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Heat shock proteins in *Varroa destructor* exposed to heat stress and in-hive acaricides

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Abstract

Varroa destructor is one of the major pests that affect honeybees around the world. Chemical treatments are common to control varroosis, but mites possess biochemical adaptive mechanisms to resist these treatments, enabling them to survive. So far, no information is available regarding whether these pesticides can induce the expression of heat shock protein (Hsp) as a common protective mechanism against tissue damage. The aims of this study were to determine differences in heat shock tolerance between mites collected from brood combs and phoretic ones, and to examine patterns of protein expression of Hsp70 that occur in various populations of *V. destructor* after exposure to acaricides commonly employed in beekeeping, such as flumethrin, *tau*-fluvalinate and coumaphos. Curiously, mites obtained from brood cells were alive at 40 °C, unlike phoretic mites that reached 100% mortality, demonstrating differential thermo-tolerance. Heat treatment induced Hsp70 in mites 4 × more than in control mites and no differences in response were observed in phoretic versus cell-brood-obtained mites. Dose–response assays were carried out at increasing acaricide concentrations. Each population showed a different stress response to acaricides despite belonging to the same geographic region. In one of them, coumaphos acted as a hormetic stressor. Pyrethroids also induced Hsp70, but mite population seemed sensitive to this treatment. We concluded that Hsp70 could represent a robust biomarker for measuring exposure of *V. destructor* to thermal and chemical stress, depending on the acaricide class and interpopulation variability. This is relevant because it is the first time that stress response is analyzed in this biological model, providing new insight in host-parasite-xenobiotic interaction.

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Introduction

The ectoparasitic mite *Varroa destructor* (Mesostigmata: Varroidae) is considered one of the major threats that affect managed honeybee colonies worldwide (Le Conte et al. 2010; Rosenkranz et al. 2010). This mite is the etiologic agent of varroosis and constitutes a vector of viral diseases (Sumpter and Martin 2004).

Due to anthropogenic pressure, *V. destructor* shifted from the original host, *Apis cerana*, to *Apis mellifera* (Oldroyd 1999), causing major economic losses in productive hives and indirectly affecting feral honeybees due to drift. In most cases, colony survival depends on primary disease control by means of the reduction of mite populations by chemical treatments including a wide range of acaricides belonging to various chemical classes. Most of these substances persist in apicultural matrices promoting the selection of resistant mites. Resistance cases have been reported in several countries (Milani 1995, 1999; Hillesheim et al. 1996; Elzen et al. 2000; Elzen and Westervelt 2002; Pettis 2004; Maggi et al. 2009).

The mite life cycle comprises two distinct phases: a phoretic phase, where adult female mites stay on honeybees and feed on hemolymph, and a reproductive phase taking place inside bee brood cells (Donzé and Guerin 1994). During their in-cell development, *Varroa* mites prefer temperatures between 26 and 33 °C (reviewed in Rosenkranz et al. 2010), and can tolerate the temperature on bee brood, which is higher. In regions showing marked seasons, the temperature inside the hive can vary greatly. During summer, the brood area is usually maintained between 32 and 37 °C (Free and Spencer-Booth 1958; Jay 1964), while in the broodless areas of the hive, temperature ranges from 28 to 33 °C (Kaya et al. 1982). In winter, honeybees maintain their nests at 20–30 °C (Free and Spencer-Booth 1958; Simpson 1961; Butler 1974) but the temperature may drop occasionally. To our knowledge, biochemical changes in the form of stress response to heat or chemical treatments have not been studied in *V. destructor*.

Heat shock proteins (Hsp) act as molecular chaperones to protect cells from the harmful effects of protein denaturation under adverse conditions. For that reason, their expression levels increase in response to different stressors (Hightower 1991; Sanders 1993), although under normal conditions they are also expressed constitutively (heat shock cognates, Hsc) (Ashburner 1982). There are several families of Hsps that can be classified according to their molecular weight and sequence homology (Carper et al. 1987). The major heat shock protein Hsp70 is ATP-dependent and interacts with other proteins, promoting essential processes such as protein synthesis, cell signalling, transcription, and metabolism (reviewed in King and MacRae 2015). Besides, Hsp70 families have been a subject of many evolutionary studies which have demonstrated that despite the fact that they are highly conserved molecular chaperones among phylogenetically distant organisms (Lindquist 1986; Feder and Hofmann 1999), natural selection often acts on their expression levels (Hoffmann et al. 2003), DNA sequences (Starrett and Waters 2007), and their number of copies (Tsai et al. 2013) in order to adapt to the local environment. Stress tolerance measured by Hsps involves integrated cellular signalling pathways that regulate cell survival and apoptosis (Beere 2004; Morimoto et al. 1994). Nevertheless, evidence indicates that Hsp70 expression patterns vary according to stress responses, environmental conditions and developmental stages (Rinehart et al. 2000; Lakhotia and Prasanth 2002; Mahroof et al. 2005; Pae et al. 2007).

The aim of the present study was to investigate the expression levels of Hsp70 in *V. destructor* mature imago, in response to temperature and increasing concentrations of acaricides from four populations. As a first approach, parasites were exposed to heat stress in order to establish specificity of monoclonal antibodies by Western blot technique.

Materials and methods

Mites

Two trials were performed in order to assess the effect of two main treatments: temperature (heat shock) and synthetic acaricides. To perform trial 1, three brood combs obtained from Langstroth hives, with intermediate prevalence of *Varroa*, emplaced in an experimental apiary (38°10′06″S, 57°38′10″W), were maintained at 29 °C and 34% RH. No chemical treatment was applied to the colonies since 1 year before and during the experiment. Female *Varroa* mites were collected during their reproductive phase from manual uncapping brood cells; otherwise phoretic mites were sampled randomly from nurse bees of heavily infested colonies. In order to avoid starvation, mites were placed over bee larvae on Petri dishes during the collection process. Mites that seemed newly moulted, weak or abnormal were discarded.

For trial 2 mites were obtained from hives of various apiaries in the south of Buenos Aires Province, Argentina, which presented also intermediate prevalence of *Varroa*. The apiaries were located in Orense (38°40′00″S, 59°47′00″W, population I), Tres Arroyos (38°22′00″S, 60°16′00″W, population II), Balcarce (37°50′47″S, 58°15′20″W, population III) and Mar del Plata (38°10′06″S, 57°38′10″W, population IV). Female mites were collected by manually opening individual honeybee brood cells and removing adults from bee larvae and non-pigmented pupae.

Trial 1. Acute thermal treatments

Adult female mites were exposed to different increasing temperatures in order to evaluate the Hsp70 expression under heat shock. Mites collected from inside the cells of brood combs or phoretic mites were exposed for 30 min to 29 °C (control), 40 or 50 °C. To that end, 20 mites were released in Petri dishes (9 cm diameter, 2 cm high), whose walls were covered with foam to avoid mites from coming out. Three replicates per treatment were employed. After the heat exposure, mites were immediately processed for protein analysis (see below).

Trial 2. Chemical treatments

Apiary therapeutic treatment records

Acaricide treatments were chosen based on historical therapeutic treatments received up to 2 years back from the beginning of the experiment. Dose selection was made according to 50% lethal concentration (LC_{50}) values reported in the literature (Maggi et al. 2008). The active components employed in population I (PI) were pyrethroids, therefore flumethrin was chosen as a treatment. In population II (PII) coumaphos was used, so this acaricide

was selected as a treatment, because it was suspected to generate resistance in mites, and also *tau*-fluvalinate, because mites would probably be susceptible to this drug and therefore have a higher response to stress exposure. Toxicity of coumaphos was evaluated in populations III (PIII) and IV (PIV), as hives never received this acaricide.

Chemicals

Technical grade *tau*-fluvalinate, flumethrin and coumaphos (Pestanal®; Sigma Aldrich, St. Louis, MO, USA) were employed.

Acaricide treatments

Effects of exposure to increasing doses of flumethrin (PI mites), both *tau*-fluvalinate and coumaphos (PII), and coumaphos (PIII, PIV) were evaluated according to the modified Ruffinengo et al. (2005) technique of complete exposure (Maggi et al. 2009). Two instances of sampling were performed in PIII, one in spring (PIIIA), the other in autumn (PIIIB).

Synthetic acaricides were diluted in 96% (wt/vol) of ethanol and decreasing concentrations were prepared. One ml of solution was applied to the bottom of a Petri dish (9 cm diameter, 2 cm high) which was kept uncovered until ethanol had evaporated. Depending on the number of mites obtained, 5–12 mites per replicate per treatment were placed in each Petri dish. One h after these mites were in contact with the acaricides, three healthy newly emerged bees were introduced in each dish to feed the mites. Bees and mites were exposed to acaricides during 24 h. Honeybees were fed with 3 g of candy (mix of powder sugar and water) placed on aluminium foil, and incubated at 29 °C and 40% RH during the experiment. Control treatments were only exposed to the evaporated solvent. Five replicates were done per treatment. Mite mortality was checked under a stereoscopic microscope, considering dead the immobile individuals, non-responding to touch stimulus. LC_{50} values and 95% confidence limits were estimated by using Minitab software according to USEPA (1986). Natural mortality values were adjusted according to Abbott (1925). After chemical exposure, mites were immediately processed for protein analysis.

Sample preparation

After exposure, *V. destructor* individuals from each group were sampled and pooled to constitute a triple observation for trial 1 and a single observation for trial 2. Whole bodies were homogenized with anticoagulant II buffer (Mead et al. 1986) adjusted at pH 6.5 (12 µl of buffer per collected mite). Then, 0.5 µl from a 200 mM solution of protease inhibitor (PMSF) was added to the homogenate in order to avoid protein degradation. To disaggregate tissues, the sample tubes were submerged in liquid nitrogen and ground using plastic micropestles attached to a power drill. This was carried out 3 × per sample for 5 min. The homogenates were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatants were used for total protein determination and analysis of Hsp70 expression. Determination of total protein in samples was performed in triplicate by using the method in Bradford (1976) at 595 nm and bovine serum albumin as the protein standard. To store homogenates for protein analysis, Laemmli Buffer 5 × was added, then samples were boiled for 2 min and frozen at − 20 °C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples denatured in a boiling water bath with 1×Laemmli Buffer (Laemmli 1970) for 2 min, and equal quantities of protein (20 µg of total protein/well) were loaded on 12% gels (0.75 mm thick) by using a Mini-PROTEAN apparatus (BioRad, Hercules, CA, USA). Electrophoresis was carried out at 170 V for 1 h in 1×running buffer. Pre-stained molecular mass standards ranging from 26.6 to 180 kDa (Sigma-Aldrich) were run in each gel.

Western blot analysis of Hsp70

The proteins resolved on the SDS-PAGE were transfered onto nitrocellulose membrane (RPN303C Hybond C, GE Healthcare, Amersham, UK) at 15 V for 25 min by employing Semi-dry BioRad Transblot with 1×transfer buffer. Blotted proteins were stained by Ponceau S solution in order to check the efficiency of transfer and consistent blotting procedures. Then membranes were washed with distillate water. In order to immuno-detect Hsp70, membranes were incubated for 2 h with blocking solution (3% non-fat dry milk-TBS) following an incubation o/n at 4 °C with primary monoclonal anti-bovine brain Hsp70 antibody produced from mouse (H5147; Sigma-Aldrich), diluted 1:2500. The secondary antibody was goat anti-mouse IgG conjugated with horse-radish peroxidase (Santa Cruz) diluted 1:5000. Membranes were incubated during 2 h, followed by the addition of NBT and BCIP for enzymatic signal detection.

Image analysis and quantification of Hsp70 expression

Relative immunoreactivity was determined by densitometry (Image J). Densities from mites exposed to heat shock or chemical treatments were normalized to the density of the Hsp70 band quantified in the control treatment. Two technical replicates were made in order to test the reproducibility of the methodology.

Results

Antibody specificity

The results show that heat shock and acaricides induce a protein with a molecular weight of around 70 kDa that highly cross-reacts with anti-Hsp70 antibodies. Clear and single bands were present on the immunoblotted membranes (Fig. 1).

Mite survival and Hsp70 expression under heat exposure

All mites exposed at 29 °C, including those obtained from cell and phoretic ones, were alive after 30 min. The same result was registered also for mites obtained from brood cells exposed to 40 °C. However, 100% mortality was recorded in phoretic mites at 40 °C and in mites from both origins at 50 °C. In any case, dead or live mites were collected for protein analysis.

According to our results, heat treatment induces Hsp70 in *V. destructor*. Also, basal level response was recorded in control individuals. Instead, the expression of Hsp70 at 40 °C was 4×higher compared to controls (Fig. 1), then its level declined at 50 °C. No

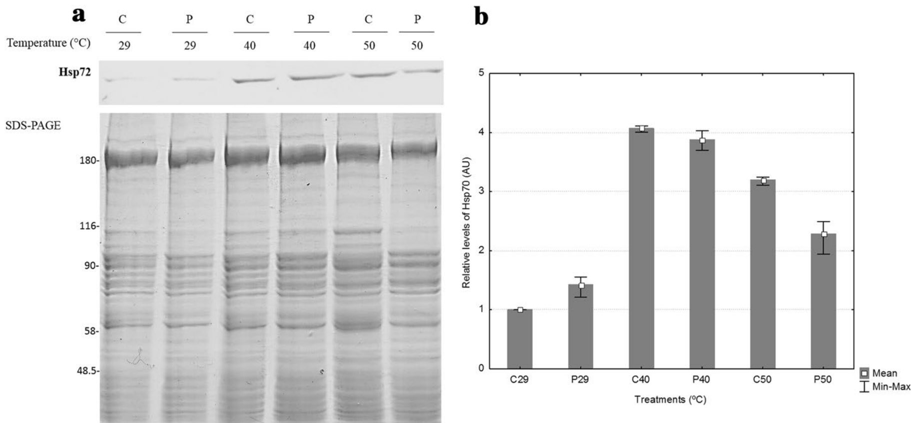


Fig. 1 Western blotting analysis of Hsp70 in the homogenates of pools of whole bodies of *Varroa destructor* exposed to heat shock. Cross-reactivity of primary antibodies, monoclonal anti-Hsp70 with *Varroa* proteins. **a** SDS-PAGE, no protein degradation was observed in dead mites. Densitometric pattern of Hsp70 levels, in arbitrary units (AU), relative to controls (n = 3). **b** Western blot, the bands correspond to Hsp72. C29: mites from cells incubated at 29 °C (control), the mean temperature inside hives. P29: phoretic mites exposed to 29 °C. C40: mites from cells incubated at 40 °C, a high temperature. P40: phoretic mites exposed at 40 °C. C50: mites from cells exposed at 50 °C, an extremely high temperature. P50: phoretic mites exposed at 50 °C

differences in protein expression were recorded between the responses obtained in mites from cells or phoretic ones under the same heat exposure.

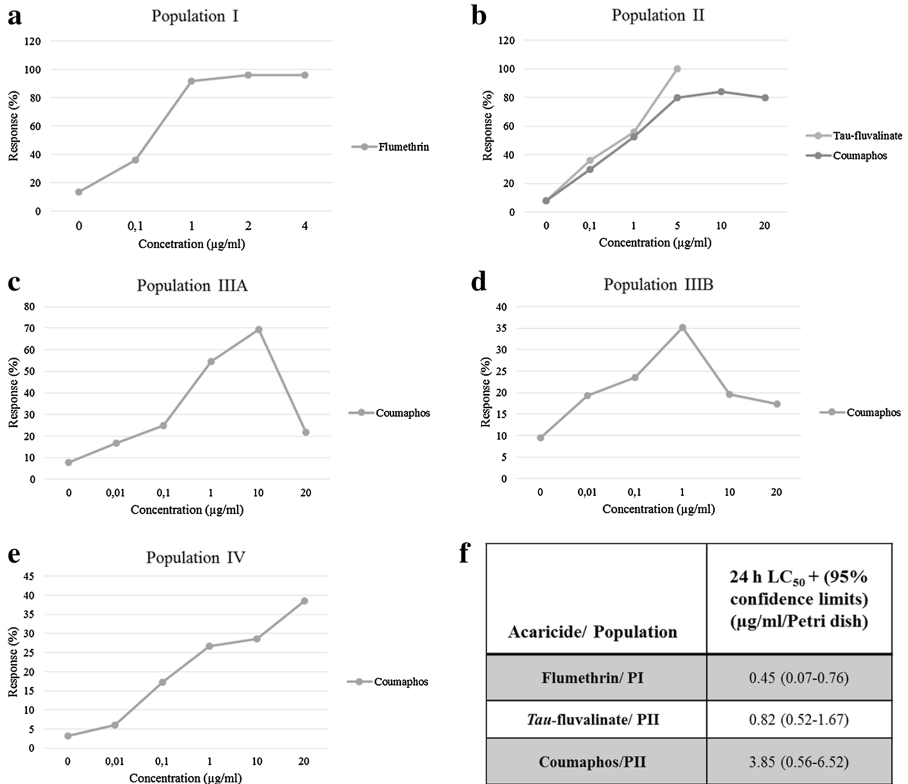
Mite mortality under chemical stress

In all bioassays, mortality in the control treatment was lower than 14%. LC_{50} values were obtained in PI and PII (Fig. 2a, b) but not in PIII (Fig. 2c, d) and PIV (Fig. 2e), because data did not fit a dose–response curve, or because the response was lower than 50%, as was recorded for the highest concentration of coumaphos in PIV (Fig. 2e). Given the strange behaviour in the dose–response curve obtained in PIIIA, the experiment was repeated in autumn, increasing the sample size (PIIIB).

Despite the historical use of pyrethroids in the apiary source of PI (Fig. 2a), high mortality of mites was recorded under exposure to flumethrin and protein expression could only be evaluated in two concentrations. For mites from PIII and PIV, a high rate of survival under coumaphos was shown. Nevertheless, PIII had never received coumaphos as a therapeutical treatment.

Hsp70 expression in *Varroa destructor*

Hsp70 expression was variable in each population, indicating a different response compared to chemical stress. Females from PI proved susceptible to the tested concentrations of flumethrin. In this population, stress response to the lowest concentration was 2.5 × greater than in the control treatment (Fig. 3). Hsp70 induction is considered significant if it is expressed at least 1.5 × higher than in non-treated individuals (Snutch et al. 1988; Requena et al. 1992; Qin et al. 2003).



Acaricide/ Population	24 h LC_{50} + (95% confidence limits) (μg/ml/Petri dish)
Flumethrin/ PI	0.45 (0.07-0.76)
***Tau*-fluvalinate/ PII**	0.82 (0.52-1.67)
Coumaphos/PII	3.85 (0.56-6.52)

Fig. 2 Dose–response curves performed with raw data. **a**–**e** Mortality (%) of *Varroa destructor* obtained from each population exposed to different chemical agents at increasing concentrations during 24 h. **f** LC_{50} values from curves that fit to PROBIT model for mites exposed during 24 h

Mites from PII exposed to coumaphos elicited a response at ≥ 1 μg/ml per Petri dish. Besides, minor changes in the relative expression of Hsp70 were revealed among the greatest concentrations (Fig. 4a, b). Mites from this population treated with *tau*-fluvalinate also showed differences in response to chemical stress. At both concentrations (0.1 and 1 μg/ml per Petri dish) Hsp70 expression was significantly higher than in controls (Fig. 4c, d).

Results were similar in PIIIA and PIIIB. Hsp70 increased its expression along with the increase of concentration of the acaricide from 0.1 to 1 μg/ml, after which a notorious drop was recorded (at 10 μg/ml). At the next concentration (20 μg/ml per Petri dish) relative expression rate increased again (Fig. 5a, b). However, in PIIIB (Fig. 5c, d) in non-treated mites and in mites treated with the lowest concentration, minimal basal response was observed.

In mites from PIV, again a response 1.5 × higher than in the control was elicited from 0.1 μg/ml. Here, the expression of Hsp70 increased linearly with increasing concentration of coumaphos (Fig. 6).

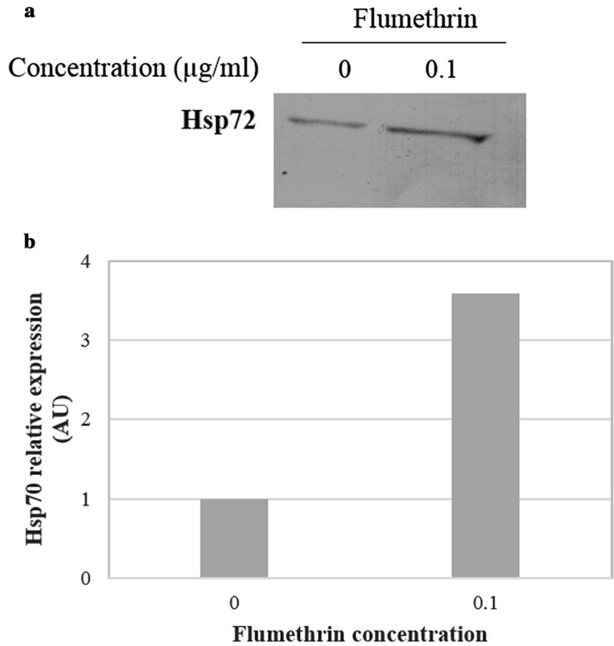


Fig. 3 Hsp70 expression relative to controls, in arbitrary units (AU). Surviving *Varroa destructor* collected from PI exposed to flumethrin. **a** Western blot, band indicates specific union with primary antibody anti-Hsp70. **b** Plot of data obtained by densitometry

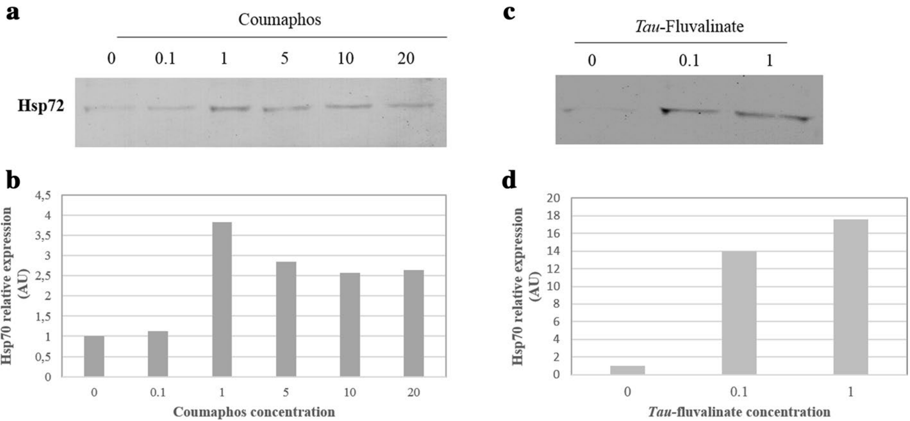


Fig. 4 Hsp70 expression relative to controls, in arbitrary units (AU). Surviving *Varroa destructor* collected from PII. **a** Western blot of proteins of mites exposed to increasing concentrations of coumaphos. **b** Plot of data obtained by densitometry. **c** Western blot of proteins of mites exposed to increasing concentrations of *tau*-fluvalinate. **d** Plot of data obtained by densitometry

Discussion

Heat shock proteins (HSPs) are molecular chaperones, and their overexpression enhances the survivability and stress tolerance of the cell. Hsp70 expression under adverse conditions was described in many animal species, including insects (King and MacRae 2015); however, this is the first report in *Varroa* mites.

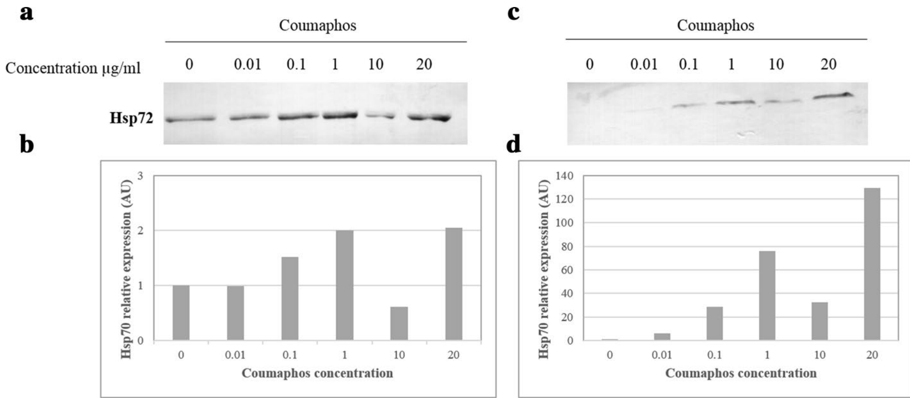


Fig. 5 Hsp70 expression relative to controls, in arbitrary units (AU). **a** Western blot of proteins of surviving *Varroa destructor* collected from PIIIA (spring sampling) exposed to increasing concentrations of coumaphos. **b** Plot of data obtained by densitometry. **c** Surviving mites collected from PIIIB (autumn sampling) exposed to increasing concentrations of coumaphos. **d** Plot of data obtained by densitometry

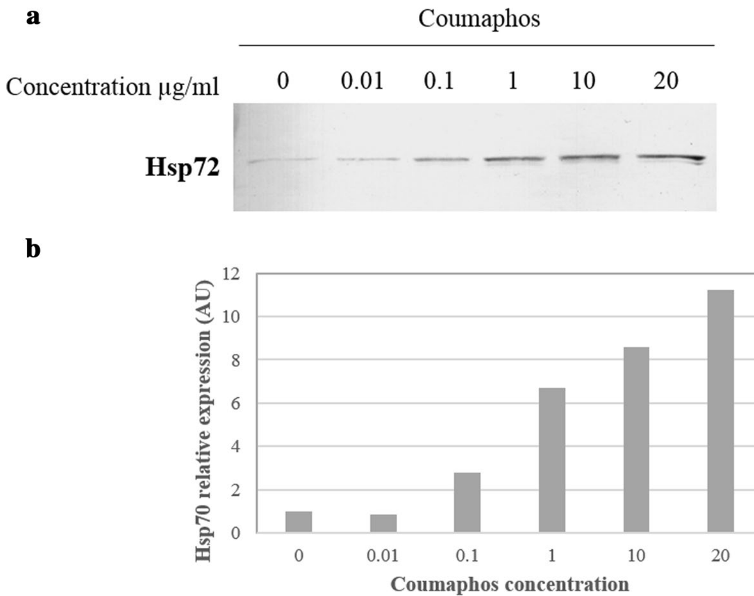


Fig. 6 Hsp70 expression relative to controls, in arbitrary units (AU). **a** Surviving *Varroa destructor* collected from PIV exposed to increasing concentrations of coumaphos. **b** Plot of data obtained by densitometry

The use of elevated temperatures has been widely recognized as a strategy to control *Varroa* mites inside hives by means of air flow at 43–48 °C during 5–15 min (Karpov and Zabelin 1978). As expected, in our assay the thermal stress at 50 °C induced 100% of mortality in mites, both obtained from bee brood cells or from the body of the bee (phoretic mites). However, a differential thermo-resistance was registered in the 40 °C exposure treatment. Interestingly, at this temperature all phoretic mites died, whereas those obtained

from the capped brood cells remained alive after 30 min of exposure. We suggest that this response to thermal stress is consistent with ambient conditions during mite ontogeny, as during the developmental stages inside the sealed brood, mites have to tolerate a constantly elevated temperature (Becher and Moritz 2009; Rosenkranz et al. 2010) and to adapt to new environmental conditions. Nest temperature is regulated by honey bee workers in order to complete the brood development. Bauer et al. (2018) demonstrated that infestation by *V. destructor* leads to a slightly increased temperature of the infested brood cells and workers are able to detect little variation of temperature in order to decide cell removal. It seems that the host-parasite association results in a 'behavioural fever' (Campbell et al. 2010).

No marked differences were found in Hsp70 expression between the origins of the mite sampling. However, our results demonstrate that exposure to 40 °C caused the highest peak of protein expression in all mites and expression levels decreased as temperature increased (50 °C), possibly due to the beginning of proteolysis affecting Hsp70 integrity. Furthermore, high temperature may cause disruption in mechanisms of protein synthesis impairing Hsp70 expression (Boutet et al. 2003), as previously reported in the mite *Tetranychus cinnabarinus* (= *T. urticae*) (Li et al. 2009).

Qualitative Hsp70 expression studies constitute an interesting tool to assess host-parasite interactions. Nevertheless, it will be interesting to conduct more experiments regarding thermo-tolerance in the reproductive phase of *V. destructor* because, although resistance is beneficial to parasites, it may come at an energetic cost of other life-history traits (Krebs and Feder, 1997). For example, Zhang et al. (2015) have demonstrated a trade-off between thermal tolerance and insecticide resistance in a lepidopteran species.

After adjusting methodology using specific antibodies in *V. destructor*, we proceed to analyse the response to chemical stressors. Evidence indicates that Hsp70 varies according to the kind of stressor, external conditions and developmental stages (Rinehart et al. 2000; Lakhotia and Prasanth 2002; Mahroof et al. 2005; Pae et al. 2007). Our results indicate that different mite populations respond differently to acaricide exposure, independent of the historical therapeutic treatment received; therefore, we can infer that genetic factors are also involved. As a result, several degrees of tolerance to acaricides could be obtained despite the proximity of apiaries. For that reason, when planning experiments the origin of a mite population must be clear, avoiding mixtures.

Although traditionally dose–response studies were based in lineal models (Murado and Vázquez 2007), the alternative hormetic model is considered a real and reproducible biological phenomenon (Calabrese and Baldwin 2003a, b). Hormesis has been observed in several response variables in uni- and multi-cellular organisms (Calabrese and Baldwin 2003a, b; Calabrese and Blain 2005). In some cases, our results fit a biphasic hormetic model, characterized by a stimulation of response at low doses of a xenobiotic and an inhibition of response at high rates (Calabrese and Blain 2005; Calabrese 2010). Hormesis has also been reported in the spider mite *Tetranychus urticae* exposed to DDT, methyl parathion and permethrin pesticides (Cohen 2006). Cohen suggested that hormesis fits in situations when parasites cannot be controlled by pesticides, if lethal or high doses exceed quantities detected at field conditions. Although all mite populations displayed resistance to coumaphos, in most of the populations we found no linear dose–response in survival at 24 h, except in PII, and limitations with it as mite mortality did not exceed 85% at the highest concentration, nor in Hsp70 expression (except in PIV). Cohen (2006) highlights that the end points, such as concentration values or lethal doses, must be clearly determined. Here, we detected a reduction in Hsp70 expression at 10 µg/ml of coumaphos per Petri dish in PIIIA and PIIIB—two assays conducted in different seasons, with different sample

sizes, yet similar results were obtained. This kind of response has been called 'inverted U' (Hranitz et al. 2009), and further experiments are necessary to study this phenomenon developing a mathematical model that describes results in a generalized form. It is also necessary to confirm whether Hsp70 are endogenously produced by *V. destructor* or partially obtained from honeybee haemolymph during feeding.

In conclusion, when designing integrated pest management programs, seasonal fluctuation in temperature should be considered to maximize the effect of acaricides and minimize costs and residues of controlling mites. This report offers a new tool and perspective to study the stress response in mites with great abilities to adapt to changing environments.

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