization by treatment" ($F_{2,36} = 0.9$, $P = 0.4$). No significant differences were found between the replicates ($F_{18,36} = 1.3$, $P = 0.25$). The

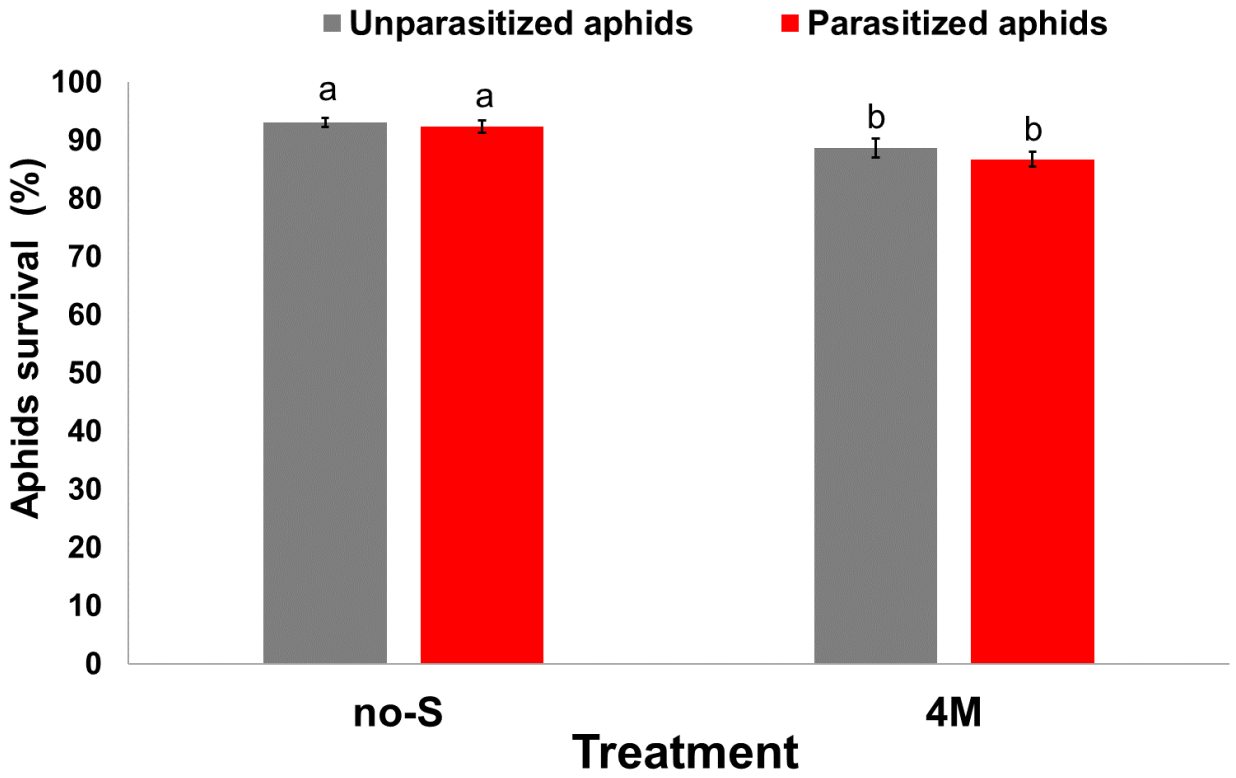

Figure 2. Mean values (± standard errors) of aphid survival 24 hours after heat shock at 35 °C (4M) for an exposure time of 30 minutes. The heat shock was performed on aphids of 4 days (third nymphal instar; 3 hours after parasitization in the parasitized groups). Means with different superscript letters differ significantly (Tukey test, α = 0.05).

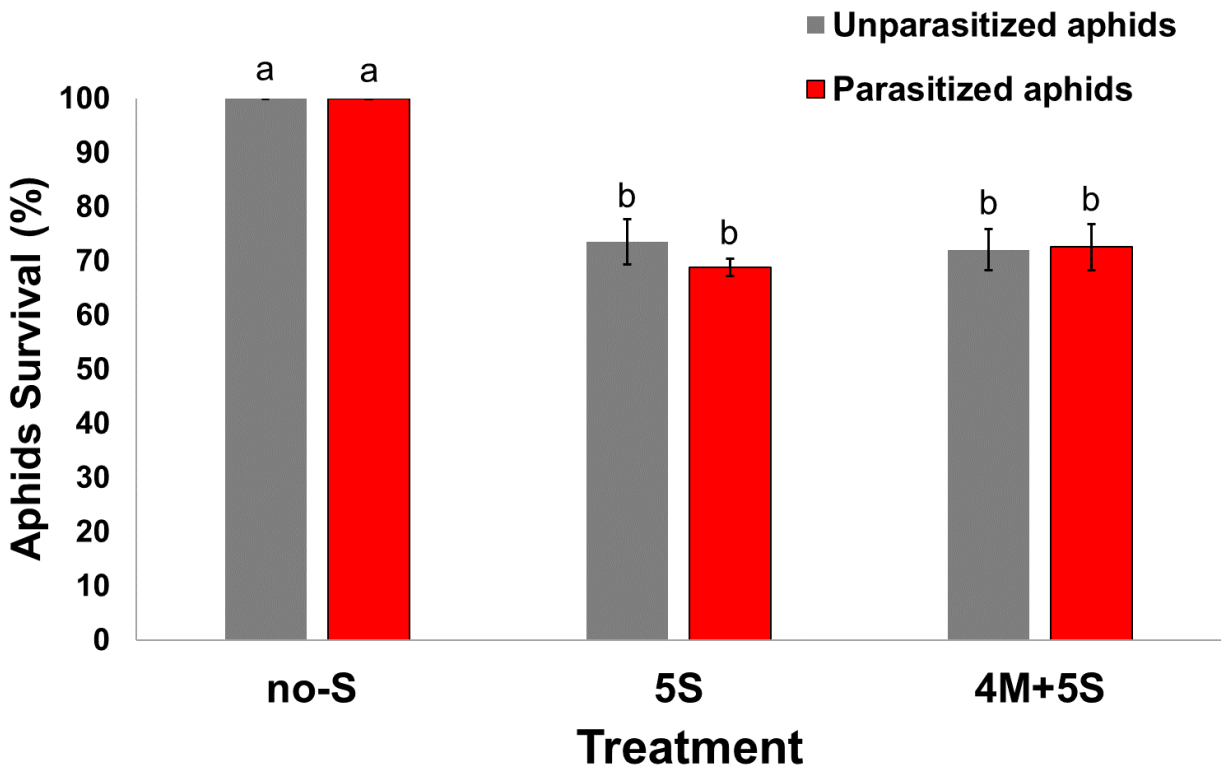

Figure 3. Mean values (± standard errors) of aphid survival 24 hours after heat shock at 39.1 °C (5S) on 5 days old aphids (end of the third nymphal instar; 24 hours after parasitization for the parasitized groups). Means with different superscript letters differ significantly (Tukey test, $\alpha = 0.05$).

5S HS (figure 3) reduced aphid survival in a similar way for parasitized (−31.1%) and unparasitized aphids (−26.4%). Approximately the same reduction in survival was observed when parasitized and unparasitized aphids were previously exposed to moderate 4M stress (−27.4% and −27.9%, respectively).

Aphid survival 24 hours after HS treatment at 39.1 °C on 9-days-old aphids (9S)

The ANOVA performed on the survival values of the aphid recorded 24 hours after the 9S showed significant differences related to treatments ($F_{4,11} = 72.9$, $P < 0.001$), between parasitization ($F_{1,11} = 24.6$, $P < 0.001$) and for the interaction "parasitization by treatment" ($F_{4,11} = 7.08$, $P < 0.01$). No significant difference between replicates was found ($F_{28,11} = 0.6$, $P = 0.87$).

The 9S HS (figure 4) alone reduced aphid survival in a different way for parasitized (−43.7%) and unparasitized aphids (−96.9%). In the parasitized groups, a similar reduction in survival was observed when aphids were previously exposed to the 4M stress, to the 5S stress or to both stresses 4M + 5S (−41.5%, −48.6% and −43.8% respectively, figure 4). In the unparasitized groups, the survival recorded 24 hours after the 9S treatment increased in relation to the type of stress to which the aphids have previously been subjected (4M + 9S: −68.6%; 5S + 9S: −58.9%; 4M + 5S + 9S: −51.9%, figure 4). It should be noted that the sample size of parasitized and unparasitized aphids decreased when one or more heat stresses were previously applied.

Effects of HS treatment on aphids and parasitoids at the end of the experiment

The survival of aphids and parasitoids checked 14 days after the establishment of synchronous colonies as related to the treatments applied is shown in figure 5. No winged aphids were observed in the unparasitized groups.

For the survival of aphids belonging to the unparasitized groups, the ANOVA showed significant differences related to treatments ($F_{7,7} = 99.7$, $P < 0.001$, figure 5), but not among replicates ($F_{23,7} = 3.05$, $P = 0.067$). The 4M HS alone did not greatly affect the survival of 14-day-old unparasitized aphids, which appears to be similar to that recorded in the control group (figure 5). The survival of 14-day-old unparasitized aphids subjected to the 5S or 9S HS (and all their combinations) was much lower compared to the control or with the respective survival at 24 hours. For the 14-day-old unparasitized groups, even survival after 9S treatment increased in relation to the type of stress to which the aphids were previously subjected (figure 5).

Parasitoid survival, measured as the number of mummies on the number of parasitized aphids, was statistically different among thermal treatments ($F_{7,6} = 18.1$, $P < 0.01$, figure 5), but not among replicates ($F_{23,6} = 3.41$, $P = 0.066$). The rates of parasitoid emergence from aphid mummies were also affected by the different thermal treatments ($F_{7,6} = 33.6$, $P < 0.001$, figure 5), but it is similar with the data recorded for the number of mummies. The 4M HS alone reduced parasitoid survival. Parasitoid survival was also reduced when parasitized aphids were subjected to a single stress during the early stages of their

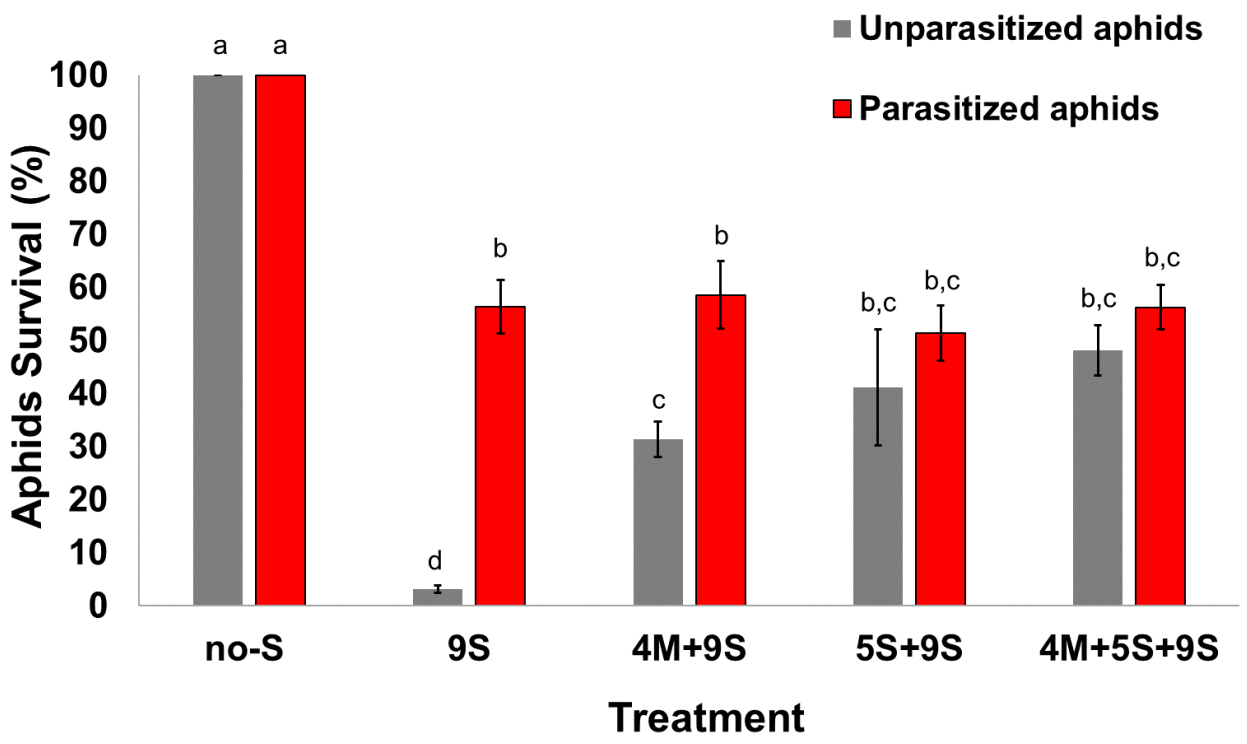

Figure 4. Mean values (± standard errors) of aphid survival 24 hours after heat shock at 39.1 °C on adult aphids (9S). For the parasitized groups, the heat shock was performed 5 days after parasitization. Means with different letters differ significantly (Tukey test, α = 0.05).

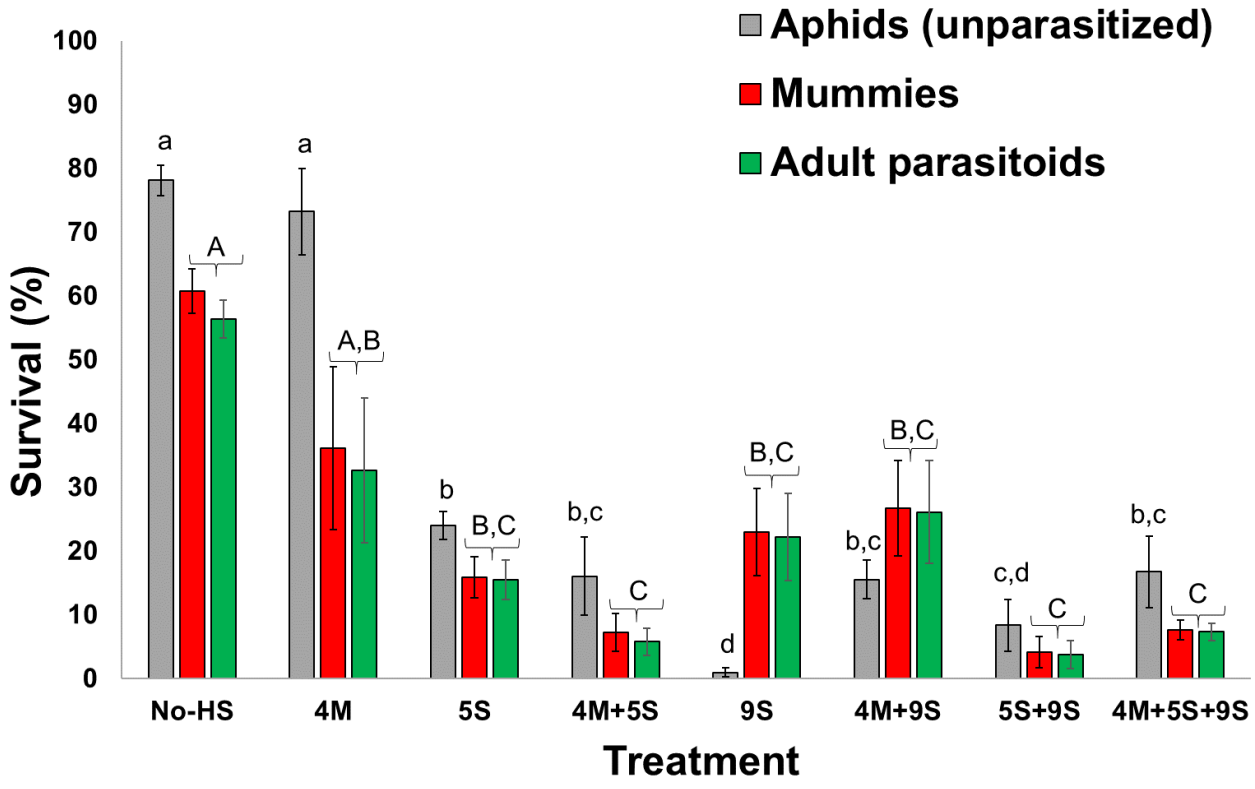

Figure 5. Mean values (± standard errors) of the survival rate of 14-day-old aphids of the mummies / initial number of aphids and adult parasitoids / number of mummies after different heat shock treatments. Means with different superscript letters differ significantly. Lowercase letters refer to differences between unparasitized aphids, while uppercase letters refer to differences between mummies or parasitoids. (Tukey tests, α = 0.05).

development (5S) or to a combination of the two stresses (5S and 9S). In the latter case, no mummies were observed in two replicates.

Discussion

Multiple stressors can influence performance independently (that is, being additive) or interact to reduce (or enhance) performance in a nonlinear and unpredictable way (Todgham and Stillman, 2013). This study considers the consequences after two different time intervals within a generation of single and repeated HS at different stages of development in a trophic model system consisting of the parasitoid *A. ervi* and its host *A. pisum*. We cannot exclude the possibility that the absence of thermal variation in the rearing of our laboratory populations could have selected a population with a lower thermal tolerance than natural populations, which would affect our results. If the laboratory rearing conditions influenced the genetic variation of a population via inbreeding or genetic drift, it is expected to observe a loss of adaptive responses when novel environmental conditions are subsequently encountered (Hoffmann and Ross, 2018) but not in its trophic interactions. Also, aphids and parasitoids are organisms that reproduce both sexually and asexually within the same lifecycle and often face a very high level of inbreeding in natural populations. We do not expect host-parasitoid relationships have changed in their form, even when the two species were subjected to stressful conditions, as some adaptive differences in life history traits among natural populations persist in spite of laboratory adaptation (Trotta *et al.*, 2006; Hoffmann and Ross, 2018). Furthermore, the mobility of apterous aphids (Ben-Ari *et al.*, 2015) allows them to search for low-temperature microclimates to reduce thermal injury through behavioural thermoregulation, which increases the thermal tolerance of insects in the field. However, studies performed using laboratory adapted populations contribute to our understanding of the impact of heat waves on predatory insects that are reared for release to provide pest control in agricultural settings (Hoffmann and Ross, 2018). Our results suggest that the 4M could be considered a mild heat stress for *A. pisum* (figure 2). Interestingly, we found that adult *A. ervi* survival was negatively affected by this mild heat stress applied at the egg stage (figure 5). Therefore, excluding mortality due to parasitism side-effects (Durán Prieto *et al.*, 2018), a mild 35 °C heat stress had a differential effect on the complex host-parasitoid system. The host response to thermal stress can also directly affect parasitoid survival. Successful parasitism of *A. ervi* strongly depends on factors of maternal and embryonic origin and on the immune response of the host (Quicke, 1997; Digilio *et al.*, 2000; Pennacchio and Strand, 2005; Grossi *et al.*, 2016). Since parasitism can affect host thermal sensitivity (Trotta *et al.*, 2018), the host response to the HS could interfere with the normal function of maternal and embryonic parasitoid factors or strengthen the response of the aphid immune system.

The 5S treatment reduced in a similar way the immediate survival (24 hours) in both the experimental groups of parasitized and unparasitized aphids (figure 3). The same reduction in survival of the third nymphal instar aphids was observed when the two groups of aphids were previously exposed to a moderate heat stress. The moderate 4M heat stress does not induce any beneficial hardening effect in third nymphal instar aphids. Unlike other insects, the time interval (24 hours) between the two treatments appears to be insufficient to induce a measurable hardening effect in third nymphal instar aphids, confirming that the extent of response to hardening can vary at the species level (Hoffmann *et al.*, 2003).

A strong reduction in survival due to the 5S treatment was recorded for both 14-day-old aphids and parasitoids (figure 5). The 5S stress (like the other severe stress combinations performed in this study) caused a permanent decrease in aphid survival due to the physiological damage and/or to the fitness costs arising from exposure to an extremely high temperature that are carried over the aphid lifespan. As a consequence of its life cycle, a koinobiotic parasitoid is affected by the stress suffered by its host and its survival is linked to the host survival. In agreement with other experiments on Lepidoptera (Mironidis and Savopoulou-Soultani, 2010) and Diptera (Xie *et al.*, 2008), in our model system heat injuries seem to accumulate during development, resulting in delayed death. However, the parasitoid survival after 5S stress was lower compared to unparasitized aphids, confirming that a heat stress had a differential effect on the complex host-parasitoid system (Machekano *et al.*, 2018; Biale *et al.*, 2020). Hardening causes physiological changes that, although not important for aphids, are added to the negative internal stress caused directly by the parasitization (Quicke, 1997), reducing the survival of the parasitoid itself.

The results of this study suggest a link between parasitization and Heat Shock Proteins (HSPs) expression (Rinehart *et al.*, 2002; Shim *et al.*, 2008). Temperature resistance in unparasitized *A. pisum* can be influenced by a previous brief exposure to a thermal stress, as a possible consequence of the induction of the stress response (Hoffmann *et al.*, 2003). Under our experimental conditions, the 9S thermal stress occurred 4-5 days after the previous experimental treatments, allowing aphids to activate the stress response, reducing the negative effect of HS on survival. Our results agree with Ma *et al.* (2018), confirming the importance of non stressful days in driving the effect of heat waves in aphids.

We found that survival after 24 hours of parasitized aphids exposed to 9S thermal treatment did not increase with hardening (figure 5). The resistance to parasitism is in part mediated by the activation of the host immune system (Martinez *et al.*, 2016) and by an upregulation of several aphid and symbiont proteins, including the early increase of HSPs (Rinehart *et al.*, 2002; Nguyen *et al.*, 2008; Shim *et al.*, 2008). It is possible that parasitization, such as hardening to high temperature, induces the aphid stress response. As a consequence, in the short term, parasitized aphids are more resistant to high temperatures compared to unparasitized ones. Parasitized aphids also showed the same immediate survival rates independently of the previous stressful temperatures experienced during their life (different combination of thermal treatments), indicating that the effects of parasitization and hardening

on aphid survival are not additive. The latter result suggests that the response to parasitization and HS may have some shared adaptive mechanisms of the stress response that confer protection against different stressors once activated (Kaunisto *et al.*, 2016).

We found that, as for the 5S stress combinations, a strong reduction in survival was recorded for 14-day-old aphids and parasitoids subjected to 9S treatment alone and in combination with the other two treatments (figure 5). Insects are capable of surviving a series of non-lethal lesions, but at a certain point the lesions accumulate to a critical level and cause death. It is known that heat-responsive mechanisms may have a high metabolic cost and long-term detrimental effects on the organism surviving stress (Zhang *et al.*, 2015; Zhao *et al.*, 2017). Our results emphasize the importance of time scales used to assess survival in heat shock experiments, as well as the importance of the timing of heat events.

Interestingly, we found that parasitoid survival was dramatically reduced if they experienced two severe heat stresses during their immature stages, although survival of parasitized aphids measured 24 hours after the HS does not appear to be strongly reduced (figure 5). Even if a common initial response mechanism is shared, the overall impact on parasitoid survival could depend also on synergistic interactions among parasitization (a biotic stressor) and high temperatures.

Adult parasitoid eclosion from mummies was not influenced by the previous heat treatments applied in this study, suggesting that parasitoid survival after mummification is not affected by the previously experienced HS.

Conclusion

Our results are consistent with previous studies in other insects documenting how high temperature events or heat waves can reduce growth, survival, and reproduction (Zhang *et al.*, 2015; Ma *et al.*, 2018). Furthermore, we observed that aphid thermal tolerance is positively influenced by heat hardening, provided that a certain amount of time elapses between the stress events, confirming the importance of non-stressful days in driving the effect of heat waves in aphids. We also observed that the effects of parasitization and hardening on host survival appear not to be additive, suggesting that they may have shared mechanisms of the stress response. Other experiments are needed to investigate the mode of action and the interactive effects of two stressors (parasitization and heat shock) in aphids to fully understand the mechanisms behind the thermotolerance capacities of parasitoids and their hosts.

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