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Susceptibility to entomopathogens and modulation of basal immunity in two insect models at different temperatures

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ABSTRACT

In this work, we analysed the efficacy of different commercial bio-insecticides (*Steinernema feltiae, Steinernema carpocapsae, Heterorhabditis bacteriophora* and *Bacillus thuringiensis*) by valuating the mortality induced on two insect models, *Galleria mellonella* (Lepidoptera) and *Sarcophaga africa* (Diptera) after exposure to different temperatures (10, 20 and 30 °C). Moreover, we investigated the effects of temperature on the basal humoral immunity of the two target insects; particularly, phenoloxidase (PO) and lysozyme activity. Our results show that *G. mellonella* is susceptible to all bio-insecticides at all the examined temperatures, except when infected at 10 °C with *S. carpocapsae* and at 30 °C with *S. feltiae* and *B. thuringiensis. S. africa* is more susceptible at 30 °C to all bioinsecticides; whereas, when infected at 10 and 20 °C, *H. bacteriophora* is the most efficient. Temperature modulates PO activity of both *G. mellonella* and *S. africa*, otherwise variations in lysozyme activity is observed only in *G. mellonella*. Except for a possible correlation between the increased lysozyme activity and the delayed *Bt* efficacy recorded on *G. mellonella* at 30 °C, a different resistance to bio-insecticides at different temperatures does not seem to be associated to variations of the host basal immunity, probably due to immunoevasive and immunodepressive strategies of these entomopathogens.

1. Introduction

In recent years, a large expansion of the home range of many species of so-called harmful insects has been observed (Lindgren et al., 2012; Aguilar-Fenollosa and Jacas, 2013; Baylis, 2017). The main cause of this worldwide phenomenon has been ascribed to changing climatic conditions (Abram et al., 2017). In particular, the modification of insects' distribution in latitude and elevation and in the timing of their life cycles is related to long-term, annual variations in temperature (Chen et al., 2011). These changes would easily lead to an uncontrolled spread of pests (i.e., phytophagous and vectors species) in different areas around the world where few enemies and natural competitors are present (Okulewicz, 2017; Renault et al., 2018).

According to the current integrated pest management practices (Chandler et al., 2011; Barratt et al., 2018), any action aimed to control the increased diffusion of vectors or phytophagous insects should not increase the use of synthetic chemical pesticides due to their well-known adverse impacts. Otherwise, environmentally-friendly methods based on the use of bio-insecticides such as predatory insects and mites, parasitoids, parasites and microbial pathogens should be intensified. However, like their target insects, these organisms are susceptible to variations in the environmental temperature. It is thus necessary to reconsider the physical conditions in which bio-insecticides are highly effective.

Among the most widely used bio-insecticides are entomopathogenic nematodes (EPNs) and *Bacillus thuringiensis* (*Bt*). EPNs are insect endoparasites belonging to the genera *Steinernema* (Rhabditidae, Steinernematidae) and *Heterorhabditis* (Rhabditidae, Heterorhabditidae). They are commercially available as inundative control agents against a variety of pests (Shapiro-Ilan et al., 2006; Lacey et al., 2015). These nematodes are mutualistically associated to bacteria of the genera *Xenorhabdus* and *Photorhabdus,* respectively (Eleftherianos et al., 2010). The combined action of both worms and bacteria results in the elusion from host immune surveillance and in a marked immune depression that culminates in the death of the target insect (Castillo et al., 2011; Eleftherianos et al., 2017). Castillo et al. (2011) have described the mechanisms by which the nematode and its symbionts interfere with the immunological processes of recognition and elimination of non-self, triggered by the host when in the presence of foreign invaders. Since overcoming the host defences is an essential prerequisite for a successful infection/parasitisation, many entomopathogens have evolved strategies to bypass host receptor-mediated immune

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recognition and/or directed to inactivate the host effector mechanisms (Brivio et al., 2004, 2006; Mastore and Brivio, 2008; Eleftherianos et al., 2010).

Bt is a Gram-positive, spore-forming bacterium which, after ingestion by the insect, releases δ-endotoxins. These toxins, interacting with epithelial cells, drastically affect the absorption of insect midgut (Gill et al., 1992). The damage to the midgut epithelium allows the passage of the bacterium to the haemocoel cavity, exposing the pathogen to the host immune system (Jung and Kim, 2007; Dubovskiy et al., 2008). When it reaches the haemocoel cavity, by means of its secondary metabolites, it can immune depress the host. Thanks to their high lethality, *Bt* sub-species and their toxins are widely used to control insect pests (Lacey et al., 2015). Although commercial *Bt* var. *kurstaki* is generally targeted against susceptible Lepidoptera pests, recent works demonstrated its efficacy against Diptera (Lonc et al., 2001; Lysyk and Selinger, 2012; Cossentine et al., 2016).

Among various environmental factors, temperature is certainly one of those most affecting the efficacy of both EPNs and *Bt*. Temperature variations can directly influence the physiology of nematodes and, consequently, the outcome of their life cycle. In particular, low temperatures can cause the reduction of metabolic levels impairing motility and rate of host penetration (Hazir et al., 2001; Bornstein-Forst et al., 2005; Gingold et al., 2013; Ali and Wharton, 2015). Despite literature data being sometimes contradictory, the lethality of *Bt* is affected by temperature variations and various hosts show a different susceptibility to the bacteria at different temperatures. *Bt* reduces its effects against the Diptera *Stomoxys calcitrans* as the temperature increases (Lysyk and Selinger, 2012). The Lepidoptera *Choristoneura rosaceana* is more vulnerable to the bacteria at 25 °C with respect to lower temperatures (Li et al., 1995); conversely, the pine processionary *Thaumetopoea wilkinsoni* (Lepidoptera) showed the highest mortality when *Bt* was administered at 15 °C (Yilmaz et al., 2013). The lethal effects of the bacteria occurred sooner and progressed rapidly with increasing temperature in *Lymantria dispar* (Lepidoptera), although the highest level of mortality decreased with increasing rearing temperature (van Frankenhuyzen et al., 2008).

After parasite or bacteria intrusion, insects can trigger defensive responses as nodulation and melanotic encapsulation. Specifically, the latter represents the fastest and most effective process to neutralise and eliminate intruders, and both these mechanisms depend on the activation of the proPO system cascade that, in turn, cleaves the prophenoloxidase into its active form (Marmaras et al., 1996; Cerenius et al., 2008). Moreover, the presence of bacteria such as *Bt* or EPNs symbionts in the haemolymph can be also counteracted by the action of specific enzymes such as lysozymes, i.e., a constitutive factor that quantitatively increases in the haemolymph because of an infection (Yu et al., 2002; Andrejko et al., 2008).

Changes of environmental temperature can also affect the immune responses of insect hosts (Adamo and Lovett, 2011; Wojda and Taszłow, 2013; Wojda, 2017). In some cases, warmer temperatures increase insect immune defences by means of the increase in activity of both phenoloxidase (i.e., PO activity) and lysozyme, other than trigger cell-mediated immunity (Catalán et al., 2012; Murdock et al., 2012). As observed in *Drosophila*, cold exposure also modulates basal immunity processes, increasing antibacterial peptides and decreasing PO activity, though resistance to pathogens and parasites seem to not be affected (Salehipour-Shirazi et al., 2017).

Insect resistance involves broad aspects of host physiology and conditions, which are shaped by both genetic and environmental variations that often interact in nonlinear ways (Schulenburg et al., 2009). From works on various host-parasite systems, minor changes in temperature can have striking effects on the outcome of their interactions; indeed, the environmental temperature can deeply affect overall resistance to a wide diversity of parasites such as nematodes (Menti et al., 2000) and bacteria (Lazzaro et al., 2008).

A correct use of bio-insecticides like EPNs and *Bt* in areas with different environmental temperature ranges and changing climatic conditions should consider the possible differences in their efficacy and the possible change of insects' physiology due to temperature variations.

In order to acquire useful information on host-parasite relationships in the context of climate change, we aimed to obtain further data on the performance of EPNs and *Bt* and on basal immunity of two insect models when reared at different temperatures (i.e., 10, 20 and 30 °C). We first compared the action of three EPNs (i.e., *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora*) and *Bt* against *Galleria mellonella* (Lepidoptera) and *Sarcophaga africa* (Diptera); two insect models known as honeybee pest and vector of human and animal diseases respectively. In addition, we studied the modulation of the basal humoral immunity of the insects' host at the different temperatures investigated. Specifically, considering the central role of the insect proPO system in the host immune responses and the activity of lysozyme in bacterial clearance, we evaluated the influence of the temperature on the PO and lysozyme activity in the host haemolymph. We think that this work may provide knowledge about the influence of temperature on the host susceptibility to different entomopathogens and on the host immune system, and that, in turn, this knowledge may be used to improve biological control techniques.

2. Materials and methods

2.1. Reagents and instruments

Reagents and instruments were purchased from Bio Rad Laboratories (Detroit, MI, USA), Sigma Chemicals (St. Louis, MO, USA), Merck Millipore Ltd (Tullagreen, Cork, Ireland). All buffers and solutions were heat sterilised or filtered on 0.22 μm syringe filters.

2.2. Bio-insecticides and target insect models

For propagation assays we used commercially available EPNs and *Bt*: *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* were supplied by Bioplanet srl (Cesena, Italy) as Nemopak® (SF, SC, and HB, respectively); *Bt* sub-species *kurstaki* was purchased from Chemia SpA (Sant'Agostino, Italy) as a product commercially named Rapax®.

EPNs, provided at the third infective juvenile stage, dehydrated, and mixed with inert material, were stored at 4 °C to keep parasites in cryptobiosis. *Bt* was supplied as a suspension: 100 gr of product contain 18.8 gr of bacteria. For all the assays *G. mellonella* and *S. africa* were used as insect models as previously mentioned. Both the species were fed with sugar and water ad libitum and kept under controlled conditions: 26 ± 1 °C temperature, 70 ± 10% relative humidity, and 12:12 h of photoperiod. All the assays were carried out with healthy individuals at comparable growth stages (4th–5th instar).

2.3. Propagation assays

As natural infestation method, we implemented a white trap tool to evaluate the lethality of the three EPNs (i.e., *S. feltiae*, *S. carpocapsae* and *H. bacteriophora*) and *Bt* against *G. mellonella* and *S. africa*. Briefly, two grams of nematodes were diluted in 100 mL of dechlorinated water, or 150 μL of *Bt* were diluted in 50 mL of dechlorinated water. The EPNs solution (6 mL) was dispensed on a filter paper placed in a 9 cm diameter Petri dish (i.e., white trap), otherwise the *Bt* solution (6 mL) was spread on food (honey plus cereal for *G. mellonella* or meat for *S. africa*) and placed in the white trap; then, ten larvae were transferred in each white trap. All the insects were kept under controlled conditions (70 ± 10% relative humidity, 12:12 h of photoperiod) in thermostatic incubators (Memmert GmbH + Co. KG, Schwabach, D) at different temperatures (i.e., 10, 20, and 30 °C) and the mortality rate was evaluated at intervals of 12 h, for 60 h. For each bio-insecticide and temperature investigated, three replicates were carried out. As controls, in

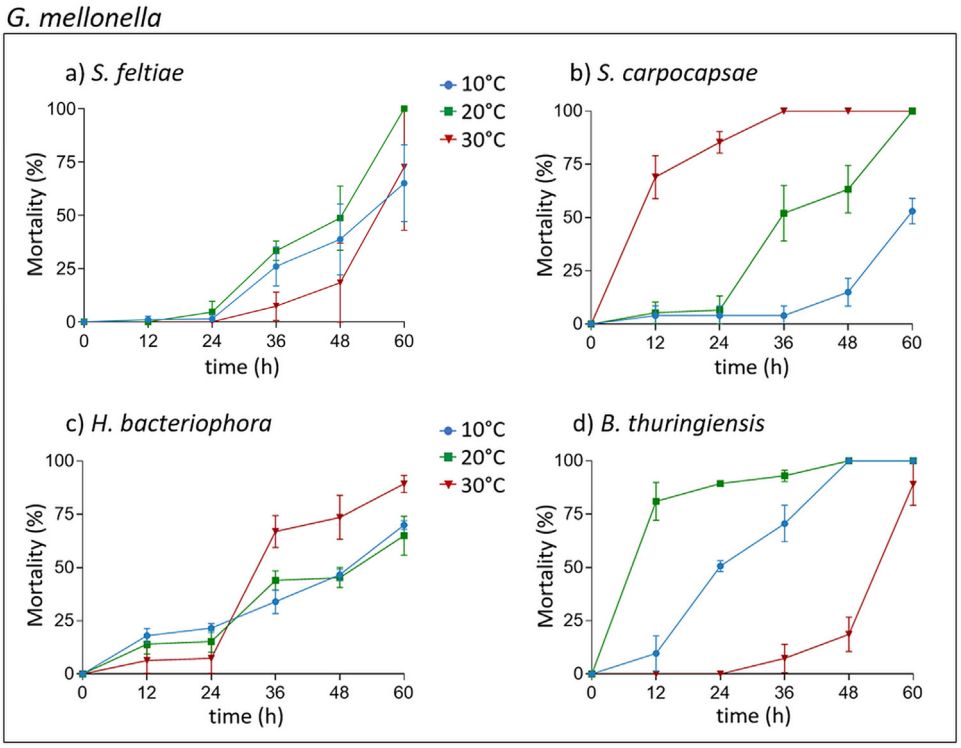


Fig. 1. Mortality rate of *G. mellonella* after exposure to *S. feltiae* (a), *S. carpocapsae* (b), *H. bacteriophora* (c), and *Bt* (d) at different temperatures (10, 20, 30 °C) for 60 h. Larvae in control assays showed a 0% mortality.

all the assays dechlorinated water without bio-insecticides was used.

2.4. Haemolymph collection

Haemolymph, 30 μL/larvae, was bleeded from punctured prolegs of last instar larvae. To avoid the undesired activation of prophenoloxidase, samples analysed for PO activity were flushed out in ice-cold Eppendorf tubes, while samples analysed for lysozyme activity were supplemented with a few crystals of phenylthiourea. Whole haemolymph samples were processed by two low-speed centrifugations (200*g* for 3 min) to remove cells and tissue debris by a refrigerated Eppendorf 5804 R (Eppendorf, Hamburg, Germany). The supernatants cell-free fractions (CFF) were immediately assayed for enzymatic activity.

2.5. PO activity

The PO activity in the haemolymph from untreated *G. mellonella* and *S. africa* larvae incubated at different temperatures (10, 20 and 30 °C) for 24 h was evaluated by spectrophotometric analysis of CFF samples, using L-Dopa as substrate. Time courses of PO activity in the haemolymph from *G. mellonella* and *S. africa* were recorded as formation of dopachrome from L-Dopa substrate, to determine the relative activity of the enzyme. All the assays were carried out with 10 μL of CFF in 1 mL of L-Dopa buffer (8 mM L-Dopa in 10 mM Tris–HCl, pH 7.2). 200 μL of sample/well were read for absorbance (Abs) in a flat-bottomed 96 well-plates by Bio Rad iMark™ Microplate Absorbance reader. Changes in Abs were recorded at 450 nm, at 5 min intervals for 45 min, at 20 °C. The L-Dopa buffer was used as blank, and in all samples PO relative activity was defined as $\Delta Abs_{450} \cdot 45\ min^{-1} \cdot 10\ \mu L^{-1}$. For each temperature three replicates were carried out; each replicate was composed of the haemolymph from three larvae. For each replicate three Abs readings were performed and then the average value was considered.

2.6. Lysozyme activity

An enzymatic assay was carried out to measure the rate of lysozyme activity (Units min^{-1} mL^{-1}) in the haemolymph samples from untreated *G. mellonella* and *S. africa* larvae incubated at different temperatures (10, 20 and 30 °C) for 24 h. One unit of lysozyme activity is defined as the change of 0.001 units of Abs at 450 nm per minute of a suspension of the Gram-positive bacteria *Micrococcus luteus* (ΔAbs_{450}). The lysozyme present in the haemolymph damages the bacterial cells wall, decreasing the turbidity of the sample and therefore its Abs. Briefly, a suspension of *M. luteus* (0.45 mg mL^{-1} in 30 mM phosphate buffer, pH 7.2) with 0.6–0.7 of optical density was used as substrate, then 25 μL of CFF were added to the bacteria suspension. The Abs of samples was read at 450 nm and recorded at 30 s intervals for 2.5 min, in a flat-bottomed 96 well-plates by a Bio Rad iMark™ Microplate Absorbance reader. For each temperature three replicates were carried out; each replicate was constituted of the haemolymph from three larvae. For each replicate four Abs readings were performed and then the average value was considered. *M. luteus* without haemolymph addition was used as control.

2.7. Statistical analysis

For each bio-insecticide (*S. feltiae*, *S. carpocapsae*, *H. bacteriophora*, and *Bt*) and temperature (10, 20, and 30 °C) through the time interval analysed (12, 24, 36, 48 and 60 h), we calculated the insects' mortality as average ± standard deviation of the three replicates. For each bio-insecticide-target insect pair, we applied the two-way analysis of variance (ANOVA) considering temperature and time as variables and the percentages of insect mortality as observations. Before the ANOVA the percentage data were *arcsin*-transformed. After the ANOVA we used the Tukey HSD *post-hoc* test to detect possible significant ($p < 0.05$) differences of insect mortality at different temperatures and times. Moreover, we applied the one-way ANOVA followed by the Tukey HSD *post-hoc* test to detect possible significant ($p < 0.05$) differences of the

PO and lysozyme activity at the three temperatures investigated. All the statistical analyses were performed using XLSTAT2011 software.

3. Results

3.1. Effects of temperature on the performance of entomopathogens

In all cases, statistically significant differences of the larvae mortality were recorded at different temperatures and durations of exposure to the four bio-insecticides, and for the interaction between these two variables (two-way ANOVA, all $p < 0.05$, see Tables SM1 and SM2 in the Supplementary material), except for the assays with *S. feltiae* against *G. mellonella* and *S. africa*. In these two cases, significant differences were only related to the period of treatment and, for the former, also to temperature (Tables SM1 and SM2).

The susceptibility of *G. mellonella* to *S. feltiae* is evident, mainly at 20 °C (Fig. 1a). At this temperature, after 48 h of treatment, the mortality was 48% on average, and after 60 h it reached 100% (Fig. 1a). However, a significant difference was only recorded at 60 h between 10 and 20 °C (Tukey test, $p = 0.015$, Table SM3). The Diptera *S. africa* was less affected by *S. feltiae*; the mortality rate never exceeded 50% on average and this value was reached at 30 °C after a prolonged exposure time (i.e., 60 h) (Fig. 2a).

Conversely, the other EPN species belonging to the *Steinernema* genus (i.e., *S. carpocapsae*) was significantly more lethal at the highest temperature tested for both the targets (Figs. 1b and 2b) (Tukey test, $p < 0.05$ in most cases, see Tables SM3 and SM4). After 36 h of exposure at 30 °C, *G. mellonella* showed a mortality of 100% (Fig. 1b). In the case of *S. africa* (Fig. 2b) an average mortality of approximately 75% was detected at 60 h.

The third EPN investigated (Figs. 1c and 2c), *H. bacteriophora*, had a behaviour similar to that of *S. carpocapsae* as the average larvae mortality resulted higher at the highest temperature tested. At 30 °C after 60 h of exposure, the mortality of both insects (Figs. 1c and 2c) was over 80% on average and significantly different from that detected at lower temperatures (Tukey test, all $p < 0.05$ except for 30 vs 10 °C in case of *G. mellonella*, see Tables SM3 and SM4).

The results of the *Bt* assays (Figs. 1d and 2d) show a significantly higher efficacy of the bacteria at 10 and 20 °C for *G. mellonella* and at 30 °C for *S. africa* (Tukey test, $p < 0.05$ in most cases, see Tables SM3 and SM4). At 20 °C, the mortality of *G. mellonella* reached approximately 80% in only 12 h (Fig. 1d). *Bt* was ineffective against *S. africa* at both 10 and 20 °C even though, after 48 h of exposure at 30 °C, it caused the mortality of more than 65% of the Diptera larvae (Fig. 2d).

3.2. Effects of temperature on the PO activity

As shown in Fig. 3a, the PO relative activity in the cell-free haemolymph samples of *G. mellonella* significantly differed between all the three temperatures investigated (one-way ANOVA, $F = 95.075$, $p < 0.0001$, Table SM5). In particular, the PO activity was significantly higher at 30 °C (Tukey test, 30 vs 20 °C: $p < 0.0001$; 30 vs 10 °C: $p = 0.001$, Table SM5), though it was also significantly higher at 10 °C than at 20 °C (Tukey test, $p = 0.001$, Table SM5). In general, *S. africa* (Fig. 3b) was less influenced by the conditioning at different temperatures (one-way ANOVA, $F = 16.736$, $p = 0.004$, Table SM5). However, a significantly higher activity was also recorded at 30 °C in this case (Tukey test, 30 vs 20 °C: $p = 0.007$; 30 vs 10 °C: $p = 0.005$, Table SM5); conversely the difference between the activity at 10 and 20 °C did not yield significant results (Tukey test, $p = 0.904$, Table SM5).

3.3. Effects of temperature on the lysozyme activity

In all the lysozyme assays with *G. mellonella* and *S. africa*, an Abs decrease corresponds to a higher lysozyme activity (Fig. 4). As shown in Fig. 4a, the relative activity of the haemolymph lysozyme of *G. mellonella* significantly increases with the temperature (one-way ANOVA, $F = 51.965$, $p < 0.0001$, Table SM6). A negligible activity was recorded at 10 °C ($3.44 \cdot 10^2$ Units mL^{-1} min^{-1}) since no significant difference was

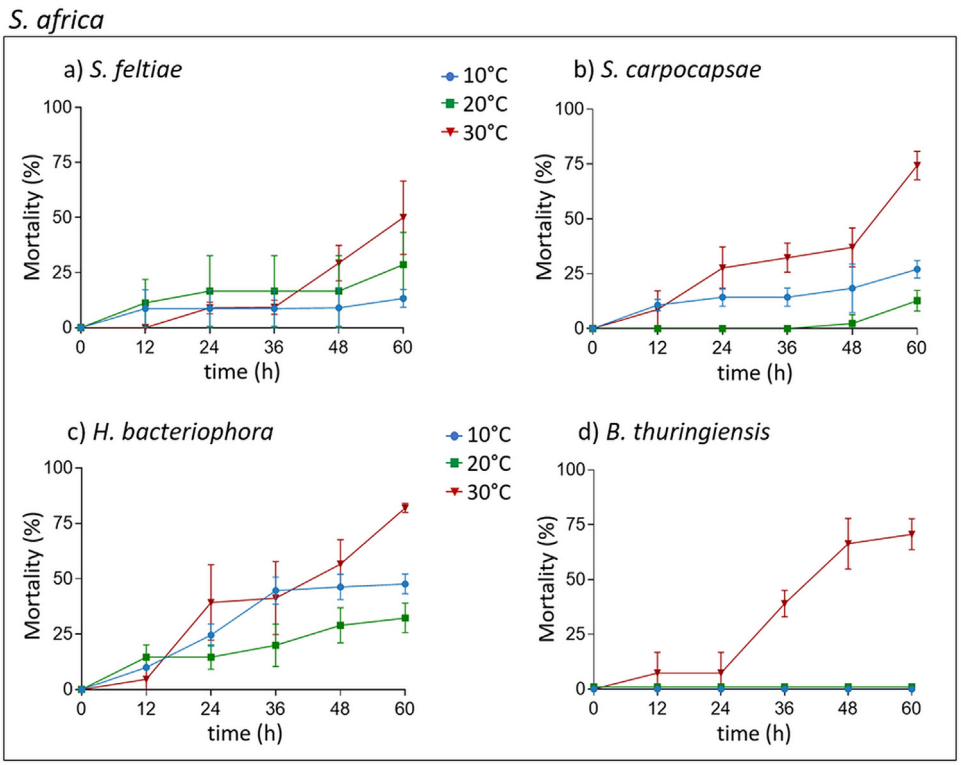


Fig. 2. Mortality rate of *S. africa* after exposure to *S. feltiae* (a), *S. carpocapsae* (b), *H. bacteriophora* (c), and *Bt* (d) at different temperatures (10, 20, 30 °C) for 60 h. Larvae in control assays showed a 0% mortality.

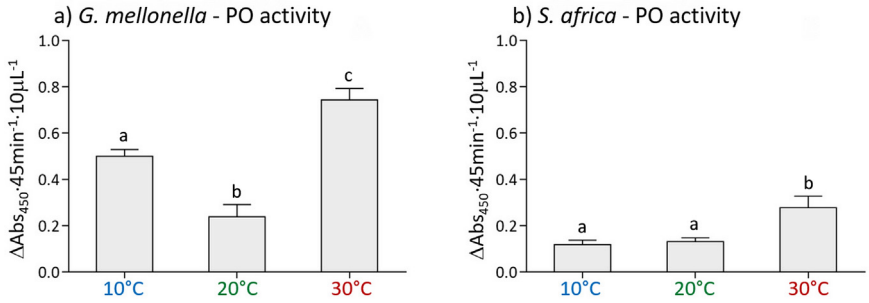


Fig. 3. PO relative activity in haemolymph of *G. mellonella* (a) and *S. africa* (b) after conditioning at various temperatures (10, 20, 30 °C). Different letters above the bars (a, b, and c) indicate a statistically significant ($p < 0.05$) difference between samples.

found with respect to the control (i.e., Tukey test, *M. luteus* vs 10 °C: $p = 0.675$, Table SM6), while significantly higher activities (Tukey test, all $p \leq 0.005$, see Table SM6) were detected at 20 °C ($1.27 \cdot 10^3$ Units mL^{-1} min^{-1}) and overall at 30 °C ($2.28 \cdot 10^3$ Units mL^{-1} min^{-1}). Unlike *G. mellonella*, *S. africa* (Fig. 4b) possesses a negligible lytic activity at any temperature tested (i.e., $1.62 \cdot 10^2$; $1.04 \cdot 10^2$ and $1.68 \cdot 10^2$ Units mL^{-1} min^{-1}, for 30, 20, and 10 °C treatments, respectively) and no significant differences were recorded between all the samples (one-way ANOVA, $F = 2.202$, $p = 0.165$, Table SM6).

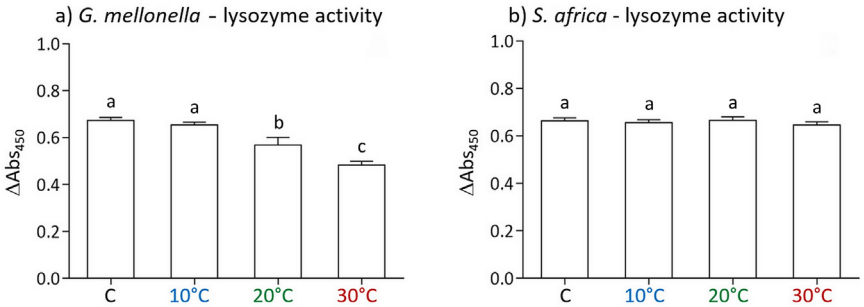
4. Discussion

As we mentioned in the introduction, the current climate change is leading to the expansion and modification of the habitat for many insect pests (Bebber, 2015) and to shifts in host-parasite interactions (Rohr and Palmer, 2013). Most host-parasite interactions are indeed mediated by physiological (mainly immunological) processes which, in turn, can be influenced by environmental temperature. From the current literature, it is clear that establishing common models of response to changes in environmental temperature is difficult, since there are discordant data even within ectotherm species. One of the reasons for this inhomogeneity is related to the maintenance conditions in laboratories in which physical parameters cannot exactly reflect those of the habitat of the species, other than the different experimental procedures performed (Terblanche et al., 2007; Santos et al., 2011; Gallego et al., 2016).

In this work, we first analysed the performance at different temperatures of four entomopathogens (*S. feltiae*, *S. carpocapsae*, *H. bacteriophora,* and *Bt*), used as bio-insecticides, against the Lepidoptera *G. mellonella* and the Diptera *S. africa*, belonging to orders comprising many phytophagous and vectors species. Since it is well known that temperature modulates biological processes of both hosts and parasites and that they function optimally within certain temperature ranges (Kung et al., 1991; Mahar et al., 2005; Shapiro-Ilan et al., 2006; Lacey, 2007; Lee et al., 2016), we expected a different infectivity level of the entomopathogens at the three different temperatures investigated (10, 20 and 30 °C).

Our assays showed a higher susceptibility of *G. mellonella* to all entomopathogens with respect to *S. africa*, reaching higher average mortality values within 60 h of treatment. *S. carpocapsae* already showed good infectivity (i.e., > 50% mortality) at high temperature (30 °C) at 12 h of treatment, likely due to a higher activity of the parasite and a better growth condition of its symbionts, *Xenorhabdus nematophila* (Chen et al., 1996; Wang et al., 2008). The infectivity of *S. carpocapsae* significantly decreased at lower temperatures (20 and 10 °C). Grewal et al. (1994) observed that this nematode is able to penetrate insects also at temperatures up to 10 °C but remains inactive within its hosts for long periods and resumes its life cycle only at warmer temperatures. Despite belonging to the same genus, *S. feltiae* had a good level of infectivity against *G. mellonella* only at 60 h of treatment at all the investigated temperatures, and caused the 100% of host mortality only at 20 °C. This result is partially in agreement with the literature which suggests that this nematode is more lethal at temperatures near to 20 °C (Chen et al., 2003; Mahar et al., 2005; Laznik et al., 2009). *S. feltiae* is indeed a temperate species that has evolved as an active nematode at cool temperatures (Hominick and Briscoe, 1990; Wright, 1992). However, some authors (Wright, 1992; Grewal et al., 1994) suggest that, thanks to its features, *S. feltiae* could also affect insects that are more active during winter. The Canterbury isolated strain, for instance, is infective at low temperatures, being able to kill *Wiseana cervinata* (Lepidoptera) at 10 °C (Wright and Jackson, 1992). Unlike *S. feltiae*, *H. bacteriophora* has a tropical or sub-tropical origin, thus its thermal preferences are specific to warmer climates (Flanders et al., 1996; Stuart et al., 2015). Accordingly, we observed that *H. bacteriophora* had significantly higher activity against *G. mellonella* at 30 °C, starting from 36 h of treatment.

EPNs are also reported to be lethal against Diptera (Jagadale et al., 2004; Jess et al., 2005; Georgis et al., 2015). Several works have showed the adverse effects of *Steinernema* spp. against the Diptera



lysozyme relative activity (Units · mL^{-1})

T= 10°C	T= 20°C	T= 30°C
$3.44 \cdot 10^2$	$1.27 \cdot 10^3$	$2.28 \cdot 10^3$

lysozyme relative activity (Units · mL^{-1})

T= 10°C	T= 20°C	T= 30°C
$1.68 \cdot 10^2$	$1.04 \cdot 10^2$	$1.62 \cdot 10^2$

Fig. 4. Lysozyme activity in haemolymph of *G. mellonella* (a) and *S. africa* (b) after conditioning at various temperatures (10, 20, 30 °C). The lytic activity of lysozyme was recorded as decrease in turbidity of a *M. luteus* suspension (control, C). Different letters above the bars (a, b, and c) indicate a statistically significant ($p < 0.05$) difference between samples. Tables below the graphs show the lysozyme relative activities as Units mL^{-1}.

Bactrocera oleae, Ceratitis capitata and *Rhagoletis indifferens* (Lindegren et al., 1990; Gazit et al., 2000; Yee and Lacey, 2003). However, in the literature, there are no studies on the pathogenicity of EPNs on *S. africa*. According to our results, when *S. carpocapsae* or *H. bacteriophora* were administered at 30 °C, both revealed a good level of infectivity on *S. africa*, even if they required a treatment of 60 h. As confirmed by the literature (Rohde et al., 2010; Pervez et al., 2015), *S. carpocapsae* and *H. bacteriophora* display the highest pathogenicity between 25 and 30 °C. Specifically, *S. carpocapsae* is highly lethal within this temperature range against the larvae of the Diptera *C. capitata*, *Dacus dorsalis,* and *Dacus cucurbitae* (Lindegren and Vail, 1986; Patterson Stark and Lacey, 1999), and *H. bacteriophora* against the Diptera *Lucilia cuprina* (Molyneux, 1984). Unlike these two EPNs, *S. feltiae* showed a low level of infectivity (i.e., ≤ 50% mortality) at all the temperatures tested within 60 h of treatment. The average mortality values were lower at 10 °C, although not significantly different from those detected at 20 and 30 °C. In other species of Diptera, such as *D. melanogaster*, *S. feltiae* was highly infective at 25 °C, showing a low lethal effect at lower temperatures, while it was able to kill *Delia radicum* larvae at 10 °C (Patterson Stark and Lacey, 1999; Chen et al., 2003; Linder et al., 2008).

In general, other than changes in EPNs infectivity, variations in temperature can cause changes in host-seeking behaviour and in the time necessary to infect the host (Molyneux, 1984). Also, the attractive stimuli produced by the insect can vary with temperature, affecting the chemotaxis processes (Pye and Burman, 1981; Kaya, 1993). Moreover, the different levels of infectivity described for *Steinernema* spp. and *Heterorhabditis* spp. at various temperatures can be due to the physiology of their symbionts, as bacteria health and growth are affected by temperature variations (Dunphy and Webster, 1988).

As for *Bt*, its infectivity at different temperatures had an opposite trend in the two target insects. The bacterium had a significantly higher lethality on *G. mellonella* at 20 and 10 °C than at 30 °C, while when it was administered to *S. africa*, significantly higher mortality was observed at just 30 °C. This higher lethality at 30 °C can be expected, since the optimal growth of *Bt*, the solubility and absorption of its crystal toxins, and the feeding rate increase with the temperature (Wraight et al., 1981). The influence of temperature on the bacterial growth has also been suggested by van Frankenhuyzen (1994), who evaluated the pathogenesis of *Bt* on *Choristoneura fumiferana* (Lepidoptera).

The main results of the assays carried out on *G. mellonella* and *S. africa* with the four entomopathogens at various temperatures and within 48 h of treatments are summarized in Table 1. In the context of climate change, these results show that entomopathogens like *S. carpocapsae* and *H. bacteriophora* could be favoured by the predicted increase in the global surface temperature (Björkman and Niemelä, 2015), while the survival of hosts such as *S. africa* could be affected by a wider range of parasites. Our results may also constitute basic guidelines useful to select the adequate bio-insecticide under varying climatic conditions for field applications.

Concomitantly with the study of entomopathogens' lethality, we have examined the effects of temperature on the basal immunity of the target insects to investigate the possible association with an altered activity of the host immune system. Although the immune defences are energetically costly, the insects' immune system can be modulated to operate at different temperatures to counteract parasites or bacteria with different thermal performances (Moret and Schmid-Hempel, 2000; Adamo, 2004; Xu and James, 2012). Thus, our assays have been carried out to evaluate how thermal conditioning modulates the basal PO and lysozyme activity, both present in the humoral fraction of the insects' haemolymph and known as the fastest mechanisms and most effective defence processes against metazoan parasites and microorganisms (Hultmark, 1996; Gao and Fallon, 2000; Brivio et al., 2002; Lemaitre and Hoffmann, 2007; Cerenius et al., 2008).

Table 1
Cumulative table of the efficacy (referred to 48 h post administration) of *S. feltiae* (Sf), *S. carpocapsae* (Sc), *H. bacteriophora* (Hb) and *B. thuringensis* (Bt) on *G. mellonella* (Gm) and *S. africa* (Sa), after conditioning at 10, 20 and 30 °C.

	10 °C				20 °C				30 °C			
	Sf	*Sc*	*Hb*	*Bt*	*Sf*	*Sc*	*Hb*	*Bt*	*Sf*	*Sc*	*Hb*	*Bt*
Gm	+	–	+	+ + +	+	+ +	+	+ + +	–	+ + +	+ +	–
Sa	–	–	+	–	–	–	+	–	+	+	+ +	+ +

–: Mortality < 25%; +: 25 ≤ Mortality ≤ 50%; + +: 50 ≤ Mortality ≤ 75%; + + + : Mortality > 75%.

Our data show the influence of temperature mainly on the *G. mellonella* PO and lysozyme activity. The PO activity increased moderately at 10 °C and strongly at 30 °C, whereas at 20 °C it kept close to the physiological activity (Zdbicka-Barabas and Cytryńska, 2010). In *S. africa,* the PO activity significantly increased only at 30 °C. Moreover, in *G. mellonella* haemolymph, we recorded a significant increase of the lytic properties from 10 to 30 °C, while in *S. africa* we did not observe significant variations of the lysozyme activity at any temperature assayed. As we found in *G. mellonella*, an influence of the temperature on the physiological PO and lysozyme activity in the haemolymph was also observed in the Orthoptera *Gryllus texensis* (Adamo and Lovett, 2011), the Coleoptera *Tenebrio molitor* (Catalán et al., 2012), the Diptera *Anopheles stephensi* (Murdock et al., 2012), the Lepidoptera *Ephestia kuehniella* (Mostafa et al., 2005), and other species.

Unlike from studies that have examined individual components or processes of the complex system entomopathogen-host (Laughton et al., 2017), in our work we used a wider approach to investigate the effects of temperature on both hosts and entomopathogens, also considering their synergistic relationships. Unlike *Bt*, which is a single micro-organism, an in-depth understanding of the EPNs' relationships with their hosts is complicated by the presence and the role played by their symbionts bacteria, which combine with the nematode their lethal effects in a tripartite interplay (Sicard et al., 2004; Hallem et al., 2007; Toubarro et al., 2013; Mastore et al., 2015; Brivio et al., 2018). Along with the changes of temperature, the complex interactions among all these species and the various processes involved can cause different immune responses, sometimes leading to divergent results (Björkman and Niemelä, 2015). Even when in the presence of an intensified immune response of the host, as we detected in *G. mellonella* at 30 °C, the evasive and depressive strategies of EPNs such as *S. carpocapsae* and *H. bacteriophora* could be responsible for a successful parasitisation. Considering the increase of PO activity at higher temperatures, we expected a better efficiency of the hosts to encapsulate parasites (Dubovskiy et al., 2016); conversely, the observed increase did not produce the expected effects. The elusive strategies of the parasites, in particular their ability to evade non-self recognition by means of the peculiar properties of their cuticle (Peters et al., 1997; Castillo et al., 2011; Cooper and Eleftherianos, 2016; Brivio and Mastore, 2018), probably defused the potential of the increased immune activity of the host. Otherwise, the delayed effects of *Bt* against *G. mellonella* and the high mortality observed in *S. africa* at 30 °C could be associated to the different lysozyme activity observed in the two insects at this temperature. The survival of *G. mellonella* could be due to the action of the enzyme on the wall of this Gram positive bacterium (Hultmark, 1996; Callewaert and Michiels, 2010) leading to a reduced bacterial proliferation in the host haemolymph.

5. Conclusions

Our data suggest that temperature changes can alter the relationships between entomopathogens and their hosts, thus affecting the efficacy of the bio-insecticides on the target insects in specific ways in different species. Temperature can also influence the host basal immune response even if, in some instances, the enhancement of immune activity does not seem to be the main factor responsible for an increased

resistance to entomopathogens. Therefore, the balance between the success of a bio-insecticide and the insect survival can differ as a result of environmental variations, which could be responsible for physiological changes of both the host and the entomopathogen.

Although we are aware that further studies are required to deepen the complex relationships between entomopathogens and their hosts in a context of climate change, we believe that our work may provide useful evidence from both a theoretical and applied viewpoint.

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtherbio.2018.11.006.

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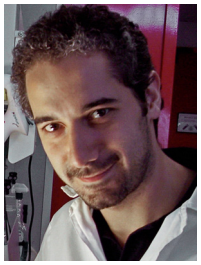

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