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ORIGINAL PAPER

When warmer means weaker: high temperatures reduce behavioural and immune defences of the larvae of a major grapevine pest

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Abstract

Phytophagous insects evolving in agroecosystems express numerous defences when faced with myriads of natural enemies. Such defensive traits might impair the effectiveness of biological control relying on these natural enemies, mostly parasitoids. In the case of parasitoid threat, these defences consist of the avoidance of the parasitoid (reduced exposure to antagonists through shortening of developmental time), hindrance of oviposition (evasive behaviours and morphological protection) or destruction of the parasitoid eggs (encapsulation and melanisation by means of the immune system). Previous works focused on one defensive trait only when investigating the effects of temperature on host resistance. By doing so, they assumed that all defensive traits would respond uniformly to a change occurring in thermal environment, which remains an undocumented fact. To test this assumption in the context of global warming, we adopted a global overview of host resistance by examining the effects of rising temperatures on multiple defensive traits used by the grape pest, *Lobesia botrana*, against its larval parasitoids. Although warmer conditions led to reduced exposure to parasitoids by accelerating larval development, warmer conditions also elicited extensive weakening of behavioural and immune defences. These results confirm that temperatures might differently modulate the levels of expression of several defensive traits. An increase in growth rate and pupal mass also occurred, especially for females, which may contribute to greater pest fecundity in the future. However, the decline of *L. botrana* resistance might enhance the efficiency of the biological control naturally exerted by parasitoids in vineyards, thereby limiting the damage to crops.

Keywords Climate change · Defensive behaviours · Host–parasitoid interactions · Host resistance · Immunity

Key message

- We studied the effects of temperature on several defensive traits in caterpillars of the major grapevine pest *L. botrana*.
- We found that all defensive traits did not respond similarly to temperature: warming reduced the duration of the larval stage by accelerating development but downregulated behavioural and immune traits.
- In the context of global warming, these results indicate a shortened period of *L. botrana* vulnerability to larval parasitoids and decreased *L. botrana* resistance during this window of vulnerability.

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Introduction

In agroecosystems, parasitoids are a major biological cause of mortality of phytophagous insects (Hawkins et al. 1997). Such natural biocontrol agents significantly modulate the abundance and the composition of herbivore communities and, ultimately, the damage caused by phytophagous pests to crops (Thomson et al. 2010; Henri et al. 2012; Reineke and Thiéry 2016). In response to this selective pressure, hosts have evolved various protective systems based on several successive lines of defence (Gross 1993; Greeney et al. 2012). In the first instance, faster host development during its vulnerable stage leads to higher host resistance because it shortens the window of host vulnerability during which parasitism may occur ('slow-growth-high-mortality hypothesis', Clancy and Price 1987). In this regard, a host perceiving parasitoids may shorten its developmental time to avoid oviposition attempts (Vogelweith et al. 2013b). If this so-called developmental barrier fails to prevent parasitoid contact, defensive behaviours such as biting the parasitoid, shaking or twisting act as a frontline defence to reduce the risk of oviposition (Greeney et al. 2012; Vogelweith et al. 2014). Oviposition can also be limited by morphological adaptations, such as toughened integument that forms a defensive barrier between the internal environment of the insect and external biotic stress (Greeney et al. 2012; Moret and Moreau 2012; Vogelweith et al. 2014). In case of oviposition, a rapid and appropriate immune response based on cellular processes may kill the parasitoid eggs (Hoffmann et al. 1996; Siva-Jothy et al. 2005). These mechanisms include the recruitment of haemocytes (immune cells circulating in the haemolymph) involved in encapsulation and melanisation of the parasitoid eggs through the action of phenoloxidase (Lavine and Strand 2002; Cerenius and Soderhall 2004).

As ectotherms, phytophagous insects are particularly sensitive to temperature, which is known to affect several aspects of performance in a curvilinear and asymmetric way (Martin and Huey 2008; Angilletta et al. 2010). As the mean temperature of the regime experienced increases, the performance gently rises up to a maximal value of performance associated with a thermal optimum. Once mean temperature exceeds this thermal optimum, the performance abruptly collapses (Martin and Huey 2008; Angilletta et al. 2010). Many life history traits that influence performance vary according to temperature and among them are several defensive traits used against parasitoids such as aggressive behaviours (Le Lann et al. 2014; Moiroux et al. 2016) and encapsulation/melanisation processes (Delava et al. 2016; Seehausen et al. 2017a). Temperature is now recognised as a crucial determinant of host–parasitoid interactions as it influences host resistance to natural enemies on the one hand, and the ability of parasitoids to overcome host resistance (e.g. parasitoid virulence, host selection or intra-patch foraging behaviour) on the other (Hance et al. 2007; Jeffs and Lewis 2013; Moiroux et al. 2015, 2016). Hence, ongoing climate change and the associated 0.3–4.8 °C increase in global mean surface temperature by the end of this century (Intergovernmental Panel on Climate Change 2014) are likely to affect the ability of phytophagous insects to resist parasitism, thereby influencing the outcomes of host–parasitoid interactions and the pressure exerted on crops (Jeffs and Lewis 2013; Harvey 2015; Furlong and Zalucki 2017). Indeed, there is accumulating evidence that the effectiveness of biological control provided by parasitoids under field conditions may be linked to variation in temperature across years (e.g. local manifestations of global warming) (Evans et al. 2013; Meisner et al. 2014) or localities following a natural thermal gradient (Romo and Tylianakis 2013; Vogelweith et al. 2013a).

To date, studies dealing with the effects of temperature on host defence systems have focused on one type of defensive trait, most commonly defensive behaviours or immunological traits (Le Lann et al. 2014; Moiroux et al. 2016; Seehausen et al. 2017a). For instance, in the host–parasitoid complex constituted by larvae of the spruce budworm *Choristoneura fumiferana* (Lepidoptera: Tortricidae) and their solitary parasitoid *Tranosema rostrale* (Hymenoptera: Ichneumonidae), an increase in mean temperature has been shown to enhance the efficiency of the hosts in encapsulating and killing the parasitoid eggs (Seehausen et al. 2017a). A more thorough understanding of the consequences of temperature for host–parasitoid interactions would, however, require further insights into the thermal modifications of the array of developmental, behavioural, morphological and immune traits used sequentially to prevent parasitisation. This is especially true since these defensive traits might respond differently to warming conditions as a result of two non-mutually exclusive explanations. First, different traits often display different thermal optima in a given species (Laughton et al. 2017), such that defensive traits could differ regarding their thermal optima. For defensive traits displaying a relatively high thermal optimum, an increase in mean temperature might enhance the host resistance by bringing thermal conditions closer to the optimum for these traits (Le Lann et al. 2014; Seehausen et al. 2017a). Conversely, for defensive traits characterised by a relatively low thermal optimum, the warming experienced might exceed the thermal optimum for the expression of these traits, and negative effects on host resistance can be expected (Fischer et al. 2011; Karl et al. 2011). Thus, an increase in mean temperature might imply different consequences for host resistance, depending on whether or not this warming exceeds the

thermal optimum for the defensive trait considered. Second, the investment in several defensive traits is costly to the host, and hence trade-offs are expected between defensive traits that serve a common function (Parker et al. 2011; Vogelweith et al. 2014). Convincing examples entail trade-offs between immunity and defensive behaviours (Kortet et al. 2007; Zylberberg et al. 2013) or between morphological traits and defensive behaviours (Steiner and Pfeiffer 2007; Vogelweith et al. 2014). As a result, the variation observed in one defensive trait following exposure to different temperatures might greatly affect the expression of other defensive traits. Still, to our knowledge, no study has so far explored the consequences of rising temperatures on different defensive traits to test whether they might respond differently to warming conditions.

In this study, we quantified the effects of an increase in mean temperature on several defensive traits expressed by *Lobesia botrana* (Lepidoptera: Tortricidae) larvae face with simulated parasitoid attack. Caterpillars of this grapevine pest feed on floral buds and berries (Thiéry and Moreau 2005), affecting the vine stock both directly (through consumption) and indirectly, by increasing its susceptibility to fungal diseases such as grey mould (*Botrytis cinerea*) and black mould (*Aspergillus niger, A. carbonarius*) (Cozzi et al. 2006). One larva is able to infest from 2 to 10 berries when developing on a group of berries called a bunch, and the density might reach up to 20–30 larvae per bunch in seriously affected vineyards (Delbac and Thiéry 2016). Hence, the economic impacts of this pest on wine activity have been rated as 'high', although still never quantified (Fowler and Lakin 2002; Thiéry et al. 2018). In vineyards, *L. botrana* is exposed to several larval parasitoid species, among them Ichneumonidae, Tachinidae, Braconidae, and Bethylidae (Moreau et al. 2010; Thiéry et al. 2011; Scaramozzino et al. 2017). This species represents a good candidate to adopt a global overview encompassing the thermal response of the whole defensive system, because caterpillars are exposed to a high parasitism pressure in the field, as indicated by parasitism rates of approximatively 40% or more, and have developed a defensive system including all of the defensive traits described above (Xuéreb and Thiéry 2006; Moreau et al. 2010; Vogelweith et al. 2013a). We therefore considered developmental defences (time until pupation, as chrysalises are protected against oviposition of larval parasitoids); three behaviours (flee, twisting and dropping); two morphological traits (integument thickness and resistance) and two immune traits (haemocytes concentration and phenoloxidase activity) involved in defence of fifth instar larvae against parasitoids. We focused on this last larval instar because it is the most damaging to grape flowers and berries (Delbac and Thiéry 2016). We expected the defensive traits expressed when faced with a simulated parasitoid attack to differ with regard to their thermal optima and/or be linked to energetic trade-offs, such that these traits may respond in different ways to gradual climate warming.

Materials and methods

Insect origin and maintenance

The larvae used in this experiment came from an inbred and diapause free stock maintained at the French National Institute for Agricultural Research (INRA) (Villenave d'Ornon, France) for more than 10 years. This strain arises from a great number of caged adults (several thousand per week) to which wild adults were periodically added to maintain genetic diversity. Similar patterns in parasitoid escape behaviour, morphological defences and basal immunity have been found between inbred stock and wild populations sampled in French vineyards (Vogelweith et al. 2014). Adults were maintained in a large cage under controlled conditions (22 ± 1 °C, 60 ± 10% relative humidity, photoperiod of L15: D8 and 1 h of dusk). Luminosity reached 1000 lx during the first 15 h of photophase and then fell to 25 lx during dusk. Two bands of waxed paper (4×6 cm) were hung for oviposition. After 2–3 days, these papers were collected and transferred to plastic boxes covered by a moist piece of paper towel to avoid desiccation until hatching.

A total of 1410 newly hatched larvae (age < 24 h) were continuously collected throughout the experiments with a fine brush and isolated in Eppendorf tubes filled ad libitum with 1.5 ml of artificial diet (composition for 1000 ml: 1000 ml water, 15 g agar, 86.6 g maize flour, 41.3 g wheat germ, 45.5 g beer yeast, 6 g ascorbic acid, 3.4 g mineral salt, 0.32 g Scala®, 2.7 g benzoic acid, 2.8 g Nipagin® and 5 ml 95% ethanol). Isolation avoided introducing any bias due to larval competition (Moreau et al. 2006a). Tube caps were drilled (Ø = 8 mm) to allow air circulation, and covered with a mesh fabric to prevent larvae from escaping. Larvae were placed in climate chambers (Exo-Terra® PT2445) and randomly distributed between three constant thermal regimes (22, 25 or 28 ± 0.1 °C, 60 ± 10% relative humidity, L16: D8 photoperiod, 500 lx) to reduce maternal and paternal effects. Thermal conditions selected corresponded to summer days in southern France (Perpignan: 42°69′N 2°90′E): 22 °C being the average temperature in July–August (from 1981 to 2010) and 28 °C being the average temperature observed during the warmest month over that period (data from http://www.meteofrance.com). Importantly, 28 °C is also the predicted mean summer temperature for Mediterranean vineyards in 2100 (Leibar et al. 2017). We mimicked such summer climatic conditions because *L. botrana* exhibits high levels of activity during these months, which are associated with the highest crop damage (Varela et al. 2010; Thiéry et al. 2011). In accordance with previous recent works, we

used and compared constant thermal regimes as a way to assess the thermal response of different traits to an increase in mean temperature (Laughton et al. 2017; Seehausen et al. 2017b), and therefore to test whether these thermal responses might differ between defensive traits. Three times per week, larvae and their associated thermal regime were switched between climate chambers in order to minimise potential climate chamber effects. Additionally, their relative location was also changed within the new incubator. Temperature inside the incubators was regularly monitored (once per week) with HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA).

Larval development was monitored when the larvae were switched between climate chambers. Throughout the experiments, fifth instar caterpillars were randomly divided between three groups, because the different measurements cannot be taken on a single individual. In Group 1, larvae were allowed to develop until pupation in order to measure developmental time, pupal mass and growth rate. In Group 2, we chose fifth instar larvae to study the display of protective behaviours followed by morphological defences. In Group 3, we used fifth instar larvae to examine immunocompetence. For measurements of behavioural/morphological and immune defences, we only selected active caterpillars twisting and fleeing when manipulated in their rearing tube.

Group 1: developmental time, pupal mass and growth rate

A total of 1171 larvae reached the pupal stage ($n = 347$ individuals at 22 °C; $n = 325$ individuals at 25 °C; $n = 499$ individuals at 28 °C). Once individuals pupated, we first recorded the developmental time as the time elapsed between hatching and pupation. Pupae were carefully removed from their cocoon and weighed (± 0.01 mg) with a microbalance (Sartorius Quintix 35-15, Goettingen, Germany). We then calculated the growth rate, expressed as the ratio between pupal mass and developmental time. Individuals were sexed after emergence.

Group 2: behavioural and morphological defences

In accordance with a previous study, we focused on the three main defensive behaviours expressed by larvae, called 'flee', 'twisting' and 'dropping' (Vogelweith et al. 2014), which were measured in 94 fifth instar larvae ($n = 28$ individuals at 22 °C; $n = 33$ individuals at 25 °C; $n = 33$ individuals at 28 °C). Behavioural tests were always carried out in the same order ('flee', 'twisting' and 'dropping') to reflect the sequence of defensive behaviours used by *L. botrana* larvae to escape from a parasitoid in natural conditions. For these behavioural measurements, each larva was tested at an ambient temperature strictly identical to the temperature of the constant regime within which the larva developed (either 22, 25 or 28 °C). Hence, testing temperatures differed according to the thermal regime of the tested larva. These testing temperatures were controlled by HOBO data logger. Once each test was run, 20 min of rest were given to larvae (in a clean Eppendorf tube pierced to allow air circulation) before the next test started.

The first behavioural defence ('flee') describes how fast larvae can escape a parasitoid without leaving the bunch they are feeding on. To quantify this behaviour, each individual was placed in the centre of a squared plastic sheet (84×116 cm, squares size: 1×1 cm) and acclimated for 30 s under the lid of a Petri dish. Once the lid was removed, the number of lines crossed by the larva was counted for 60 s. A preliminary experiment established that it took a minimum of 60 s for a larva to exit the squared sheet. As this behaviour typically occurs before the physical contact between caterpillar and parasitoid, no stimulus was needed to cause larvae to flee (Vogelweith et al. 2014).

The second behaviour ('twisting') corresponds to the ability of caterpillars to quickly and repeatedly twist in an attempt to hinder parasitoid oviposition. In our experimental setup, each larva was placed in a plastic box and acclimated for 30 s under the lid of a Petri dish. The lid was then removed and the larva touched with a fine brush in the dorsal part of the abdomen to mimic a parasitoid attack. An entire sequence of 1 min including 4 contacts (one per 15 s) was recorded (camera SONY HDR-CX220E, Tokyo, Japan) and analysed in slow motion with Kinovea 0.8.15 software to count the total number of twists over the 60 s.

The third behaviour ('dropping') describes how the caterpillar weaves a silk yarn and drops from the parasitised patch to land on another bunch, thereby escaping the foraging parasitoid. To mimic this situation, each larva was deposited at the top of a 100-cm high bracket, and touched with a fine brush (as described in the previous paragraph) until it dropped. The length of the yarn, which is supposed to be positively linked to escape ability of the caterpillar (Vogelweith et al. 2014), was measured with a ruler (precision ± 0.5 mm) after 5 s of stabilisation. As silk production is costly, yarn length can be linked to the investment of each larva in 'dropping' defence.

After completing three behavioural tests, all the larvae were frozen at −20 °C to further assess their morphological defences. The effectiveness of the integument acting as a physical barrier against a parasitoid sting relies on two features: mechanical resistance and thickness (Vogelweith et al. 2014). Mechanical resistance was quantified by penetrometry: larvae previously stored at −20 °C were thawed 15 min prior to the measurements being taken, and placed on an aluminium sheet fixed to a scale (precision ± 0.1 mg). A steel needle (TERUMO® 0.8×40 mm, Tokyo, Japan) mounted on a drill (PROMAC® 210 Z, Gonesse, France) was slowly

lowered until it penetrated the dorsal and ventral cuticle of the larva and touched the aluminium sheet. The contact between needle and sheet caused the illumination of a light device, and the scale indicated the physical pressure required to breach the integument. Immediately following integument resistance, we measured integument thickness (±0.01 mm) using a thickness gauge (Teclock SM-112, Milano, Italy). For each larva, two measurements of mechanical resistance and thickness were taken at two different locations to determine an average value for each of these integument features. Larvae were then stored in 70% ethanol for subsequent measurements of head capsule width. These measurements were taken using a stereo-microscope at 20× magnification (Nikon SMZ1500, Badhoevedorp, Netherlands), considering the distance between the most distant margins of the capsule as the most reliable estimation of larval body size in this species (Delbac et al. 2010). Nine individuals (4 larvae originating from the 25 °C treatment and 5 larvae originating from the 28 °C treatment) had been damaged during freezing process and were therefore removed from measurements of integument features and head capsule width.

Group 3: immune parameters

Immune parameters were assessed on 144 larvae ($n=48$ individuals for each thermal regime) without a prior immune challenge, because *L. botrana* caterpillars have a very high basal immunity and do not significantly respond to an infection (Vogelweith et al. 2014). Fifth instar larvae were chilled on ice for 30 min, and a 2 µl sample of haemolymph was obtained by pricking the posterior part of the dorsal surface, using a sterile glass capillary for collection (Hirschmann®Laborgeräte, Eberstadt, Germany). After haemolymph collection, caterpillars were preserved in 70% ethanol for subsequent measurements of head capsule width using the same method as described above.

These 2-µl samples of haemolymph were transferred into microcentrifuge tubes containing 20 µl of filtered phosphate-buffered saline (PBS, 8.74 g NaCl, 1.78 g Na_2HPO_4, 1000 ml distilled water, pH 6.5). Ten microlitres of this solution was immediately withdrawn to estimate the concentration of haemocytes, using a Neubauer-improved haemocytometer under a phase contrast microscope at 400× magnification (Nikon Eclipse E200, Amsterdam, Netherlands). The remaining solution (12 µl) was diluted in 10 µl of filtered PBS and stored at −20 °C for later measurements of activity of phenoloxidase (PO). We distinguished the activity of naturally functional PO (PO activity) and the activity of the proenzymes added to PO activity (total PO activity potentially involved in immune defence); both parameters were estimated using a spectrophotometry method based on Vogelweith et al. (2011). To assess total PO activity required the activation of proenzymes by adding chymotrypsin. For these measurements, each sample of diluted haemolymph solution was allowed to thaw very slowly on ice and subsequently centrifuged (4000 *g*, 15 min, 4 °C). Five microlitres of supernatant was deposited in a microplate well, either with 160 µl of diluted PBS solution (35 ml ultrapure water, 5 ml filtered PBS) for PO activity or with 160 µl of chymotrypsin solution (35 ml ultrapure water, 5 ml filtered PBS, 2.45 mg trypsin) for total PO activity. Finally, 20 µl of L-dopa solution (40 ml ultrapure water, 160 mg L-dopamine) was added to each well. The enzyme reaction proceeded for 3 h at 30 °C in a microplate reader (Versamax Molecular Devices, Sunnyvale, CA, USA), after which the readings were taken every 15 s at 490 nm. Data were subsequently analysed using the SOFT-Max®Pro 4.0 software (Molecular Devices, Sunnyvale, CA, USA). Maximal enzyme activity (V_{max}) was estimated as the slope of the absorbance curve during its linear phase and reported to the activity of 1 µl of pure haemolymph.

Statistical analyses

For larvae assigned to Group 1, developmental time was compared between sex and thermal regimes with a generalised linear model (GLM) based on Gamma distribution, which fit the data best, followed by Tukey post hoc test to identify differences among thermal regimes. Pupal mass and growth rate were normally distributed (Shapiro–Wilk test), and their variances were homogenous between sexes and thermal regimes (Levene test). Hence, pupal mass and growth rate were compared between sexes and thermal regimes by performing a two-way ANOVA followed by Tukey post hoc test, which helped to describe the significant interaction between sex and thermal regime we observed for these two measurements.

For larvae assigned to Group 2, no transformation allowed data normalisation for behavioural measurements. We estimated any confounding effect of larval body size on the behavioural measurements with Spearman's rank correlation test. Only the total number of twists was significantly correlated with larval body size. Hence, we corrected these data through division by size. All behavioural measurements were then compared between thermal regimes using Kruskal–Wallis test followed by Nemenyi post hoc test to identify differences among thermal regimes. Measurements of integument features were normally distributed and their variances were homogenous between thermal regimes. These data were therefore analysed with ANCOVA integrating thermal regime (as a factor) and larval body size (as a covariate), followed by Tukey post hoc test.

For larvae assigned to Group 3, no transformation allowed data normalisation for immune parameters. We tested for an effect of larval size on immune parameters with Spearman's rank correlation test. No size correction

was needed here because none of the immune parameters was significantly correlated with larval body size. All the immune parameters were compared between thermal regimes with Kruskal–Wallis test followed by Nemenyi post hoc test. Error in Tukey post hoc tests was controlled with Bonferroni correction. A tie correction was applied to Nemenyi post hoc tests when several observations shared the same value in dataset (behavioural measurements and haemocytes concentration). All statistical analyses were carried out using R 3.2.3 software (R Development Core Team 2016). For all tests, the alpha significance threshold was set at $\alpha = 0.05$, unless adjusted as described above to prevent Type I errors.

Results

Group 1: developmental time, pupal mass and growth rate

Developmental time was affected by the additive effects of sex and thermal regime: males developed faster than females, and both sexes developed faster as mean temperature increased (Fig. 1, Table 1). Mean values of developmental time and their 95% confidence interval ($CI_{95\%}$) were 24.7 days for males ($CI_{95\%} = [24.0; 25.5]$, $n = 622$ individuals), and 27.5 days for females ($CI_{95\%} = [26.5; 28.5]$, $n = 549$ individuals). Pupal mass and growth were impacted in the same way by the interactive effect between sex and thermal regime (Table 1). In females, pupal mass and growth rate increased more steeply with temperature compared with males. Hence, individuals reared at 28 °C displayed higher pupal mass and growth rate than those reared at 22 and 25 °C, and these differences were more pronounced for females than males (Fig. 2a, b).

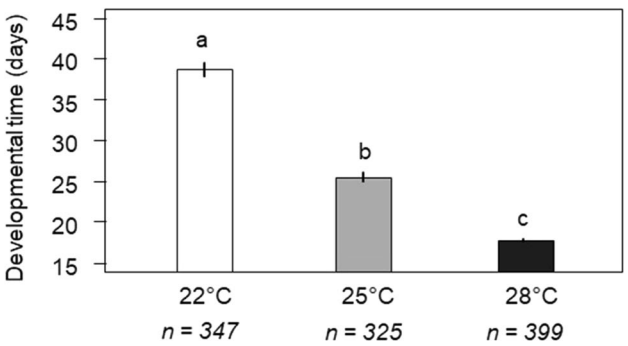

Fig. 1 Effect of thermal regime (22, 25 or 28 °C) on the mean (±95% confidence interval) developmental time of male and female *L. botrana* larvae. Means associated with the same letter are not statistically different ($P > 0.05$) and numbers below the x-axis refer to sample sizes

Table 1 Effects of sex, thermal regime (22, 25 or 28 °C) and their interaction on developmental time (from hatching to pupation), pupal mass, and growth rate (pupal mass/developmental time)

Factor	Test value	*P*
Developmental time[a]		
Sex	$\boldsymbol{\chi^2_2 = 30.310}$	**< 0.001**
Thermal regime	$\boldsymbol{\chi^2_2 = 1753}$	**< 0.001**
Sex × Thermal regime	$\chi^2_2 = 0.027$	0.870
Pupal mass[b]		
Sex	$\boldsymbol{F_{1,1165} = 1177}$	**< 0.001**
Thermal regime	$\boldsymbol{F_{2,1165} = 37.284}$	**< 0.001**
Sex × Thermal regime	$\boldsymbol{F_{2,1165} = 13.925}$	**< 0.001**
Growth rate[b]		
Sex	$\boldsymbol{F_{1,1165} = 284}$	**< 0.001**
Thermal regime	$\boldsymbol{F_{2,1165} = 548}$	**< 0.001**
Sex × Thermal regime	$\boldsymbol{F_{2,1165} = 35.298}$	**< 0.001**

Subscripts associated with statistics indicate degrees of freedom of the corresponding test. Bold values indicate significant differences ($P < 0.05$)

[a]Generalised linear model based on Gamma distribution

[b]Two-way ANOVA

Group 2: behavioural and morphological defences

Number of lines crossed (flee behaviour) was not impacted by thermal regime (Fig. 3a, Table 2). Total number of twists was negatively correlated with larval body size (Spearman's $\rho = -0.284$, Table 2), such that bigger larvae twisted significantly less than smaller larvae. After size correction, total number of twists was influenced by thermal regime (Table 2), such that larvae reared at 28 °C twisted less than those reared at 25 and 22 °C (Fig. 3b). Length of the silk yarn (dropping behaviour) also responded to thermal regime (Table 2), such that caterpillars wove shorter silk yarns at 28 °C in comparison with 22 °C (Fig. 3c).

Integument resistance was positively correlated with larval body size ($F_{1,81} = 5.174$, $P = 0.026$), such that bigger larvae displayed a more resistant integument than smaller larvae, but was not affected by thermal regime ($F_{2,81} = 3.000$, $P = 0.055$). Mean values of integument resistance were 2.58 mg at 22 °C ($CI_{95\%} = [2.29; 2.86]$, $n = 28$ individuals), 2.32 mg at 25 °C ($CI_{95\%} = [1.99; 2.67]$, $n = 29$ individuals), and 2.96 mg at 28 °C ($CI_{95\%} = [2.57; 3.36]$, $n = 28$ individuals). Integument thickness was not influenced by larval body size ($F_{1,81} = 0.952$, $P = 0.332$) or thermal regime ($F_{2,81} = 1.901$, $P = 0.155$). Mean values of integument thickness were 17.1 µm at 22 °C ($CI_{95\%} = [15.4; 18.7]$, $n = 28$ individuals), 15.9 µm at 25 °C ($CI_{95\%} = [14.5; 17.5]$, $n = 29$ individuals), and 17.9 µm at 28 °C ($CI_{95\%} = [16.7; 19.2]$, $n = 28$ individuals).

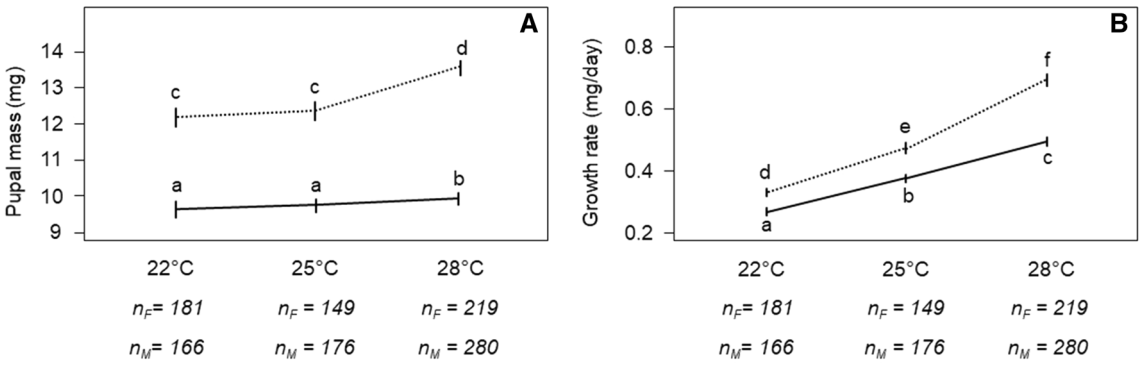

Fig. 2 Interactive effect between sex and thermal regime (22, 25 or 28 °C) on the mean (±95% confidence interval) of two life history traits for males (solid lines) and females (dotted lines): **a** pupal mass, and **b** growth rate. Means associated with the same letter are not statistically different ($P > 0.05$) and numbers below the *x*-axis refer to sample sizes (n_F for females, n_M for males)

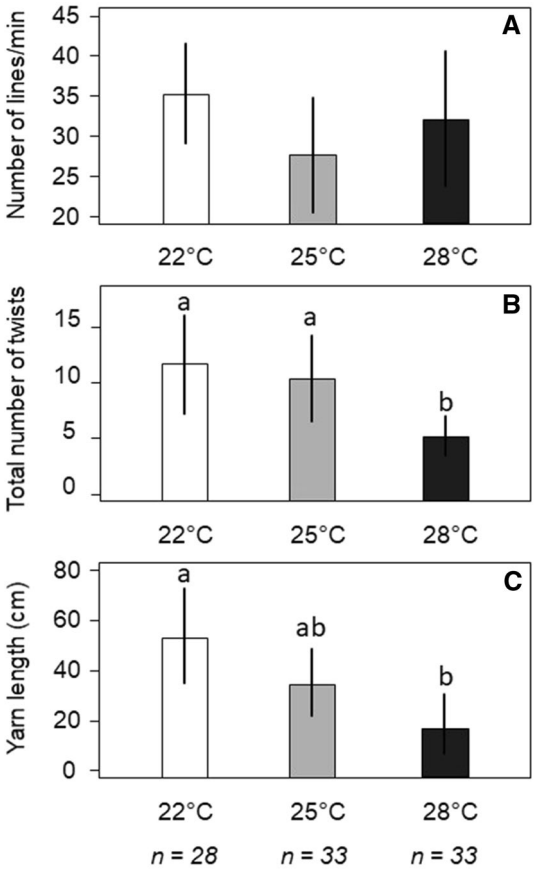

Fig. 3 Effect of thermal regime (22, 25 or 28 °C) on the mean (±95% confidence interval) of three evasive behaviours expressed by *L. botrana* larvae: **a** number of lines crossed, **b** total number of twists, and **c** length of the yarn spun. Means associated with the same letter are not statistically different ($P > 0.05$) and numbers below the x-axis refer to sample sizes

Table 2 Effects of larval body size and thermal regime (22, 25 or 28 °C) on behavioural and immune defences displayed by fifth instar *L. botrana* larvae

	Larval body size		Thermal regime	
	Test value	*P*	Test value	*P*
Behavioural defences				
Number of lines crossed	$\rho = 0.086$	0.434	$\chi^2_2 = 2.534$	0.282
Number of twists	$\boldsymbol{\rho = -0.284}$	**0.008**	$\boldsymbol{\chi^2_2 = 6.520}$	**0.038**
Yarn length	$\rho = -0.088$	0.422	$\boldsymbol{\chi^2_2 = 12.393}$	**0.002**
Immune defences				
Haemocytes concentration	$\rho = -0.012$	0.884	$\boldsymbol{\chi^2_2 = 17.232}$	**< 0.001**
Total PO activity	$\rho = -0.060$	0.474	$\boldsymbol{\chi^2_2 = 6.783}$	**0.033**
PO activity	$\rho = -0.015$	0.855	$\chi^2_2 = 0.919$	0.632

ρ values refer to Spearman's rank correlation test and χ^2 to Kruskal–Wallis test. Subscripts associated with statistics indicate degrees of freedom of the corresponding test. Bold values indicate significant differences ($P < 0.05$)

Group 3: immune parameters

None of the immune parameters measured were impacted by larval body size (Table 2). Haemocyte concentration and total PO activity were both affected by thermal regime (Table 2), such that larvae reared at 28 and 25 °C possessed significantly lower concentration of haemocytes than those reared at 22 °C (Fig. 4a). Similarly, total PO activity was lower for larvae reared at 28 °C compared with 22 °C (Fig. 4b). The PO activity did not respond to thermal regime (Table 2). Head capsule width (larval body size) was not impacted by thermal regime (one-way ANOVA, $F_{2,226} = 0.952$, $P = 0.388$).

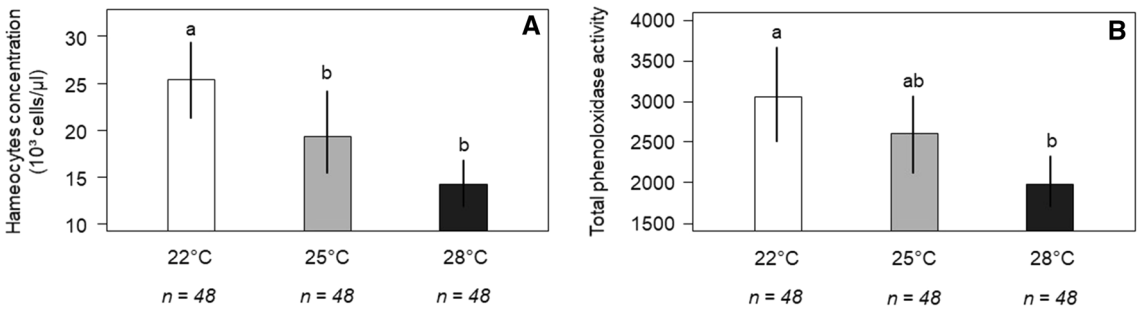

Fig. 4 Effect of thermal regime (22, 25 or 28 °C) on the mean (±95% confidence interval) of two immune parameters in one microlitre of pure haemolymph from *L. botrana* larvae: **a** haemocytes concentration, and **b** total phenoloxidase activity. Means associated with the same letter are not statistically different ($P > 0.05$) and numbers below the *x*-axis refer to sample sizes

Discussion

In this study, we assessed the response of several defensive traits displayed by fifth instar *L. botrana* larvae against parasitism when larvae were reared under different thermal regimes. Our study provides new evidence that a realistic increase in mean temperature, according to the predictions for simulated year 2100 in Mediterranean vineyards (Leibar et al. 2017), may impact the defensive system used by an herbivorous insect against parasitoids. Additionally, we showed that defensive traits responded differently to an increase in rearing temperature: some defensive traits were upregulated (developmental time), some were downregulated (evasive behaviours and immunity), and some were not influenced by this gradual warming (integument features). These results illustrate that considering the thermal response of only one defensive trait might be misleading when attempting to predict pest response to its natural enemies. More specifically, these findings have two major consequences for the resistance of *L. botrana* caterpillars to larval parasitoids and the outcomes of the interaction between these two trophic levels in the context of climate change.

First, we found that higher temperature diminished developmental times from an egg hatching to its chrysalis (e.g. duration of larval stage), which is a widely observed pattern in ectothermic animals (Angilletta et al. 2004). According to the 'slow-growth-high-mortality' hypothesis, faster development during a vulnerable stage limits the amount of time during which parasitism may occur (Clancy and Price 1987; Benrey and Denno 1997; Vogelweith et al. 2013b). In vineyards, most of the parasitoids known to attack *L. botrana* are larval endoparasitoids (Scaramozzino et al. 2017). Therefore, the shorter larval stage we observed under warmer conditions is likely to reduce larval exposure to a large proportion of its natural enemies.

Second, despite this potential increase in the ability of *L. botrana* caterpillars to avoid the encounter with parasitoids, one of our most striking results was a widespread decrease in larval defensive behaviours (twisting and dropping) and immune effectors involved in destroying parasitoid eggs (haemocytes concentration and total PO activity) at 28 °C compared with cooler mean temperature (25 or 22 °C). These two defensive strategies have been identified as the two major components of the defensive system used by *Lobesia botrana* caterpillars against parasitoids (Vogelweith et al. 2014). Thus, global warming could substantially increase parasitoid success by limiting both the ability of host larvae to actively reduce parasitism rates (e.g. avoiding parasitoid oviposition by means of defensive behaviours) and parasitoid emergence (e.g. killing parasitoid eggs by means of immune processes).

Some critical issues must, however, be considered before inferring that parasitism success is linked with thermally induced changes in host development, behaviour and immunity. First, insects experience temperatures that fluctuate daily over the course of their development in natural conditions (Colinet et al. 2015). Such thermal fluctuations are known to affect defensive traits involved in host resistance, like those related to immune function (Fischer et al. 2011). The effects of thermal fluctuations on performance can be predicted and understood once the thermal response of the trait considered has been established, as we did in our study by comparing constant regimes. Daily fluctuations in temperatures may affect performance in different ways depending on the mean temperature around which they occur, and whether or not these fluctuations include stressful temperatures that depress performance (Colinet et al. 2015). More specifically, thermal fluctuations might exert negative effects on host resistance if they expose the organism to temperatures remaining above the thermal optimum for defensive traits during the warmest hours of the cycle. In the context of global warming, such negative effects of thermal fluctuations on host resistance are more likely to occur as mean temperatures rise (Colinet et al. 2015). For this reason, we expect that daily fluctuations in temperatures experienced in hotter environments will also lead to decreased host resistance,

as we observed to result when hosts were exposed to an increase in constant temperature during development.

Second, to what extent direct measurements of host immunocompetence can predict host resistance to natural enemies is under debate (Adamo and Lovett 2011; Enríquez-Vara et al. 2012; Yin et al. 2014). Many larval endoparasitoids belonging to the Ichneumonidae and Braconidae families inject viruses that suppress the host immune system and protect parasitoid eggs from encapsulation and melanisation (Asgari et al. 1996; Beckage 1998). Interestingly, the ability of the parasitoid to circumvent the immune function of its host depends on thermal conditions: higher temperatures have been shown to downregulate the expression of viral genes transmitted by the parasitoid to its lepidopteran host (Seehausen et al. 2017a). In vineyards, ichneumonid and braconid species frequently parasitise *L. botrana* larvae (Scaramozzino et al. 2017), and thus, it may be important to consider that host immune response may vary with temperature and parasitoid species.

Third, our results clearly indicate a complex response of several host defensive traits that is likely to modify the host–parasitoid interaction, but not in easily predictable ways. Warming conditions are known to significantly reduce the time needed to complete the development of parasitoids within their host (Duan et al. 2014; Schreven et al. 2017; Noor-ul-Ane et al. 2017). This thermal response is expected to maintain a temporal overlap between the occurrence of adult parasitoids and vulnerable hosts, even if the host is speeding up its development following exposure to warmer conditions (Klapwijk et al. 2010; Noor-ul-Ane et al. 2017). However, the ability of parasitoids to cope with a reduced period of host susceptibility will also be influenced by their temperature-dependent foraging activity and lifespan (Henri et al. 2012). For example, temperature influences locomotor activity, patch time allocation, sex allocation and host selection of parasitoids (Moiroux et al. 2015, 2016), which makes difficult to predict the way global warming may affect host–parasitoid interactions. Consequently, additional information about the thermal response of the whole host–parasitoid complex would be helpful to understand more deeply how these interspecific relationships might be reshaped by global warming (see Bahar et al. 2012; Delava et al. 2016 for some examples).

To what extent grapevine moth might impact wine production in the future will depend on the concurrent effects of hotter conditions on its population dynamics and its relationships with lower (e.g. host plant) and upper trophic levels (e.g. natural enemies such as parasitoids). The simulated warming experienced by *L. botrana* caterpillars induced increased pupal mass and growth rate, which remains in accordance with other experimental findings (as reviewed in Angilletta and Dunham 2003). Importantly, this thermal response was stronger in females. As female fecundity is positively linked to pupal mass in *L. botrana* (Moreau et al. 2006b), these results indicate a potential benefit of global warming on the fecundity of this pest. The associated detrimental impacts on wine-making activity could even be exacerbated by an increase in voltinism as observed in Mediterranean vineyards, owing to higher development rates and an extended growing season (Martín-Vertedor et al. 2010). In agroecosystems, *L. botrana* caterpillars might feed on several grapevine cultivars and alternative host plants with different nutritional values. This variation in food quality has been shown to affect both the immunocompetence of this species (Vogelweith et al. 2011; Muller et al. 2015) and the parasitism rates observed in the field (Vogelweith et al. 2013a). The geographic distribution of grape varieties is largely controlled by climatic conditions, hence global warming is expected to lead to a profound reshaping of the optimal zones for many cultivars (Fraga et al. 2016). Accurate predictions about future crop damage associated with *L. botrana* in a given area with specific changes in climate could be made when combining the predicted future optimal zones of grape varieties with the known effects of these cultivars on the biological control exerted by parasitoids. Our study provides new evidence that a realistic increase in mean temperature might modulate the levels of expression of several defensive traits used by *L. botrana* caterpillars against their larval parasitoids. In the light of these findings, we advocate to also incorporate the direct effects of future thermal conditions likely to occur in vineyards when attempting to predict the damage associated with *L. botrana* in the coming decades.

Author contribution

GM, JM and PL conceived the experimental design. GM carried out the experiments. All the authors analysed the data. CI led the writing of the manuscript. All authors gave their final approval of the version to be published.

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Data accessibility Datasets are available under request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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