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Defense strategies used by two sympatric vineyard moth pests

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ABSTRACT

Natural enemies including parasitoids are the major biological cause of mortality among phytophagous insects. In response to parasitism, these insects have evolved a set of defenses to protect themselves, including behavioral, morphological, physiological and immunological barriers. According to life history theory, resources are partitioned to various functions including defense, implying trade-offs among defense mechanisms. In this study we characterized the relative investment in behavioral, physical and immunological defense systems in two sympatric species of Tortricidae (*Eupoecilia ambiguella, Lobesia botrana*) which are important grapevine moth pests. We also estimated the parasitism by parasitoids in natural populations of both species, to infer the relative success of the investment strategies used by each moth. We demonstrated that larvae invest differently in defense systems according to the species. Relative to *L. botrana*, *E. ambiguella* larvae invested more into morphological defenses and less into behavioral defenses, and exhibited lower basal levels of immune defense but strongly responded to immune challenge. *L. botrana* larvae in a natural population were more heavily parasitized by various parasitoid species than *E. ambiguella*, suggesting that the efficacy of defense strategies against parasitoids is not equal among species. These results have implications for understanding of regulation in communities, and in the development of biological control strategies for these two grapevine pests.

1. Introduction

Natural enemies including parasitoids are the major biological cause of mortality among phytophagous insects (Hawkins et al., 1997). In response, preys have evolved a set of defenses, including behavioral, morphological, physiological and immunological barriers (Greeney et al., 2012; Gross, 1993). Behavioral mechanisms are the first line of defense, and involve a wide range of behaviors (e.g. biting, twisting, dropping) that act to reduce the risk and effects of infection by parasitoids (Greeney et al., 2012). The second line of defense in insects is the tough cuticle, which forms an efficient protective integument over the external surface (Cole, 1959; Greeney et al., 2012). If the cuticle is breached by parasitic infection, the insect's immune system has to produce a rapid and efficient response to ensure host survival. Insect immunity is innate and relies on a suite of systemic responses that include encapsulation, whereby haemocytes form a multi-layered capsule around a foreign object, such as a parasitoid egg. The agglutinated haemocytes produced melanin on the surface of the capsule, through activation of the enzymes of the prophenoloxidase cascade (Cerenius and Soderhall, 2004; Siva-Jothy et al., 2005).

As described above, the host has several defense strategies for preventing deadly infection. Dewitt and Langerhans (2003(DeWitt, 2003 #235)) proposed an integrated approach to study of the various defense traits, so as to achieve a better understanding of how natural enemies result in the formation of an arsenal of defenses in prey species. Indeed, they noted that different defenses can be either negatively correlated (trait compensation) or positively correlated (trait co-specialization). If defense strategies are costly, it is likely that hosts will evolve only a subset of those available. The cost of defense has some direct support (Flenner et al., 2009; Kraaijeveld et al., 2002; Nelson, 2007; Parker et al., 2011; Rigby and Jokela, 2000). Numerous studies demonstrate trade-offs between morphological and behavioral defenses (DeWitt et al., 2000; Hammill et al., 2010; Mikolajewski and Johansson, 2004; Parker et al., 2011; Steiner and Pfeiffer, 2007), and some a trade-off between behavioral and immunological defenses (Rigby and Jokela, 2000; Zylberberg et al., 2013). Trade-offs suggests that organisms may benefit from balancing investment in immunological and

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non-immunological defenses, because both entail costs but serve a common function. Hence, trait compensation among defense strategies appears to be a common response to predation avoidance. In contrast, few studies have demonstrated co-specialization among defense strategies (DeWitt et al., 2000; Mikolajewski and Johansson, 2004).

Studies assessing the interaction between defense traits have typically focused on two defense barriers. However, integrating behavioral, morphological and immunological defenses in single studies would greatly extend our understanding of compensation and co-specialization among multiple traits (Steiner and Pfeiffer, 2007). Considering immunological and non-immunological defenses in the same study is necessary for assessing the adaptation of hosts to their natural enemies, but only few studies have adopted this approach (Rigby and Jokela, 2000; Zylberberg et al., 2013). To obtain a complete picture of the relationships among defense traits, comparison of closely related species that share the same environment (same ecological niche and trophic resources) is useful in evaluating environmental influences on defense traits (Mikolajewski and Johansson, 2004), and assessing how generalization in insect defenses has evolved (Greeney et al., 2012).

The two sympatric grapevine moths used in this study are *Eupoecilia ambiguella* and *Lobesia botrana* (Lepidoptera, Tortricidae). These two major grapes pests of European viticulture and are ideal candidates (i.e. same ecology) for assessing how defense traits are related. These species often co-occur together in septentrional vineyards having intermediate hygrometry (dry conditions being a lethal factor for *E. ambiguella* eggs). They also share the same parasite range, comprising a few key parasitoids of their eggs and larvae (Thiéry, 2008). In this study we characterized the relative investment in behavioral, physical and immunological defense systems in laboratory strains of these Tortricidae species. We undertook a similar study using wild collected insects of each species, from a single vineyard. In addition, to assess the relative success of the investment strategies of these moth species among behavioral, physical and immunological defense systems, we estimated parasitism success by their parasitoids.

2. Material and methods

This study conformed to French legal requirements, and to accepted international ethical standards, including those relating to conservation and welfare, and to the journal's policy on these matters. All experiments conformed to the Guiding Principles in the Care and Use of Animals, approved by the Council of the American Physiological Society.

2.1. Insect models and experimental designs

E. ambiguella and *L. botrana (*Lepidoptera, Tortricidae), further referred as *E.a.* and *L.b.*, have a wide geographical distributions and mainly damage all grape bunches development stages (Thiéry, 2008). Depending on the region in Europe, *E. ambiguella* completes 2–3 broods each year and *L. botrana* completes 3–4. The first generation of eggs is laid on the flower buds in spring, and the young larvae bore into the flower buds and aggregate them with silk in larval nests called glomerulae. The second generation of larvae emerges between the end of June and the mid-July according to the climate and the third generation occurs between mid-August and the end of September. The larvae are both polyphagous and can develop on most grape cultivars, and on other plant species (Thiéry, 2008; Thiery and Moreau, 2005).

The laboratory strains of *E. ambiguella* and *L. botrana* used in this study were derived from inbred stock maintained at the French National Institute for Agricultural Research (INRA), Bordeaux, France. For each species the larvae were cultured in groups and maintained under standard laboratory conditions (22 ± 1 °C; 70 ± 10% relative humidity; light/dark photoperiod: 16:8). The larvae were fed *ad libitum* with a semi-artificial diet (Vogelweith et al., 2011). The study involved a total of 502 newly hatched larvae (age < 24 h) of *E.a.* and 433 of *L.b*. These were reared individually in centrifuge tubes containing 1.5 mL of semi-artificial diet, which was sufficient for the larva to complete development (Thiery and Moreau, 2005). The lid of each tube was pierced with a needle to enable air circulation. The larvae were cultured until they reached the 5th larval instar stage, when they were used in experiments to assess defense strategies (physical, behavioral or physiological).

Wild larvae of *L.b.* and *E.a.* were collected in May 2012 on Cabernet Franc grapevines at the Château Brillette vineyard (Médoc, Aquitaine, France). We sampled larvae at the end of larval development (5th instar) from the first generation. The 5th instar was checked by measuring the head capsule width, an easy and accurate indicator for the determination of larval stages in natural populations of these species (Delbac et al., 2010). Only silk nests with larvae inside were removed from the bunches. The two grape pest tested in this study are Tortricids (subfamily) which lay separate and spaced eggs among bunches (Thiery and Gabel, 1993). As a result larvae are not gregarious and larvae are single per nest. Collected larvae were maintained in small polyethylene boxes (60 × 40 × 21.4 cm), fed *ad libitum* on bunches collected from the same locality, and maintained at 24 ± 1 °C, 60 ± 10% relative humidity and natural photoperiod conditions until used in experiments. The larvae were then screened using a binocular microscope to estimate the parasitism rate and parasitism success. The parasitism rate was estimated by recording the presence of parasitoid stings (small melanotic patches) on the larval body surface, and was calculated as the number of larvae having parasitoid stings divided by the number of larvae screened. The parasitism rate enabled us to assess the efficacy of the behavioral and morphological defense strategies. Parasitism success was estimated by keeping larvae individually with their silk nest and cotton soaked in water in small plastic jars (30 mm × 30 mm diameter) pierced with a needle to enable air circulation. Each larva was checked daily until pupation occurred, at which time the chrysalis was carefully removed from the flower bud and placed in a glass tube (70 × 9 mm diameter) stoppered with a cotton plug, and stored under standard laboratory conditions, as described above. The chrysalids were checked daily for adult emergence. Parasitism success was calculated as: number of parasitoids/(number of adult *L. botrana* + number of parasitoids emerged). In this measure of parasitism, we considered all larval endoparasitoids emerging in order to get an overall index of the local selective pressure imposed by parasitoid community.

Field larvae with no parasitoid stings on the cuticle were used to characterize levels of investment in behavioral, physical and immunological defenses, as described above for the laboratory strains.

2.2. Behavioral defenses

We focused on three defense behaviors used by moth larvae to escape predators or parasitoids. We first considered the ability of larvae to move away ('flee') by measuring their movement speed. To this end, each larva was placed in a horizontal gridded plastic sheet (84 × 116 cm) and acclimated for 15 s under the cap of a 50 ml Falcon tube. Following removal of the cap, the number of lines crossed by the larvae was recorded for 90 s, which was the minimum time required for a larva to exit the gridded sheet, estimated in preliminary experiments.

The second defense behavior was the ability of the larvae to repeatedly and rapidly twist ('twisting' defense) in response to a stimulation mimicking a parasitoid sting (Greeney et al., 2012).

Each larva was placed on a plastic sheet (21 cm × 29.7 cm) and acclimated for 15 s under a Falcon tube cap. The larva was touched dorsally with a fine brush once every 15 s for 60 s, and the entire sequence was video recorded (JCV Everio GZ-MG21E hybrid camera). The larval response was quantified by scoring the number of twists (wave motion characterized by S-shaped twists) from which the average number of twists per larva was calculated.

The third defense behavior considered was the natural escape behavior from the bunches, larvae in danger spin a silk yarn and drop to land on another grape ('dropping' defense). The length of the silk thread determined the distance to which the larva could escape using this method. Larvae were placed on a gallows set at a height of 50 cm. Each larva was touched dorsally with a fine brush until they jump the gallows. The yarn length woven by each larva was measured with a ruler (precision ± 0.5 mm), and the number of touches required to induce the escape behavior was recorded.

2.3. Physical defenses

We investigated the mechanical resistance and thickness of the integument, as its hardness acts as a mechanical barrier to parasitoid puncture. The mechanical resistance of the integument was quantified using a penetrometry method. Larvae of both species were sacrificed in ethanol 1 h prior to measurement. Each larva was attached to a polystyrene board (30 × 40 mm) using double-sided tape, and the board was placed on a precision scale (precision ± 0.1 mg). A steel needle in a drill press was moved slowly down until it touched and penetrated the integument. The value on the scale when the needle disrupted the integument was used as a measure of the physical pressure required to breach the integument, and was recorded as its mechanical resistance. Measurements could only be performed once per larva because of the small size of the larvae (maximum head capsule width: 1.3 mm).

Measurements of integument thickness were made immediately following measurements of the mechanical resistance. Larvae were removed from the polystyrene board, dissected to isolate the integument (dorsal and ventral part), and the integument thickness was measured using a pressure-sensitive thickness gauge (Teclock SM-112, Alpa SpA, Milano, Italy; precision ± 0.01 mm). For each larva two measurements were performed at different parts of the integument, and the mean value of the two measurements was recorded.

2.4. Immune defenses

Levels of investment in the immune system were determined by measuring three immune parameters: the concentration of circulating haemocytes; the activity of the prophenoloxidase enzyme system (Marmaras and Lampropoulou, 2009); and the level of antimicrobial activity in individual samples of haemolymph.

To determine the base levels of these immune parameters, a first group of larvae of each species was used to estimate each parameter from large individual samples of haemolymph from immunologically naïve larvae. To this end the larvae were chilled on ice for 20 min, and from each larva a 2 μl sample of haemolymph was collected using a sterile glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany) from a wound made in the posterior part of the ventral side of the abdomen. Of this, 1 l of sample was transferred to a microcentrifuge tube containing 25 μl of cold sodium cacodylate/$CaCl_2$ buffer (0.01 M sodium cacodylate; 0.005 M $CaCl_2$; pH 6.5), and a 10 μl sample of this solution was immediately removed for measurement of the concentration of haemocytes, based on counts made using a Neubauer Improved Haemocytometer and phase contrast microscopy (magnification ×400). The remaining haemolymph solution was stored at −27 °C for later measurement of the PPO system. The remaining 1 μl of haemolymph in the capillary was then flushed into an internally coated (n-phenylthiourea; Sigma P7629, Sigma–Aldrich, St Louis, MO, USA) microcentrifuge tube containing 2 μl of cold sodium cacodylate/$CaCl_2$ buffer, and this solution was stored at −27 °C for later measurement of antibacterial activity.

To estimate the amplitude of the immune response in each species, a second group of larvae was used for measurement of the concentration of haemocytes and the activity of the PPO system, performed when they were immunologically naïve and 24 h following an immune challenge mimicking a bacterial infection (Vogelweith et al., 2013). This time-point was chosen based on preliminary experiments that showed that the amplitude of the immune response reached its highest level at this moment (results not shown). To obtain a haemocyte count and measure the activity of the PPO system prior to challenge, the larvae were chilled on ice for 20 min, and 1 μl of haemolymph (collected as described above) was flushed into a micro-centrifuge tube containing 20 μl of cold sodium cacodylate/$CaCl_2$ buffer. The larvae were then immediately immune-challenged with a sterile needle dipped into a concentrated suspension of heat-killed *Arthrobacter globiformis* (approximately 10^9 cells ml^{-1}; Pasteur Institute, CIP 105365) to mimic a bacterial infection; the bacterium was cultured as described by Vogelweith et al. (2011). Each challenged larva was transferred to a microcentrifuge tube and provided *ad libitum* food under standard conditions for 24 h, at which time a second 1 μl sample of haemolymph was collected to assess the hemocyte concentration and PPO system activity following challenge. As our purpose was to compare immune activity across species following experimental infection, controlling for the effects of wounding was not necessary.

The activity of the PPO system was evaluated following the method of Vogelweith et al. (2011), and involved spectrophotometric measurement of the activity of naturally-activated phenoloxidase enzymes (PO activity), and the combined activity of the proenzyme (prophenoloxidase) and PO (total-PO activity). Antimicrobial activity in the hemolymph was measured using the zone of inhibition assay as described by Vogelweith et al. (2011).

2.5. Statistical analyses

As the data were not normally distributed (Shapiro–Wilk and Bartlett's test), we used the Mann–Whitney Wilcoxon test to compare the species in relation to behavioral (flee, dropping and twisting) and physical (mechanical resistance and thickness of the integument) defenses, and their basal immunity (haemocyte concentration, PO and total-PO enzyme activity, and antimicrobial activity).

Changes in the haemocyte concentration and the PO and total-PO activities following bacterial challenge were analyzed using analyses of variance for repeated measures (repeated ANOVA), with immune challenge as the within-subject factor and species as covariate. The assumption for parametric tests was assured by natural log transformations of the immune parameters. We used Pearson χ^2 test to assess differences in parasitism measures (parasitism rate and successful parasitism) between the species. All statistical tests were performed using the R 2.15.0 (R Development Core Team 2012) software. In all the comparisons the level of significance was $\alpha = 0.05$.

3. Results

3.1. Physical defenses

In the laboratory insect lines the mechanical resistance (Fig. 1a) and thickness of the integument of larvae (Fig. 1b) were greater for

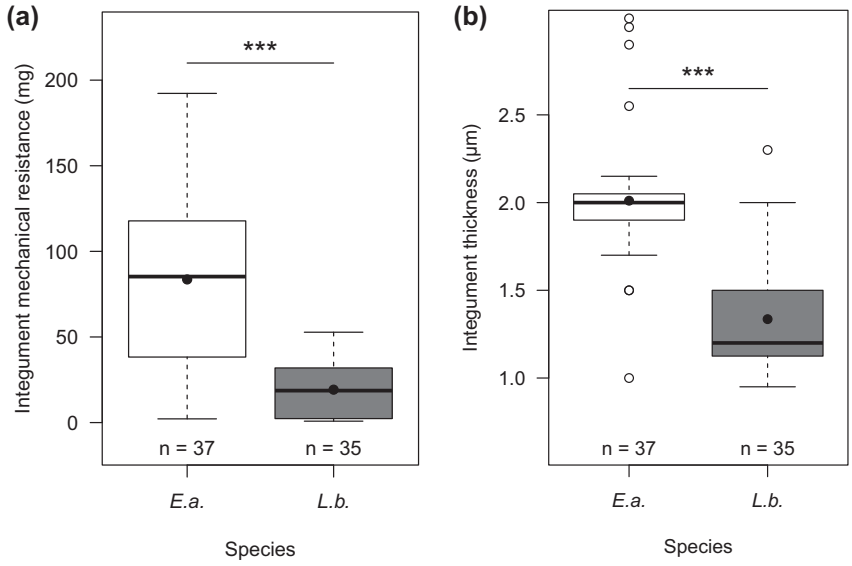

Fig. 1. Physical defense barriers in grape moths. (a) Integument mechanical resistance (mg) and (b) integument thickness (µm) in *E. ambiguella* (*E.a.*; white) and *L. botrana* (*L.b.*; grey). The edges of the boxes: first and third quartiles; the central features: the medians; maxima and minima: dashed lines; black circles: the means; white circle: the outliers. ***Highly significant difference ($p \leqslant 0.0001$); *n* (above the *x*-axis) = number of larvae of each species tested.

Table 1
Species effect on defense measures considered in the study.

		Rearing lines		Wild lines	
$Measures_{df}$		Test value	p	Test value	p
Physical barrier					
Mechanical integument $resistance_1$[a]		**21.81**	**<0.0001**	**18.01**	**<0.0001**
Integument $thickness_1$[a]		**38.02**	**<0.0001**	1.95	0.16
Behavioral barrier					
$Flee_1$[a]		**15.10**	**<0.0001**	**20.92**	**<0.0001**
$Dropping_1$[a]		**43.37**	**<0.0001**	**5.19**	**0.02**
$Twisting_1$[a]		**54.3**	**<0.0001**	**20.73**	**<0.0001**
Physiological barrier: immune parameters					
Basal immunity[a]					
Haemocyte $concentration_1$		3.66	0.055	2.79	0.09
PO $enzyme_1$		**10.14**	**0.001**	**16.13**	**0.0001**
Total-PO $enzyme_1$		**30.50**	**<0.0001**	**12.34**	**0.0004**
Antimicrobial $activity_1$		2.450	0.11	0.18	0.67
Immune challenge[b]					
Haemocyte $concentration_{35}$	*E.a.*	**3.78**	**0.0003**	–	–
Haemocyte $concentration_{19}$	*L.b.*	−0.86	0.80	–	–
PO $enzyme_{35}$	*E.a.*	**1.74**	**0.045**	–	–
PO $enzyme_{19}$	*L.b.*	0.88	0.19	–	–
Total-PO $enzyme_{35}$	*E.a.*	0.85	0.19	–	–
Total-PO $enzyme_{19}$	*L.b.*	−1.00	0.83	–	–
Parasitism measures					
Parasitism $rate_1$[c]		–	–	**21.48**	**<0.0001**
Parasitism $success_1$[c]		–	–	**5.92**	**0.015**

Significant values ($p \leqslant 0.05$) are shown in bold.
[a] Mann–Whitney Wilcoxon test.
[b] Wilcoxon signed rank test.
[c] Pearson χ^2 tests.

E.a. than for *L.b.* (Table 1). In wild caught insects the integument of the *E.a.* larvae was more resistant than that of the *L.b.* larvae, but the integument thickness was similar (Table 1).

3.2. Behavioral defenses

Except for the number of touches, all behavioral defenses measured were influenced by insect species, whether they were laboratory strains or wild caught (Table 1). The number of lines crossed (Fig. 2a), the number of twists (Fig. 2b) and the length of the silk yarn per larva were greater for *L.b.* than for *E.a.* The number of touches required to induce a dropping defense behavior was greater for *E.a.* than for *L.b.* (Table 1), but only in laboratory strains.

3.3. Immune defenses

3.3.1. Basal immunity

The haemocyte concentration and antimicrobial activity were not different between insect species, whether the larvae were from laboratory strains or wild caught (Table 1). The *L.b.* larvae had an

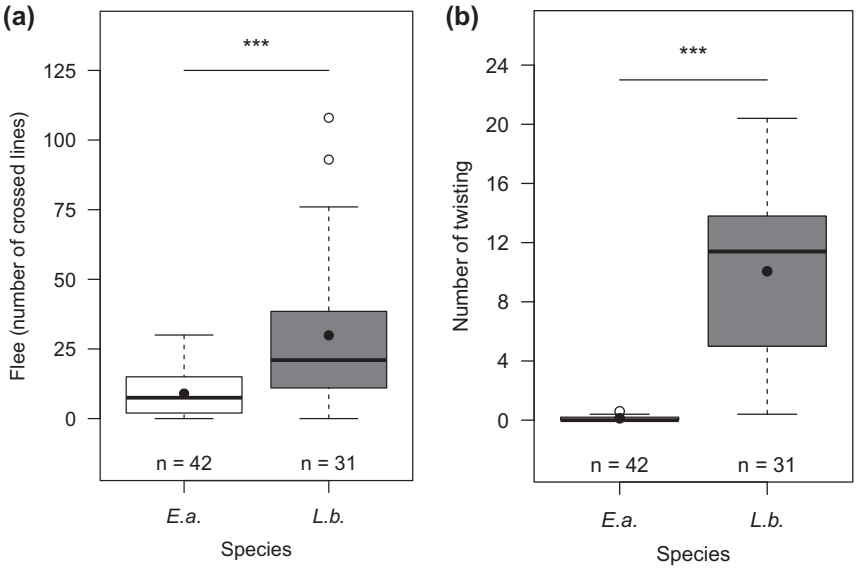

Fig. 2. Behavioral defense barriers in grape moths. (a) Flee response (number of lines crossed) and (b) twisting for reared and wild caught larvae of *E. ambiguella* (*E.a.*; white) and *L. botrana* (*L.b.*; grey). The edges of the boxes: first and third quartiles; the central features: the medians; maxima and minima: dashed lines; black circles: the means; white circle: the outliers. ***Highly significant difference ($p \leqslant 0.0001$); *n* (above the *x*-axis) = number of larvae of each species tested.

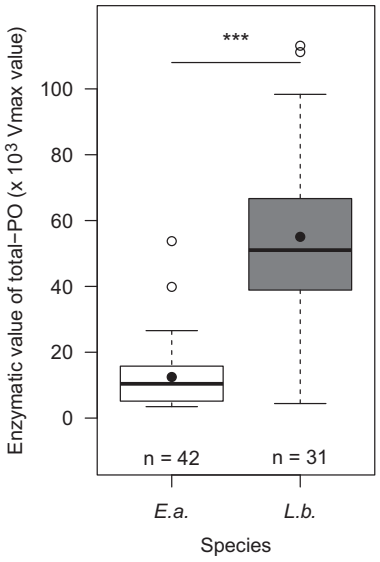

Fig. 3. Basal immunity in grape moths based on activity of the total-PO enzyme ($\times 10^3$ V_{max} value) in reared and wild-caught larvae of *E. ambiguella* (*E.a.*; white) and *L. botrana* (*L.b.*; grey). The edges of the boxes: first and third quartiles; the central features: the medians; maxima and minima: dashed lines; black circles: the means; white circle: the outliers. ***Highly significant difference ($p \leqslant 0.0001$); *n* (above the *x*-axis) = number of larvae of each species tested.

average of 13,758 ± 957 haemocytes/μl of haemolymph, whereas *E.a.* had an average 19,106 ± 1796 haemocytes/μl. The mean diameter of the zone of inhibition was 1.52 ± 0.66 mm for *L.b.* larvae and 4.07 ± 1.17 mm for *E.a.* larvae. However, the PO and total-PO enzyme activities were higher in *L.b.* larvae than in *E.a.* larvae, for both laboratory strains and wild caught insects (Table 1; Fig. 3).

3.3.2. Immune challenge

No change in immune response was observed in larvae of *L.b.* among the immune effectors considered (haemocyte concentration, PO and total-PO enzyme activities) (Tables 1 and 2). In contrast, in *E.a.* larvae the concentration of haemocytes and PO activity increased significantly following immune challenge (Tables 1 and 2; Fig. 4), but there was no significant effect on total-PO enzyme activity (Tables 1 and 2).

Table 2
Repeated measures analysis for haemocytes, and PO and total-PO activities.

	Haemocytes		PO activity		Total-PO activity	
Source	$F_{1, 53}$	*p*	$F_{1, 53}$	*p*	$F_{1, 53}$	*p*
Between subjects						
Species	**7.56**	**0.008**	**69.45**	**<0.0001**	**344.58**	**<0.0001**
Within subjects						
Time	1.68	0.20	1.91	0.17	0.02	0.87
Time*species	**7.97**	**0.007**	0.33	0.57	1.82	0.18

Significant values ($p \leqslant 0.05$) are shown in bold.

3.4. Parasitism measures

Larvae of both species differed in the rate of parasitism and the parasitism success (Table 1). A total of 28.5% of the *L.b.* larvae had cuticle stings, whereas only 4.1% of the *E.a.* larvae had stings (Table 1; Fig. 5). Parasitism by parasitoids was more successful in *L.b.* than in *E.a.*, with no parasitoids successfully developing in *E.a.* (Table 1, Fig. 5).

4. Discussion

Insect larvae protect themselves from parasitoids using a diverse suite of defense systems. In this study we compared the relative investment into behavioral, physical and immunological defense systems in two related grapevine moths, *E. ambiguella* and *L. botrana*. We demonstrated that larvae of these two species exhibit different patterns of investment into the various defense systems. The *E. ambiguella* larvae invested more into morphological defenses (more resistant and thicker integument) than in behavioral defenses (slow flee behavior, weaker twisting and dropping defenses) than did *L. botrana*. In addition, the larvae of *L. botrana* exhibited higher basal levels of immune defense (PO and PPO activity) than the larvae *E. ambiguella*. However, the latter

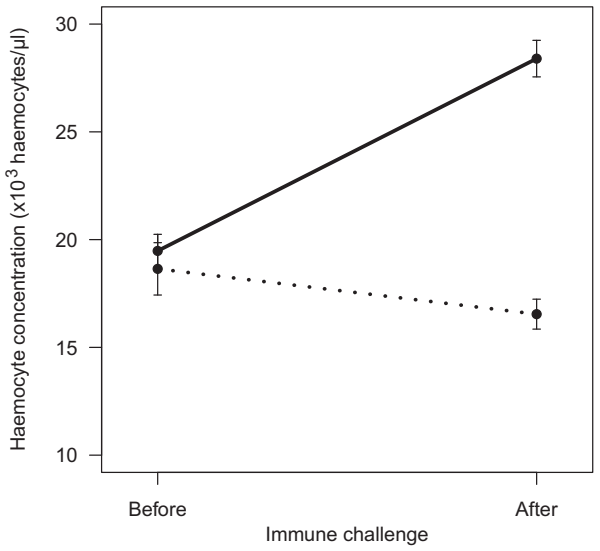

Fig. 4. Variation in haemocyte concentration ($\times 10^3$ haemocytes/µl) in *E. ambiguella* (*E.a.*; solid line) and *L. botrana* (*L.b.*; dotted line) prior to and following immune challenge with heat-killed bacteria (*A. globiformis*). Black circles represent the mean and standard error. A total of 36 *E.a.* larvae and 20 *L.b.* larvae were tested in this experiment.

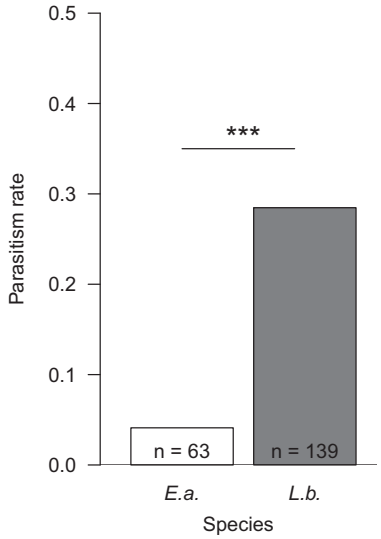

Fig. 5. Parasitism rate in *E. ambiguella* (*E.a.*; white) and *L. botrana* (*L.b.*; grey), based on the number of larvae with parasitoid stings. ***Highly significant difference ($p \leqslant 0.0001$); *n* (above the *x*-axis) = number of larvae of each species tested.

responded to immune challenge by increasing immune parameters, whereas the larvae of *L. botrana* appeared to not have the capacity to increase their immune effectors following a bacterial challenge. *L. botrana* larvae from natural populations were more parasitized by parasitoids than *E. ambiguella*, suggesting that the efficiency of the immune defense against parasitoids is not equal in these species.

A major finding of this study is that the two sympatric species have not developed complete suites of defense. As predicted by optimal defense theory, resources allocated to different defense systems should be partitioned with other fitness traits. For example, an individual investing in defense may reduce investment in fecundity (Gwynn et al., 2005). Moreover, defense systems against natural enemies are energetically costly, imposing a limit on the diversity of defense mechanisms that can be invested in. In this regard we observed a differential investment in defense systems, which varied between the insect species. *L. botrana* appears to rely primarily on behavioral and basal immunity responses, whereas in *E. ambiguella* morphological and immune responsiveness to immune insults appear to dominate. Therefore, our results suggest that tortricid moths show a set of trait co-specialization and compensation, based on the classification of DeWitt et al. (2000). Our results are consistent with the general observation that in these two closely related moths, morphological defenses are negatively traded-off with behavioral defenses (DeWitt et al., 2000; Hammill et al., 2010; Lefevre et al., 2012; Mikolajewski and Johansson, 2004; Parker et al., 2011; Steiner and Pfeiffer, 2007). Indeed, we found that the species having the stronger morphological defenses also had poor behavioral defenses against parasitoids. We also found clear trade-offs between immunological defenses within the moth species. *L. botrana* larvae showed greater basal activity of the prophenoloxidase system than did *E. ambiguella*, whereas the latter had stronger and faster immune responses to bacterial challenge, which involved an increased number of circulating haemocytes (probably released from the hematopoietic organ and/or by continued division of circulating haemocytes; (Lavine and Strand, 2002) and higher PO activity. The comparison of the immune systems of these two related species highlights two different defense strategies. *L. botrana* has a constitutive defense system that is ready to respond to an attack, whereas *E. ambiguella* has a low level of basal immunity but is capable of rapid mobilization of immune effectors to combat infection. Differential selective pressure imposed by parasitoids in vineyards may explain the co-occurrence of different defense strategies in these related species. Our field sampling showed that *L. botrana* larvae were more parasitized than *E. ambiguella*, with approximately 30% of the former having parasitoid stings on their cuticles compared with only 4% of *E. ambiguella* larvae. Maintaining high levels of investment in immune defense is costly, leading to individuals showing reduced expression of other important life history traits (Lochmiller and Deerenberg, 2000). In the absence of selective pressure by parasites, we expected a reduction in basal immunity as observed in *E. ambiguella*. *L. botrana* did not respond to immune challenge because its base level of immune defense was already high. Our study shows the importance of studies integrating different defense traits (behavioral, morphological and immunological) for understanding how these traits have co-evolved.

The two sympatric moth species live in the same habitat. Even if these species use oviposition-deterring kairomones (Thiery and Gabel, 1993), larvae sometimes share the same bunches and can be targeted by the same natural enemies. Despite these similarities, *L. botrana* was more heavily parasitized than *E. ambiguella*. This strongly suggests that the combination of defense traits in *E. ambiguella* is more efficient against parasitoids than that in *L. botrana*. While our experiment was not focused on assessing the relative contributions of behavioral, morphological and immunological traits to the risk of being parasitized, our results enable some inferences to be drawn. *E. ambiguella* larvae have thick cuticles but move little and slowly, suggesting that a thick cuticle provides better protection against parasitoid stings than does active behavior. Previous studies have shown that parasitoid oviposition is more difficult in older larvae because of cuticle thickness (Beckage and Riddiford, 1978). Most parasitoid wasps contact their hosts to oviposit inside the host body, and host behavior, including spiting and biting, may be an effective preventative mechanism (Potting et al., 1999). In addition, low physical activity reduces the risk of *E. ambiguella* being detected by searching parasitoids (Cressler et al., 2010). Many parasitoids use visual signals to find their hosts (Vinson, 1976), but chemical cues can also be involved; host chemical cues and frass are known to be important for host detection by parasitoids (Chuche et al., 2006; Mattiacci and Dicke,

1995). Thus, it remains possible that parasitoids in vineyards are able to more easily detect larvae of *L. botrana* than *E. ambiguella* using chemical signals. Among the few *E. ambiguella* larvae in which stings were detected, no adult parasitoids were observed to emerge, suggesting that physiological defenses are more efficient in this species than in *L. botrana*. Haemocytes and the PO enzyme cascade (PPO and PO activity) are the frontline of immune defense against parasites entering the insect haemocoel (Cerenius and Soderhall, 2004; González-Santoyo and Córdoba-Aguilar, 2011; Haine et al., 2008; Lavine and Strand, 2002; Wilson and Cotter, 2013), and haemocytes are the most important functional element involved in the recognition and encapsulation of pathogens (Cerenius and Soderhall, 2004; González-Santoyo and Córdoba-Aguilar, 2011; Lavine and Strand, 2002; Wilson and Cotter, 2013). The ability of insects to rapidly mobilize haemocytes and enzymes of the PO cascade following immune challenge has been shown to be associated with the ability to encapsulate parasitoid eggs, as demonstrated in *Drosophila* and *Manduca sexta* (Eslin and Prevost, 1996, 1998; Jiang et al., 2010). Therefore, we hypothesize that *E. ambiguella* has a greater ability than *L. botrana* to withstand pathogen attack during the larval stage. The absence of parasitoid emergence from *E. ambiguella* pupae supports this hypothesis. Functional studies such as that of Van Buskirk and McCollum (2000) may enable assessment of which defense traits are directly involved in parasitoid avoidance.

Lepidopteran larvae are the target of many natural enemies in the field including parasitoids, predators (including insects, birds and bats) and pathogens (Hawkins et al., 1997; Kalka et al., 2008). These have the potential to cause selection of different defenses in their prey, which act before, during and after an attack. Therefore, it is difficult to establish which kind of defense traits function against parasitoids or predators, and thus which natural enemies are causing selection of particular traits. However, previous studies have shown that some defense mechanisms act against predators, and others act against parasitoids (Barbosa and Caldas, 2007; Gentry and Dyer, 2002; Smilanich et al., 2009). In addition, many defense traits are effective against predators but ineffective against parasitoids (Gross, 1993, and references therein). In a recent study, Smilanich et al., (2009) argued that in Lepidopteran larvae the immune response is a more important defense against parasitoids than behavioral traits. The assumption behind this statement is the finding that among defense mechanisms the immune response was the best predictor of parasitism in the field (Barbosa and Caldas, 2007; Gentry and Dyer, 2002; Smilanich et al., 2009). In our study, *E. ambiguella* had greater immunity and morphological defenses, had fewer parasitoid stings, and were less parasitized than *L. botrana,* which exhibited stronger morphological defenses. Therefore, our results are consistent with the conclusion of Smilanich and collaborators (2009) concerning the importance of the immune system in defense against parasitoids.

The results of this study have implications for the management of biological control programs against these two species. *L. botrana* and *E. ambiguella* are two of the major grape pests in Europe, because of the damage they cause to grape bunches (Thiéry, 2008). Although they are mainly controlled in vineyards using pesticides, biological control is a possible future strategy against these pests because a range of parasitoids of *L. botrana* and *E. ambiguella* occurs in vineyards (Thiéry, 2008; Thiéry et al., 2001, 2011; Xuéreb and Thiéry, 2006). In this study we showed that defenses differ between *L. botrana* and *E. ambiguella*, and consequently the two species may not be subject to the same parasitoid pressure. As efficient defenses affect the success of parasitoids used in classical biological control, host defenses may influence the establishment of biological control species. Our results support the hypothesis that resistance to parasitoids differs in these two species, and that this could account for their spatial distributions. Application of biological control for these species could involve the release of parasitoids, which would be more likely to be successful against *E. ambiguella* than against *L. botrana*; however, this remains to be tested. In this context, we hypothesize that *E. ambiguella* is less amenable to biological control than *L. botrana*. Our results indicate that more research on defensive traits and their efficiency against parasitoids will be necessary to assess the potential for implementing a successful biological control program against tortricid moths. Choice experiments involving exposure of both *E. ambiguella* and *L. botrana* to female parasitoids will enable assessment of which is the preferred species. If *L. botrana* is preferred, this would suggest that it will be difficult to control *E. ambiguella* populations. As noted by others (Dyer and Gentry, 1999), there is considerable under-utilization of current knowledge in ecological theory in the implementation of successful biological control programs.

Conflict of interest

We declare that we have no conflict of interest.

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