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Heat shock proteins expression and survival at fluctuating high temperature in the larvae of two moth species, *Helicoverpa armigera* and *Thaumetopoea pityocampa*

Manuela BRANCO[1], Maria Glória ESQUÍVEL[2], Miguel CORTEZ[1,2], Elisabete FIGUEIREDO[2]
[1]*CEF -Forest Research Centre, Instituto Superior de Agronomia, Universidade de Lisboa, Portugal*
[2]*LEAF - Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, Portugal*

Abstract

Thermal stress is a critical factor when assessing responses of organisms and populations to global warming. Understanding responses to upper lethal temperature is critical to predict the effect of heat waves on populations. On the other hand, it is important to understand physiological traits allowing populations to adapt to changes in temperature. Here we compare survival to high temperatures of two moth species: a Mediterranean species *Thaumetopoea pityocampa* (Denis et Schiffermuller) and the broadly distributed *Helicoverpa armigera* (Hubner). Larvae were exposed to thermal stress, mimicking heat waves with daily maximum temperature (T_{max}) up to 45 °C. Control groups were kept at 25 °C. The changes in two heat shock proteins HSP70 and HSP90 were further studied for the second and third larval stages of *T. pityocampa* and *H. armigera*, respectively. An increase in mortality was observed on *T. pityocampa* larvae for T_{max} above 38 °C. On the contrary, larvae of *H. armigera* survived well up to 44 °C T_{max}, but survival decreased abruptly at 45 °C T_{max}. Thus, the two species differ by 6 °C on their upper threshold. For *H. armigera* HSP70 and HSP90 were both inducible by temperatures above 38 °C, steeply increasing with temperature which may account for superior adaptation to upper temperatures. In *T. pityocampa* HSP70 and HSP90 occurred constitutively in the control at 25 °C and did not increase with temperature. Results evidence major differences in HSP expression between species, which might account for differences in larval survival at the upper thermal limits.

Key words: heat tolerance, heat shock proteins (HSP), global warming, thermal adaptation, heat waves.

Introduction

Climate changes due to global warming may impose severe environmental stress to organisms and populations, with implications in their evolutionary adaptive processes (Arias *et al.*, 2011; Moritz and Agudo, 2013). Insects and other ectotherms are particularly expected to be significantly affected by global warming (Hoffmann *et al.*, 2013). Heat stress associated with climate change may particularly affect mid-latitude species (Kingsolver *et al.*, 2013). On the other hand, extreme events, such as heat waves, with extremely high maximum daily temperatures, observed in a few days, may acutely increase mortality at such extent that populations will reduce significantly locally, even if the mean temperature remains within the tolerance thermal thresholds (Arias *et al.*, 2011; Paaijmans *et al.*, 2013; Robinet *et al.*, 2013; Rocha *et al.*, 2017).

The upper temperature thresholds for insects varies greatly among species from about 25 °C up to 42 °C, in great part related to the species latitudinal geographical distribution (Deutsch *et al.*, 2008). Whereas differences on the threshold temperatures among species are well established, evolutionary adaptive responses to thermal stress, as well as for other stresses, is still poorly understood.

Heat shock proteins (HSP) are one of the most known mechanisms involved in most organisms to deal with stress, such as that caused by heat (Feder and Hoffman, 1999). HSP act as molecular chaperones repairing damage induced by stress, by stabilizing and refolding denatured proteins, and may be found in reaction to stress (Wang *et al.*, 2004). The 70 and 90-kilodalton (kDa) families, HSP70 and HSP90, respectively, are the most known and the more conservative HSP induced by heat or cold stress (Arias *et al.*, 2011; Shu *et al.*, 2011; Zhao and Jones, 2012). Genetic changes in the expression of molecular mechanisms such as HSP genes might account for differences among close species or even populations as an adaptation to its environment. In fact, recent studies suggest that strongly stenothermal organisms, living in nearly constant temperature environments, whether from cold or warm environments, lack the heat shock response (Tomanek, 2010).

In the present work we estimated the survival to high temperatures and the corresponding changes in HSP70 and HSP90 in two Lepidoptera species of the same superfamily, *Thaumetopoea pityocampa* (Denis et Schiffermuller) (Noctuoidea, Notodontidae) and *Helicoverpa armigera* (Hubner) (Noctuoidea, Noctuidae). The two species are between the most environmentally and economically important pests, in forest and agricultural ecosystems, respectively. Both species are present all over the Mediterranean basin, where global changes are expected to lead to more frequent extreme events of summer heat waves with several consecutive days with temperatures above 40 °C (Díaz *et al.*, 2006). In this context, it is important to understand how such events will affect insect populations.

T. pityocampa is a pine defoliator undergoing one generation per year, with an obligatory diapause during the pupal stage. Typically, the adults reproduce in sum-

mer and larval development takes place during autumn-winter. Thus, cold during winter has been considered the main limiting factor for the geographical distribution of this species (Buffo *et al*., 2007; Battisti *et al*., 2015; Roques *et al*., 2015). Nevertheless, extreme high temperatures were suggested also a possible cause of high mortality of early instar larvae during summer/fall heat waves as observed in France in 2003 (Robinet *et al.*, 2013).

H. armigera is a multivoltine species exhibiting up to 2-5 generations per year in the Mediterranean Basin (Meierrose *et al*., 1989; Arnó *et al*., 1994; Sanchez *et al*., 2000; Mironidis and Savopoulou-Soultani, 2008). Larval development completes in about 16 days, at 25 °C (Amate *et al.*, 2000; Jallow and Matsumura, 2001; Mironidis and Savopoulou-Soultani, 2008), taking place during warm periods. This insect is widely distributed all around the world, excluding North America. Recently, it has rapidly spread in South America and it has high potential to invade Central and North America (Kriticos *et al*., 2015). It is a key-pest in several greenhouse and open field crops (Meierrose *et al*., 1989; Lammers and MacLeod, 2007; Vojoudi *et al*., 2011; Kriticos *et al*., 2015) under climate conditions where temperatures may often rise above 40 °C (Mironidis and Savopoulou-Soultani, 2008). For *H. armigera*, the upper threshold limits for the different stages are not experimentally demonstrated, as far as we know, apart from adults (Mironidis and Savopoulou-Soultani, 2010).

In this study we aimed at comparing the survival of the two species at high temperatures, mimicking heat waves. We further intended to analyse possible molecular mechanisms involved in the larval survival near the upper thermal limits of the two species. With that purpose the protein profiling and immunoblot analysis of two heat shock proteins HSP70 and HSP90, well known to be typically inducible by heat stress, were performed in the two species.

Materials and methods

Larval rearing

Larvae of *T. pityocampa* were obtained from egg masses collected in the field in Leiria area (39°50'N 8°57'W <50 m a.s.l.), Portugal. Eggs masses were collected in September 2012 and kept in the laboratory (25 ± 2 °C) until hatching. Neonate larvae were fed with pine needles of its natural host *Pinus pinaster* Aiton until reaching second instar larval stage.

Both before and during the treatments the larvae were kept in acrylic boxes (18 × 12 × 12 cm), and fed daily with fresh branch of pine needles inserted into wet floral foam (Oasis®; Smithers-Oasis). Pine branches were previously sterilized by washing with a 0.53% bleach solution, rinsed and dried with a tissue.

H. armigera larvae were obtained in the field in processing tomato crop in Lezíria de Vila Franca de Xira, Portugal (38°58'N 8°55'W). In laboratory, adults were kept in cages, about 30-40 adults per acrylic cage (30 × 30 × 40 cm) until mating and oviposition. Eggs were laid on filter paper placed on cages walls and collected from the paper to small boxes and kept under 23 °C and high humidity. Neonate larvae were transferred to plastic Petri dishes (12 × 12 cm), and fed on an artificial maize based diet adapted from Poitout and Bues (1974), reared at 23 ± 2 °C (14L:10D) conditions until reaching third instar larval stage. For heat treatments, larvae of *H. armigera* were put inside Petri dishes 9 cm diameter with a slice of the fresh maize based diet.

Larval stages and times of exposure were adjusted to the life cycles of both species. Young larvae were used for the tests, since young larval stages are the most sensitive to temperature stress (Santos *et al.*, 2011). First and second instar larva have high sensitiveness to manipulation and low body mass weight which would imply an extreme high number of larvae for the molecular analysis. On the other hand, extracts from higher instar stages of *H. armigera* would contain a higher lipid level that would make difficult the extraction and the analysis of the heat shock proteins (authors' observation). So, 3rd instar of both species should be used but 3rd instar larvae *T. pityocampa* are urticating and thus difficult to manipulate. Therefore, we selected larvae of the 2nd instar for *T. pityocampa* and 3rd instar for *H. armigera*.

Heat treatment

Larval developmental time of the two studied species is very different. Whereas 2nd instar larvae *T. pityocampa* takes about 15 days (at c.a. 22 °C) to moult (Berardi *et al.*, 2015), 3rd instar larvae *H. armigera* takes only about 2 days (at 25 °C) (e.g. Amate *et al*., 2000; Liu *et al.*, 2004). Therefore, heat treatment times used for *T. pityocampa* and *H. armigera* were adjusted for each species.

For *T. pityocampa* larvae, thermal stress consisted of three days with high maximum diurnal temperature (T_{max}) mimicking intense hot summer days (14L: 10D). Treatments were conducted with relative humidity fixed at 60% and programmable temperature and photoperiod. Temperature program was set to begin at 10 °C below the target maximum daily temperature (T_{max}) during the scotophase. After 1 h for larval adaptation, the temperature increased at a rate of 1.2 °C h^{-1} until T_{max} was reached. T_{max} that was maintained for 4 h, declining thereafter at a rate of 1.2 °C h^{-1} until reaching minimum nocturnal temperature (figure 1).

For *H. armigera*, thermal stress consisted of 20 h treatment. In the scotophase period (10 h) temperature program was set to begin with 25 °C and after a 30 min period for larval adaptation, temperature increased during 270 min up to 10 °C below the T_{max} and this temperature was maintained for 300 min. Then, in the beginning of the photophase period (10 h), temperature was increased 1.6 °C h^{-1} during 300 min and 2.0 °C h^{-1} during 60 min up to the T_{max} that was maintained for 4 h (figure 1).

Tests were conducted in climatic chambers, models Fitoclima 700 EDTU and S600 (ARALAB, Portugal), for T_{max} = 36, 38, 40, 42 and 44 °C for *T. pityocampa* and T_{max} = 38, 40, 42, 44 and 45 °C for *H. armigera*. The temperature upper limit of the climatic chambers was 45 °C conditioning the maximum temperature tested. For both species, a control group was kept in climatic

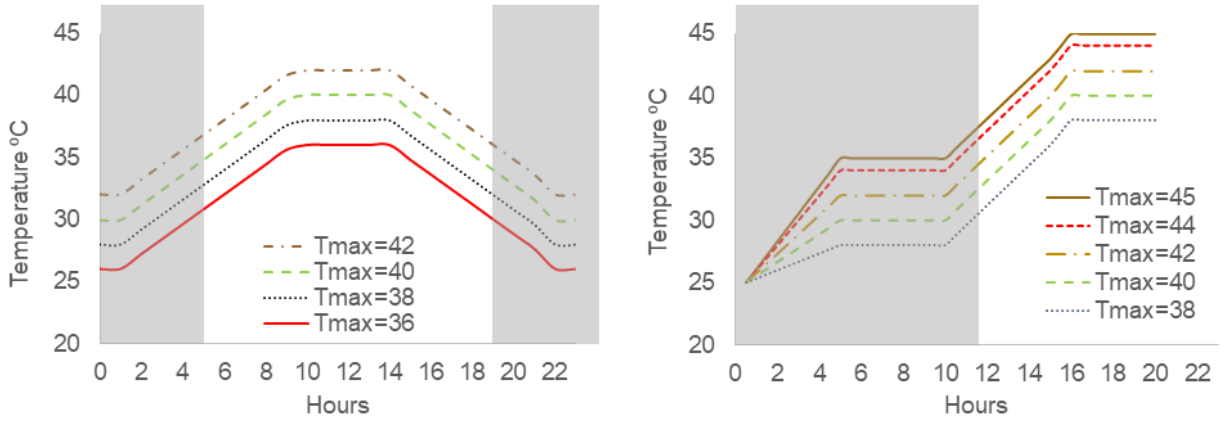

Figure 1. Daily fluctuations in temperature programed in the climatic chambers for the trials with *T. pityocampa* (left) and *H. armigera* (right). Tmax - maximum daily temperature (°C). Grey areas correspond to the scotophase (10 h).

chamber at 25 ± 1 °C for the same period and then used for survival and HSP comparison with treated groups.

Each trial consisted of test boxes, exposed to one T_{max}. For the survival trials for *T. pityocampa* each box (7 to 12 replicates) contained a sample of 20 2nd instar larvae, and for *H. armigera* it contained about 5-10 3rd newly moult instar larvae per dish to avoid cannibalism (10 to 19 replications in a total of 50-250 larvae). To guarantee genetic homogeneity among the replications on both species larvae were mixed from several origin families and then subsamples were collected at random.

Protein extraction, electrophoresis and immunoblotting

HSP70 expression was determined for heat treatment of 38 °C and 40 °C for both species. HSP90 expression was determined for temperatures of 38 °C for *T. pityocampa* and 38 °C, 40 °C and 42 °C for *H. armigera*. For both species, live larvae were collected at the end of the 4 h of T_{max}, when we expected that HSP expression should attain a peak. The live larvae were then immediately frozen in liquid nitrogen and preserved at −80 °C until being processed. In all cases, the control consisted of larvae kept at constant temperature of 25 °C. Samples for both species contained the amount of larvae needed to obtain the mass of 0.25 g for protein extraction (90-100 larvae for *T. pityocampa*; 20-40 larvae for *H. armigera*).

The desired larvae tissue samples (ca. 0.25 g per sample) were homogenized at 4 °C in extraction buffer consisting of 100 mM Tris (hydroxymethyl)aminomethane - HCl (Tris-HCl), pH 8.0; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mM 2-mercaptoethanol and 2% (w/w) polyvinylpyrrolidone (PVP). Extracts were centrifuged at 10,000 *g* for 10 min at 4 °C. The amount of protein in the resulting supernatants was quantified by the Lowry-Bensadoun method (Bensadoun and Weinstein, 1976). Aliquots of the protein homogenates were prepared for electrophoresis by heating for 4 min at 98 °C in the presence of 2% (w/v) SDS; 0.1M 2-mercaptoethanol and 10% of glycerol.

After centrifugation at 10,000 *g* for 1 min, protein extracts were loaded onto the gels and subjected to discontinuous SDS-PAGE (11% polyacrylamide in the resolving gel) as described by Laemmli (1970). Polypeptides were electrophoretically transferred from the gel to polyvinylidene difluoride (PVDF) membrane, probed with HSP70 or HSP90 mouse antibodies from Sigma and detected by colorimetry. The secondary antibody used for detection was goat anti-mouse IgG (H+L) alkaline phosphatase conjugate from Sigma.

Survival analysis

For the survival analysis, the larvae exposed to the heat treatments were first brought to room temperature and 2-3 h later were checked for larval mortality. Larvae that did not move when touched were considered as dead.

Survival data was treated by generalized linear models (GLM), with binomial distribution and logit link function, in which the dependent variable (larvae living in the end of treatment) was related to the temperature. Each species was treated separately. Tests of models effects are expressed in the form of Wald Chi-square (W χ^2) and *p*-value. This analysis was performed using the software IBM SPSS Statistics 20.0.

Results

Larvae survival

For *T. pityocampa* the survival rates differed among heat treatments (W χ^2 = 118.43, $p < 0.001$). The negative effect of thermal stress was significant at both 40 °C and 42 °C, with a decrease of 30% and 48% respectively, relatively to the control (95.0 ± 1.5%). Mortality was 100% at T_{max} of 44 °C (figure 2).

Up to T_{max} of 42 °C, thermal stress had no significant effect on *H. armigera* larval survival (figure 2). Survival decreased in comparison with control when T_{max} rose to 44 °C (−9%; $p = 0.001$) and in particular at 45 °C (−66%; $p < 0.001$) (W χ^2 = 134.402, $p < 0.001$). *H. armigera* larvae surviving exposure to the highest studied temperatures (42 °C to 45 °C) did not moult to the next instar during the treatment but the majority of the larvae exposed to 38 °C and 40 °C did.

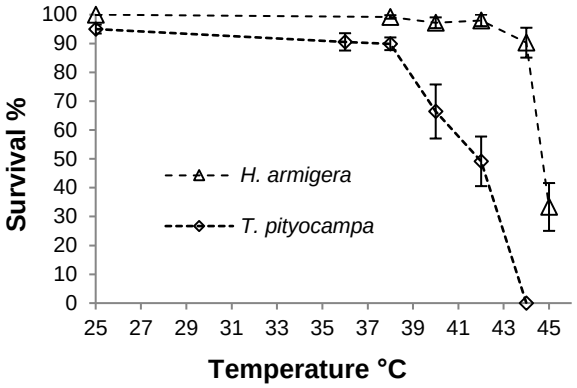

Figure 2. Larvae survival (mean ± SE) of young larvae of *T. pityocampa* and *H. armigera* at different maximum temperatures. Survival in control groups (25 °C) was 95.0 ± 4.1% and 100% for *T. pityocampa* and *H. armigera*, respectively.

Expression of HSP70 and HSP90

Commercially available antibodies to HSP70 and HSP90 (Sigma) recognized polypeptides of the appropriate apparent molecular weights on western blots from *T. pityocampa* and *H. armigera* extracts (figures 3, 4 and 5).

The western analysis demonstrated that *T. pityocampa* larvae exposed to 25 °C, 38 °C and 40 °C had similar levels of HSP70. The polypeptides (72 and 70 kDa) are visible in the western blots from the beginning of the experiment. The two bands are present in large amount emerging as a large band. However, with careful analysis it is possible to realize that they are two overlapping bands rather than one (figure 3). The HSP70 from *H. armigera,* the low-molecular-mass (HSP70) and the high-molecular-mass (HSP72) isoforms, were much more visible (figure 4, HSP70b and HPS70a respectively). A number of low-molecular-weight polypeptides were also detected by the heat shock protein HSP70 antibodies in all extracts of *H. armigera* (figure 4). However, the two HSP70 polypeptides (72 and 70 kDa constitutive and stress-inducible polypeptides, respectively) were not detected in western blots from *H. armigera* larvae collected at 25 °C. In addition, the HSP70 polypeptides were hardly detectable in *H. armigera* under the temperature of 38 °C and only increased their levels when larvae were exposed to 40 °C (figure 4a-b). The stress-inducible HSP polypeptide (70 kDa) seems to be more abundant than the 72 kDa at 40 °C (figure 4b).

Regarding the HSP90 polypeptides, they were visible in all the western blots of *T. pityocampa* and *H. armigera* (figure 4). However, HSP90 increased with thermal stress on samples of *H. armigera* originating a boost at 42 °C (figure 5).

Discussion

Variability and adaptation to thermal stress

With global warming many species and populations will be subjected to higher temperatures, which are expected to increase in most terrestrial ecosystem during this century (Moritz and Agudo, 2013). In particular, heat waves above 40 °C are expected to become more frequent in temperate and Mediterranean areas whereas in the past this type of events was rare (Díaz *et al.*, 2006). Ultimately such events may cause death of a significant part of populations. As an example, a strong population decline for *T. pityocampa* was observed in Central France following a heat wave period in 2003 (Robinet *et al.*, 2013). In evolutionary terms such mechanism may suddenly cause a strong selective pressure as only the individuals well adapted to such extreme conditions will survive.

Insects seem to be constrained in their upper thermal tolerance by a maximum for all species of about 42 °C, due to the limitations imposed by their respiratory system and water balance (Denlinger and Yocun, 1998; Pörtner, 2002; Hoffmann *et al*., 2013). Nonetheless, the upper thermal tolerance varies largely among species from about 25 °C up to 42 °C (Deutsch *et al.*, 2008). From present results, the two Lepidoptera species studied are thus among those with higher levels of tolerance to heat stress, which was about 38 °C in *T. pityocampa* and 44 °C in *H. armigera*. Since *T. pityocampa* has mostly larval winter-development, it is extraordinary that this species shows a high level of tolerance to upper temperature. However, since egg laying occurs in sum-

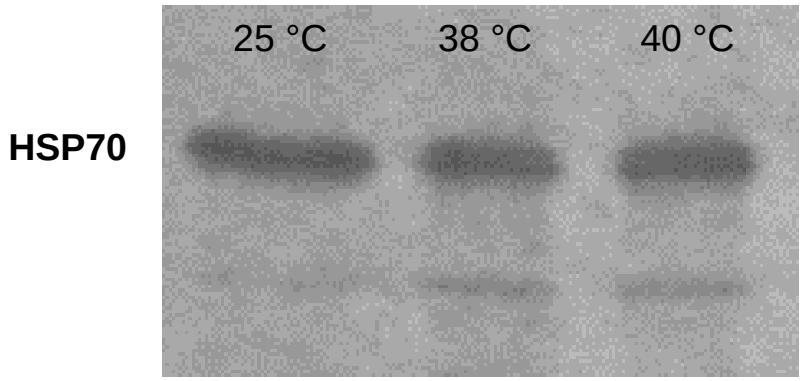

Figure 3. Western blot analysis of HSP70 polypeptides from *T. pityocampa* larvae tissue samples, collected at different temperatures (25 °C, 38 °C and 40 °C). The PVDF membranes were probed with antibodies specific against HSP70.

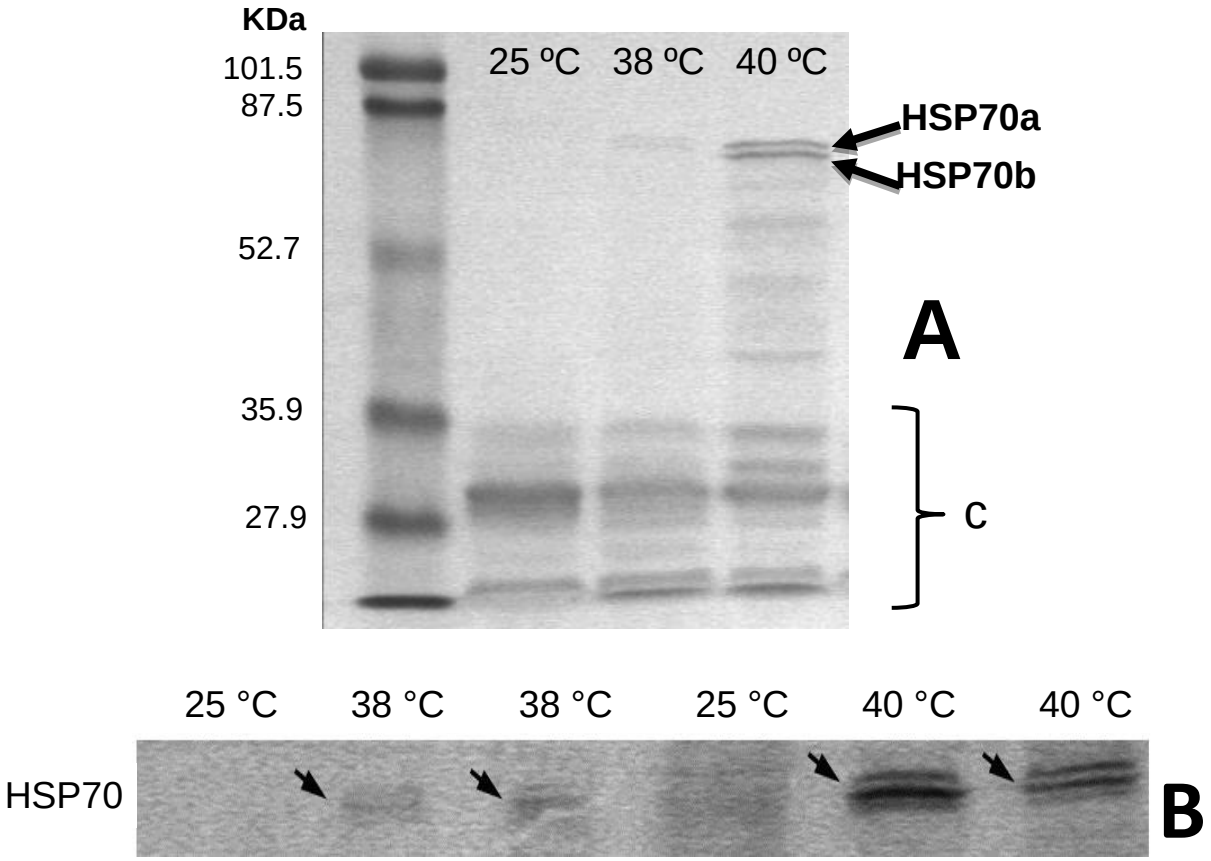

Figure 4. A) Western blot analysis of HSP70 polypeptides of larvae tissue samples from *H. armigera* collected at different temperatures (25 °C, 38 °C and 40 °C). The PVDF membranes were probed with antibodies specific against HSP70. For comparison the molecular mass standards are shown: 101.5 kDa - 27.9 kDa; HSP70a and HSP70b - the polypeptides of 72 and 70 kDa respectively; C - low-molecular-weight polypeptides also detected by HSP70 antibodies. **B**) Membrane section of western blot analysis of HSP70 polypeptides from *H. armigera* collected at the same different temperatures.

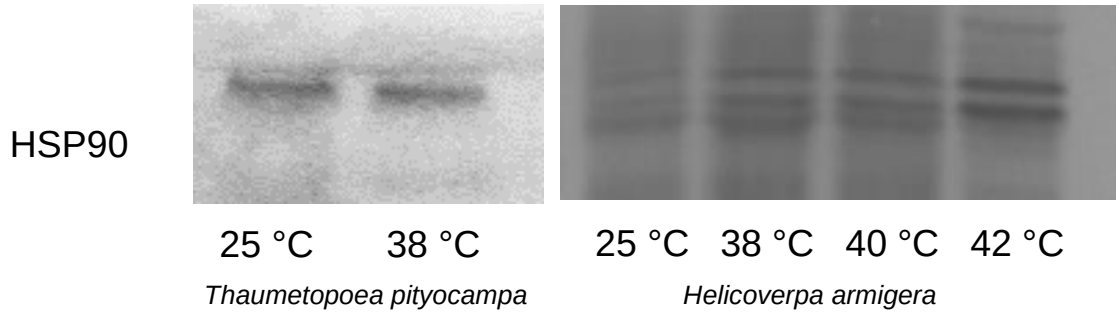

Figure 5. Western blots analysis of HSP90 polypeptides of larvae tissue from *T. pityocampa* samples, control (25 °C) and thermal heat stress (Tmax = 38 °C) and from *H. armigera* collected from control (25 °C) and thermal heat stress trials (Tmax = 38 °C, 40 °C and 42 °C). The PVDF membranes were probed with antibodies specific against HSP90.

mer, larvae born in the end of summer may be exposed to high temperatures under Mediterranean climate conditions. The tolerance observed was particularly high in *H. armigera* (44 °C) which may be justified by its distribution in tropical and Mediterranean climates (Zhou *et al.*, 2000). Still, at the highest temperatures tested a negative effect on moulting on this species was observed. By a modelling approach, Mironidis and Savopoulou-Soultani (2008) obtained upper threshold for the larvae of 41.8 °C or 43.2 °C, considering fluctuating or constant temperatures, respectively. Our data support experimentally these models and reinforces the resistance of *H. armigera* larvae to high temperatures. We did not follow development of the larvae further on and we did not evaluate other possible effects of thermal stress on other life stages or on fertility. Mironidis and Savopoulou-Soultani (2010) found that *H. armigera* did not reproduce when adults are exposed to 40 °C for 6 h.

We hypothesize that a negative effect on fertility might also happen when larvae are exposed to high temperature, which needs to be investigated.

Daily fluctuations

As critical as finding the upper thermal tolerance is its definition and the method used. For a given individual death is a function of temperature and exposure time. In thermal static methods the thermal tolerance limits are obtained maintaining organisms at constant temperature for a specific time period and their survival evaluated at the end of the trial. Naturally, results depend on the time period specified. In alternative, the dynamic methods consist in increasing the temperature at a specific rate until a lethal temperature is achieved and the organism dies (Addo-Bediako *et al.*, 2000). Again, this method depends on the rate of change and might overestimate upper thermal limits under which an organism may live (Rezende *et al.*, 2011). These experiments are frequently unrealistic in comparison with the conditions faced by organisms in nature since organisms usually not live at constant temperatures, and, especially on temperate climates, temperatures may greatly vary along a unique day. Thus, exposure to high temperatures occurs during only a part of the day and might be repeated in time in consecutive days. Effects of daily fluctuations in temperature are not usually considered when assessing the species tolerance to thermal stress. Yet, daily temperature variation may significantly increase or decrease survival compared with the constant baseline temperature (Paaijmans *et al*., 2013; Rocha *et al.*, 2017). In the present study we used a daily fluctuation with 10 °C temperature amplitude. This approximately mimics very hot days in the studied region. Mironidis and Savoupolou-Soultani (2008; 2010) showed that *H. armigera* cannot develop from egg to adult at constant temperatures outside the range 17.5-32.5 °C; however, if the adverse temperature is not constant, as in nature, it reaches adult stage facing temperatures within a wider range, 10-35 °C.

Expression of heat shock proteins

In this study, the commercial antibodies to HSP70 recognized the corresponding polypeptides from *H. armigera* and *T. pityocampa,* contrarily to what was found by Díaz *et al.* (2015) on *Drosophila melanogaster* Meigen and especially on *Bemisia tabaci* (Gennadius). The molecular weights of the constitutive (or cognate) and stress-inducible HSP polypeptides are 72 kDa and 70 kDa, respectively. The antibodies used against HSP70 also reacted with low-molecular-weight polypeptides which may be HSP70 fragments generated by protein degradation.

Among heat shock proteins, HSP90 and HSP70 are the major families reported in relation to thermal stress (Feder and Hofmann, 1999; Zhao and Jones, 2012). At cellular level a rise in these HSP may generate a shift in upper (heat stress) and lower (cold stress) onset temperatures for protein denaturation. The two species differed on the HSP expression following heat stress, evidencing differences on the molecular mechanisms involved. For *H. armigera* larvae, HSP70 was found to be thermal inducible, and HSP90 expression increased with temperature. But for *T. pityocampa* the proteins were also found present in the control group at 25 °C. HSP70 was also found to be thermal induced on *H. armigera* in salivary glands' tissue cultures of the 5th instar larvae (Singh and Lakhotia, 2000) and on pupae of *Helicoverpa zea* (Boddie) (Zhang and Denlinger, 2010), a close related species. A great increase at 42 °C in the immunoreactive HSP90 in *H armigera*, coupled with the low mortality at this temperature, indicates that at 42 °C this species has efficient mechanisms to cope with the heat stress.

The presence of HSP70 (constitutive or cognate) in *T. pityocampa* at all temperatures, without an increase on stress-inducible polypeptide, suggests a lack of an inducible response of the HSP70 in this species. The mechanistic bases for the absence of an inducible HSP response vary among taxa. In certain species this lack could be modulated at transcriptional level, where heat shock transcription factors are bound and thereby inactivated and sequestered by the constitutive HSPs (Tomanek, 2010). In other species, the rapid degradation of HSP mRNA is responsible for the absence of inducible HSP (Tomanek, 2010).

Conclusions

Our results demonstrate significant major differences between the two species on the upper thermal tolerance, when exposed to heat waves, and that the expression of HSP might be one of the molecular mechanisms involved. Thermal tolerance was extremely high for *H. armigera*, up to 44 °C, which might be accounted by the expression of both HSP70 and HSP90 on this species, particularly noticeable at the higher temperature analysed.

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Authors' addresses: Manuela BRANCO (corresponding author: mrbranco@isa.ulisboa.pt), CEF - Forest Research Centre, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal; Maria Glória ESQUÍVEL, Miguel CORTEZ, Elisabete FIGUEIREDO, LEAF - Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal.

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