

# Geographical variation in parasitism shapes larval immune function in a phytophagous insect

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**Abstract** Two of the central goals of immunoeology are to understand natural variation in the immune system among populations and to identify those selection pressures that shape immune traits. Maintenance of the immune system can be costly, and both food quality and parasitism selection pressure are factors potentially driving immunocompetence. In tritrophic interactions involving phytophagous insects, host plants, and natural enemies, the immunocompetence of phytophagous insects is constrained by selective forces from both the host plants and the natural enemies. Here, we assessed the roles of host plants and natural enemies as selective pressures on immune variation among natural populations of *Lobesia botrana*. Our results showed marked geographical variation in immune defenses and parasitism among different natural populations. Larval immune functions were dependent of the host plant quality and were positively correlated to parasitism, suggesting that parasitoids select for greater investment into immunity in moth. Furthermore, investment in immune defense was negatively correlated with body size, suggesting that it is metabolically expensive. The findings emphasize the roles of host plants and parasitoids as selective forces shaping host immune functions in natural conditions. We argue that

kinds of study are central to understanding natural variations in immune functions, and the selective forces beyond.

**Keywords** Grape varieties · Immune defense · *Lobesia botrana* · Parasitism · Tritrophic interactions

## Introduction

By altering growth, fecundity, and survival of their hosts, parasites and pathogens represent strong selective forces. Numerous lines of defense have evolved in response to such selective pressures, among which the immune system is probably the most sophisticated (Zuk and Stoehr 2002), with efficient immune systems clearly important in counteracting potentially negative effects of parasites. However, despite this obvious benefit, much variation is typically evident within and among populations with respect to susceptibility to parasites. This suggests that variable selection pressures may shape and drive adaptation of immune traits. For instance, ability of the fruit fly, *Drosophila melanogaster*, to encapsulate parasitoid eggs or resist fungal infection has been found to be geographically variable (Kraaijeveld and Vanalphen 1995; Tinsley et al. 2006), suggesting that the immune system may have adapted to local conditions, such as parasite abundance or virulence (Kalbe and Kurtz 2006; Cornet et al. 2009).

Although there have been numerous studies in ecological immunology, few have investigated variations in immune defense among natural populations, and consequently, there is inadequate information about spatial variability of immune defenses in the wild. Life history theory states that immunity is costly and imposes resource-based trade-offs with other fitness traits (Sheldon and Verhulst 1996). Three lines of evidence suggest that these trade-offs are a potential source of variation in investment in immune systems. First, when expressed at high levels, immunity is often traded off against

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other fitness parameters (Kraaijeveld and Godfray 1997). Second, mounting an immune response can impose condition-dependent fitness costs (Ilmonen et al. 2000; Moret and Schmid-Hempel 2000). Third, producing an immune response often involves release of cytotoxic chemicals that are harmful to the host (Nappi and Ottaviani 2000; Sadd and Siva-Jothy 2006). Hence, natural variation in immune function is expected to be the unavoidable consequence of balancing the relative benefits and costs of immunity.

Variation in immune defense can also result from environmental conditions, which may affect expression of the immune response and, consequently, outcome of host infection. Nutrition is becoming recognized as a critical factor in immune defense and resistance to pathogens (Lazzaro and Little 2009; Ponton et al. 2011). Experimental studies of insects have demonstrated that food deprivation in the host differentially affects immune responsiveness (Siva-Jothy and Thompson 2002; Ayres and Schneider 2009; Yang et al. 2008; Klemola et al. 2007; Kapari et al. 2006) and changes in the expression of several immunity genes (Pletcher et al. 2002). Resistance to parasite infection can also be affected by changes in food composition (Povey et al. 2009; Cotter et al. 2011). In addition to host resistance to infection, nutrition can also affect tolerance, which is a defense process by which the negative impact of infection on host fitness is reduced without reducing the parasite load (Ayres and Schneider 2012; Medzhitov et al. 2012). For instance, food-restricted *Drosophila* infected with *Salmonella* showed similar levels of bacteria to infected control *Drosophila*, but survived longer, suggesting that resistance was unchanged but tolerance was increased (Ayres and Schneider 2009). Hence, nutrition and food composition have a variable effect on immune system and may be key parameters having a reversible impact on immunity. In the long term, they could also influence the evolution of the physiology of the host, with potential consequences for the immune system.

Tritrophic systems involving insect herbivores and their host plants and natural enemies provide a useful model for addressing immunoecological issues, especially with regard to the potential combined effects of parasitism and nutrition on the evolution of host immune defenses in natural populations. Indeed, relative performance of phytophagous insects is classically constrained by selective forces imposed by both their host plants and natural enemies, imposing bottom-up and top-down selection pressures, respectively (Hairston et al. 1960). Through their relative composition of nutrients and toxic defensive compounds, host plants affect the condition of phytophagous insects (Coley et al. 2006; Smilanich et al. 2009), with major consequences for the expression of immune defense systems (Klemola et al. 2007; Bukovinsky et al. 2009; Vogelweith et al. 2011). Hence, host plants have the potential to influence either directly or indirectly the evolving physiology of the immune system of phytophagous insects. However, parasitoids are major sources of mortality among natural populations of insect herbivores (Hawkins et al. 1997; Smilanich et al. 2009). The strong selective pressure

exerted by parasitoids could well lead to higher levels of immune system investment by their hosts. In contrast, because of the costs associated with immune defense (Schmid-Hempel 2003; Siva-Jothy et al. 2005), when parasitoid selective pressure is relaxed, investment in immunity is likely to be selected against.

In this study, we investigated variations in immunity in natural populations of a phytophagous insect, as a result of selective pressures imposed by variation among host plants, and by parasitism. The study involved one of the main tortricid pests in European vineyards, the European grapevine moth, *Lobesia botrana*. The larvae of *L. botrana* are polyphagous and, in vineyards, can develop on most grape varieties present despite their different nutritional values (Moreau et al. 2006a, b). To investigate the effects of geographical area and parasitism on insect immunity, the larvae for our study were collected from several grape varieties in a number of vineyards in different regions of France.

As in other insects, immune defense relies on constitutive mechanisms involving cellular and humoral components (Hoffmann et al. 1996; Siva-Jothy et al. 2005). For example, hemocytes are immune cells that circulate in the hemolymph and are recruited in processes such as phagocytosis and encapsulation of parasites, including parasitoid eggs. This latter process often involves melanization of the foreign object through activation of the prophenoloxidase (PPO) cascade, which is a common and generalized response to invasion by a parasite (Cerenius and Soderhall 2004). Coupled with this line of defense is the induced response, which consists mainly of a suite of antimicrobial peptides (Iwanaga and Lee 2005) that are usually produced within 3 h following a microbial infection; the resulting antibacterial activity conferred on the hemolymph can last for several days (Haine et al. 2008).

We measured the baseline levels of a broad range of constitutive (hemocyte count, PPO system activity) and inducible (antimicrobial activity) immune defenses among wild larvae collected in different vineyards. To assess potential trade-offs between immune defense and other fitness-related traits, we measured the size of the larvae at the end of their development. We also measured the parasitism of local parasitoids on larvae to estimate the relative selective pressure they imposed locally (Moreau et al. 2010). We used this approach to enable estimation of potential geographical covariations (such as temperature) between parasitism, immune defenses, and other phenotypic traits among populations of *L. botrana*. If a host plant variety markedly affects the expression of immune defenses in *L. botrana*, we hypothesized that the above covariations would be influenced by the grape varieties from which larvae were sampled.

## Materials and methods

This work conforms to French legal requirements; to accepted international ethical standards, including those relating to

conservation and welfare; and to the journal's policy on these matters. All experiments have been conducted in conformity with the guiding principles in the care and use of animals' approved by the Council of the American Physiological Society.

#### Model insect, the host species

*L. botrana* (Lepidoptera, Tortricidae) is one of the major grape pests in Europe because of its wide geographical distribution and the damage it can cause to bunches (Thiéry 2008). Depending on the region, in Europe, *L. botrana* completes two to four broods each year. The first generation of eggs is laid on the flower buds, and on hatching, the young larvae form and aggregate in protective silk shelters termed glomerulae and eat the buds. The second generation emerges at the end of June–July, and the third generation occurs between mid-August and the end of September. The larvae are polyphagous and can develop on almost any grape variety and other plant species (Thiéry 2005, 2008; Moreau et al. 2006a). Each generation can cause serious damage to grape bunches; the damage can be quantitative and/or qualitative and can facilitate infection by pathogenic fungi including the grey mold *Botrytis cinerea* (Leotiales, Sclerotiniaceae) (Roehrich and Boller 1991) and black mold *Aspergillus* spp. (Thiéry 2008). Because of this, economic losses resulting from larval feeding on berries, and infection by fungi related to larval feeding, are typically attributed to the grapevine moth (Thiéry 2008).

#### Vineyard larval sampling

Six viticultural regions of France were sampled in May and June 2011 (corresponding to the end of the first moth generation): Alsace, Aquitaine, Bourgogne, Champagne-Ardenne, Rhône-Alpes, and Provence-Alpes-Côte d'Azur (Fig. 1). In each region, one or two vineyards were sampled: Cave de Beblenheim (CB) and Turckheim (Tschartke and Hawkins) in Alsace, Château Climens (CC) and Domaine INRA de la Grande Ferrade (DGF) in Aquitaine, Villiers (VI) in Bourgogne, Oeuilly (OE) and Mercier (MER) in Champagne-Ardenne, Domaine de Boisviel (DB), and Plateau de Roquemartine (PR) in Rhône-Alpes and Chiroubles–le Bourg (CBG) and Chiroubles–le Bois (CBS) in Provence-Alpes-Côte d'Azur. In each vineyard, we sampled one to five grape varieties (Fig. 1). For each variety, larvae were only collected when population monitoring indicated that there were >20 larvae per 100 bunches. Using this procedure, we were able to measure the direct effect of grape variety while avoiding the potential confounding effects of environmental variation (including temperature, light exposure, and humidity) among vineyards. We sampled larvae at the end of larval development (fifth instar stage) from the first generation on young flower buds. Larvae of *L. botrana* rarely move from one bunch to

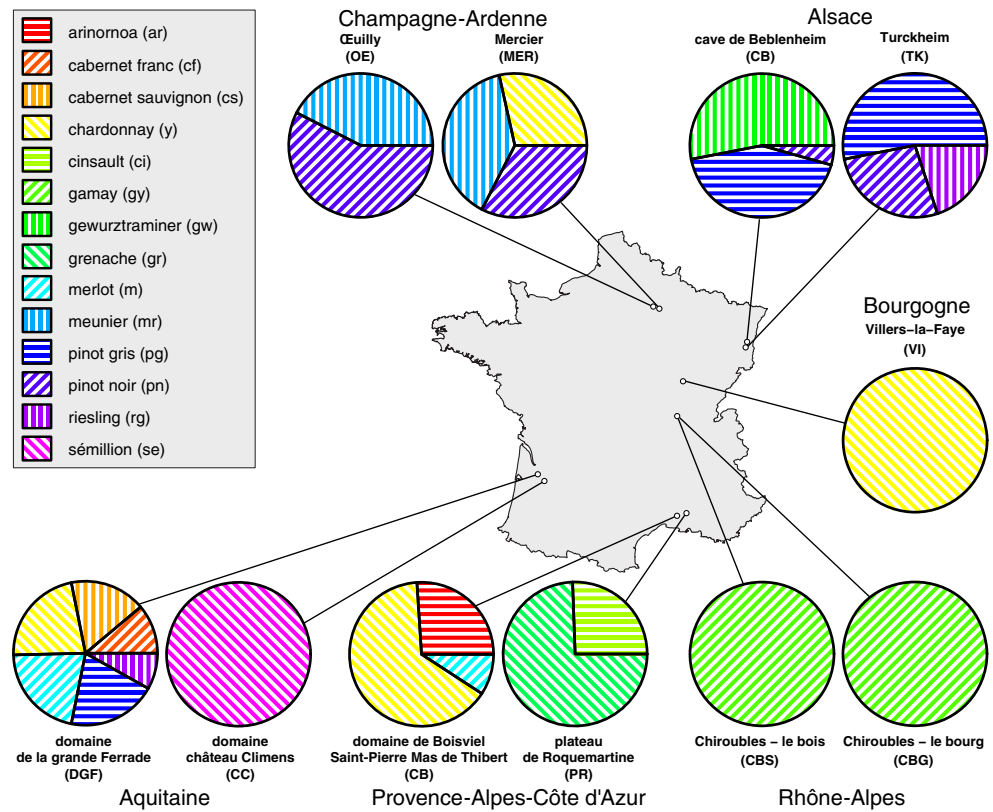
another, and consequently, each larva was assumed to have completed its development on the bunch from which it was collected (Torres-Vila et al. 1997). The collection process was nondestructive; only the silk nests containing the larvae were removed from each bunch. The grape pest used in this study is Tortricids belonging to the Olethreutinae family in which females lay separate eggs. Typically, *L. botrana* exhibit spacing oviposition behavior (Thiéry and Gabel 1993; Gabel and Thiéry 1996). As a result, larvae are not gregarious and larvae are single per nest. Until used in experiments, the larvae were maintained in polyethylene boxes (60×40×21.4 cm) at 24±1 °C, 60±10 % RH and under the ambient photoperiod conditions; they were fed ad libitum on grape bunches from the collection locality. For experiments, the larvae were randomly separated into two samples. One was used to measure parasitism (number of parasitoids emerged). This involved checking larvae daily until pupation, at which time the pupae were carefully removed from the flower buds and placed individually in glass tubes (70×9 mm diameter) closed with cotton plugs and stored at 23 °C under ambient photoperiod conditions. The pupae were monitored daily for adult emergence. Parasitism was measured a posteriori by checking adult emergence and, for each grape variety, the parasitism was calculated as: [number of parasitoids/(number of *L. botrana* + number of parasitoids emerged)]×100. In this measure of parasitism, we considered all larval endoparasitoids to get an overall index of the local selective pressure imposed by parasitoid community.

The second subsample was used to measure four key immune system parameters in each individual following hemolymph collection: hemocyte count, PO activity and total PO activity, and antimicrobial activity (see below). For each measurement, the number of individuals tested is indicated on the figures, below the *x*-axis. To test the effect of temperature on the larval immune system, we recorded the mean daily temperature over a set period of 12 days during the 2 months prior to larval sampling (March and April) and calculated the average of these temperatures for each vineyard. March is the period of adult flight and April the period of egg laying. The temperatures were obtained from the website <http://weatherspark.com/>.

#### Hemolymph collection

Individual larvae from the second subsample were chilled on ice for 20 min and then a 2 µl sample of hemolymph was collected in a sterile glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany) from a wound made in the posterior part of the ventral side of the abdomen. One microliter of the hemolymph sample was transferred to a microcentrifuge tube containing 25 µl of cold sodium cacodylate/CaCl<sub>2</sub> buffer (0.01 M sodium cacodylate; 0.005 M CaCl<sub>2</sub>; pH 6.5), and a 10-µl sample of this solution was immediately removed for hemocyte counting; the remainder was stored at -27 °C for later measurement of the PO activity. The remaining hemolymph in the capillary (1 µl) was

**Fig. 1** Map of the locations of the French vineyards sampled in the study. The *colored hatching* represents the various grape varieties sampled. The *pie charts* represent the proportion of *L. botrana* larvae sampled on the various grape varieties



flushed into a microcentrifuge tube internally coated with n-phenylthiourea (Sigma P7629, Sigma-Aldrich, St Louis, MO, USA) and containing 2  $\mu$ l of cold sodium cacodylate/ $\text{CaCl}_2$  buffer. The tube was stored at  $-27^\circ\text{C}$  for later assessment of antibacterial activity.

#### Immune parameters

Hemocyte counts were undertaken using an improved Neubauer hemocytometer counting chamber and phase contrast microscopy (magnification 400 $\times$ ). The activity of naturally activated PO enzymes (hereafter “PO activity”) and the activity of the PPO proenzymes in addition to that of PO (hereafter “total PO activity”) were measured using a spectrophotometer following the method described by Cornet et al. (2009). PO activity was quantified without further activation, while total PO activity required conversion of the PPO to PO with chymotrypsin. For each sample, the frozen hemolymph was thawed on ice and centrifuged (4,000 $\times g$ , 15 min,  $4^\circ\text{C}$ ). A sample of the supernatant (5  $\mu$ l) was added to a microplate well containing 20  $\mu$ l of PBS and either 140  $\mu$ l of distilled water to measure PO activity only or 140  $\mu$ l of chymotrypsin solution (Sigma C-7762, 0.07 mg/ml of distilled water) to measure total PO activity. A volume of 20  $\mu$ l of L-dopa solution (Sigma D-9628; 4 mg/ml of distilled water) was then

added to each well. The reaction was allowed to proceed for 40 min at  $30^\circ\text{C}$  in a microplate reader (VersaMax, Molecular Devices). Readings were made every 15 s at 490 nm, and the data were analyzed using SoftMax<sup>®</sup> Pro 4.0 software (Molecular Devices). Enzyme activity was measured as the slope ( $V_{\max}$  value: change in absorbance units per min) of the reaction curve during the linear phase of the reaction and was reported as the activity of 1  $\mu$ l of pure hemolymph.

Antimicrobial activity in the hemolymph was measured using a standard zone of inhibition assay (Moret 2006). The samples were thawed on ice and, using the assay described below, were tested for antimicrobial activity, based on the formation of zones of inhibition in agar plates seeded with the bacterium *Arthrobacter globiformis*. A single colony of *A. globiformis* from a streak plate was inoculated into a broth medium (10 g Bacto Tryptone, 5 g yeast extract, and 10 g NaCl in 1,000 ml of distilled water, pH 7.0) and incubated overnight at  $30^\circ\text{C}$ . From this culture, the bacterium was inoculated into a broth medium containing 1 % agar to achieve a final density of  $10^5$  cells  $\text{ml}^{-1}$ . This inoculated agar medium (6 ml) was poured into a Petri dish and allowed to solidify. Wells for sample addition were made in the agar using a Pasteur pipette fitted with a ball pump. For each thawed test sample solution, 2  $\mu$ l was added to a well in the agar plate. A positive control (tetracycline: Sigma T3383) was included in



each plate. The plates were incubated for 48 h at 30 °C, at which time the diameters of inhibition zones were measured.

#### Larval body size and parasitism

The body size of *L. botrana* is correlated to immunocompetence (Vogelweith et al. 2013). We estimated larval body size by measuring the distance between the most distant lateral sides of the head capsule margins (Delbac et al. 2010) using a Nikon SMZ-10A stereoscopic microscope and a VTO 232 video analysis system (Linkam Scientific Instruments). Several larval body size and instar indicators may be used. Head capsule (HC) width measurement is the most reliable measurement of body size in most Lepidoptera larvae (Godin et al. 2002; Panzavolta 2007; Delbac et al. 2010) and was, thus, used for this study. Alternative sizing or weighting of living old larvae is extremely difficult in *L. botrana* which move vigorously and are, thus, tricky to handle. In a recent study, Delbac et al. (2010) developed a simple and convenient statistical model based on HC size in order to determine the larval instars of field individuals. Interestingly, HC size varies among individuals within instar. Fourth and fifth instars were the easiest to discriminate because of nonoverlapping distributions. Thus, we selected this parameter.

Because parasitoid injuries or the presence of parasitoid eggs can challenge the immune system of larvae (Barnes and Siva-Jothy 2000), only nonparasitized larvae, or larvae with no evidence of parasitoid feeding injuries, were used in the measurement of immune parameters. To assess the status of larvae, each larva was carefully inspected using a binocular microscope (see characteristics above). After the immune collection, all larvae were dissected to control for possible effects of parasitoid eggs in the hemolymph on immune levels of larvae. Those larvae having visible infections (the presence of feeding injuries, parasitoid eggs, or encapsulated parasitoid eggs) were excluded from the analysis.

#### Statistical analysis

The grape varieties in each vineyard differed among the viticultural areas in the study. Therefore, we were unable to use nested analyses or generalized linear models with interaction terms between vineyards and grape varieties. Consequently, analysis of the immune system and parasitism required two steps. In the first step, we assessed differences in the larval immune system and parasitism among grape varieties in each vineyard. To test the effect of grape variety on hemocyte counts, PO activity, and total PO activity, we used negative binomial generalized linear model (NBGLM) because the data follow the negative binomial distribution. To assess the parasitism rate, we used Pearson's  $\chi^2$  test, and as the antimicrobial activity showed a binary distribution, we assessed differences in this parameter using Pearson's  $\chi^2$  test.

Body size was normally distributed and was assessed using analysis of variance (ANOVA). Vineyards having only a single grape variety were not included in the analysis. In the second step, to test for the effect of vineyard, we chose vineyards having a common grape variety and used the negative binomial generalized linear model (NBGLM) for comparison of hemocyte counts, the Kruskal–Wallis rank sum test for comparison of PO activity and total PO activity, and Pearson's  $\chi^2$  tests for comparison of antimicrobial activities and parasitism. None of the data were normally distributed. For analysis of body size, we used ANOVA because the data were normally distributed. Following these tests, we used Tukey's HSD post-hoc test to assess the significance of grape variety or vineyard. To investigate the relationship between the four immune parameters measured within a single population, we used Spearman's rank correlation coefficient with a confidence interval of 95 % (C.I.) because the data were not normally distributed. When the C.I. included 0, the correlation was not significant. Principal component analysis was used to summarize the information for the various immune parameters into one orthogonal principal component (the summary immune value) describing the basal level of immunity in larval hemolymph. The relationships between PC1 and parasitism, PC1 and head capsule size, and PC1 and temperature were explored using Spearman's rank correlation coefficient with a C.I. of 95 % between the different grape varieties. To check the results of these correlations, we have run a second analysis by taking into account only the vineyards. To this purpose, we calculated average levels of PC1, parasitism, and head capsule size per vineyard as the sum of these variables for each cultivar within vineyards divided by the number of cultivars within each vineyard. The temperature data were analyzed separately because of the confounding effect with the north–south gradient vineyards. Due to the controversy of adjustment following multiple comparisons tests (Moran 2003; Nakagawa 2004), no correction was made after multiple comparisons. However, the outcome of these multiple comparisons was examined by calculating the probability of having exactly one test being statistically significant due to chance alone using a Bernoulli process (Moran 2003). For all comparisons, the level of significance was set at  $\alpha=0.05$ . All statistical tests were performed using the R (version 3.0.1) software.

## Results

Effect of grape variety on immune parameters, parasitism, and larval size within a vineyard

No effect of grape variety on PO activity or total PO activity was detected in the hemolymph of larvae from within each vineyard (Table 1). However, the larval hemocyte count was dependent

**Table 1** Effect of grape variety in each vineyard on hemocyte count, PO activity, total PO activity, antimicrobial activity, parasitism, and larval body size. *P* values  $\leq 0.05$  are shown in bold. See Fig. 1 for definitions of the acronyms. The outcome of these multiple statistical tests was validated by the

probability *p* ( $p=0.002$ ) calculation of having *k* ( $k=8$ ) significant tests over *N* ( $N=49$ ) performed tests following  $p=[N!/(N-K)!K!] \times \alpha^K (1-\alpha)^{N-K}$  (Moran 2003)

Vineyards <sub>df</sub>	Hemocyte count <sup>a</sup>		PO activity <sup>a</sup>		Total PO activity <sup>a</sup>		Antimicrobial activity <sup>b</sup>		Parasitism <sup>b</sup>		Body size <sup>c</sup>	
	$\chi$	<i>P</i>	$\chi$	<i>P</i>	<i>X</i>	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	<i>F</i>	<i>P</i>
CB <sub>2</sub>	1.70	0.42	2.44	0.29	2.65	0.26	1.88	0.39	0	0	2.97	0.06
TK <sub>2</sub>	<b>8.32</b>	<b>0.01</b>	4.70	0.09	4.59	0.10	2.88	0.23	3.29	0.19	2.52	0.08
CC <sub>0</sub>	–	–	–	–	–	–	–	–	–	–	–	–
DGF <sub>4</sub>	6.32	0.18	0.57	0.96	0.71	0.95	<b>20.86</b>	<b>0.0003</b>	<b>10.06</b>	<b>0.04</b>	0.05	0.99
VI <sub>0</sub>	–	–	–	–	–	–	–	–	–	–	–	–
MER <sub>2</sub>	0.98	0.61	0.78	0.67	0.99	0.60	2.35	0.31	3.50	0.17	0.04	0.96
OE <sub>1</sub>	0.55	0.45	0.67	0.41	0.24	0.62	0.008	0.92	3.47	0.06	0.06	0.81
DB <sub>2</sub>	<b>8.64</b>	<b>0.01</b>	0.01	0.99	0.68	0.71	<b>11.94</b>	<b>0.003</b>	<b>6.19</b>	<b>0.04</b>	<b>3.63</b>	<b>0.03</b>
PR <sub>1</sub>	<b>8.72</b>	<b>0.003</b>	2.10	0.14	0.34	0.25	3.06	0.08	0.006	0.94	0.68	0.41
CBG <sub>0</sub>	–	–	–	–	–	–	–	–	–	–	–	–
CBS <sub>0</sub>	–	–	–	–	–	–	–	–	–	–	–	–

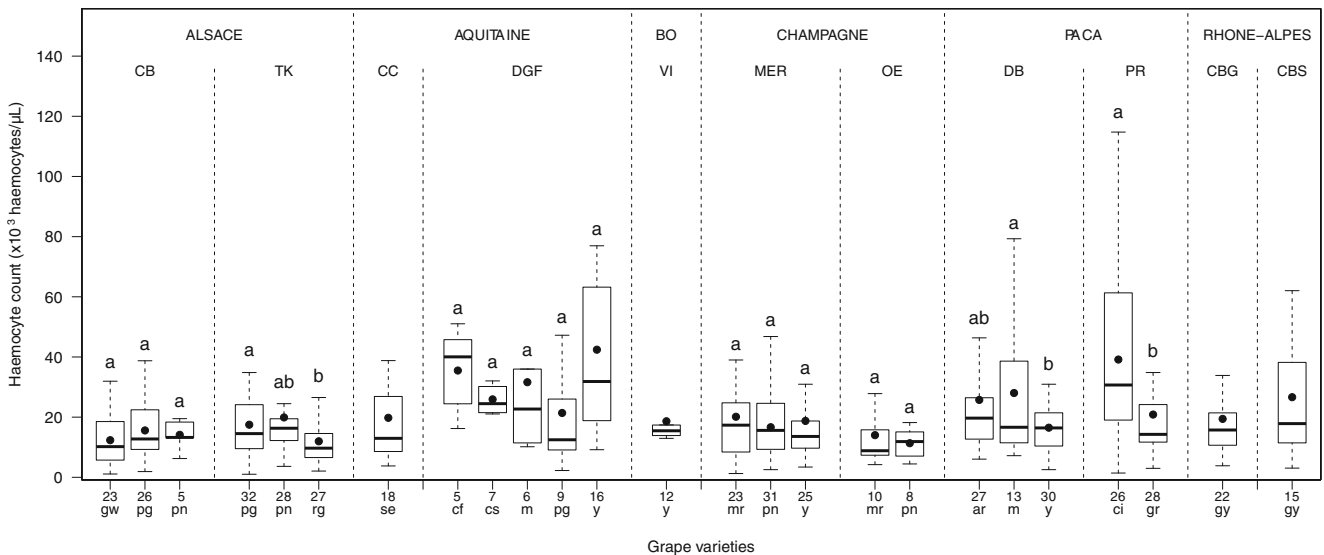
<sup>a</sup> NBGLM

<sup>b</sup> Pearson's  $\chi^2$  tests

<sup>c</sup> ANOVA

of the grape variety in three vineyards (Domaine de Boisviel, Plateau de Roquemartine, and Turckheim), with those from the Merlot, Cinsault, and Pinot Gris varieties having higher hemocyte counts than those collected on the Chardonnay, Grenache, and Riesling varieties (Table 1, Fig. 2). For the vineyards Domaine de la Grande Ferrade and Domaine de Boisviel, grape variety also affected the percentage of larvae having antimicrobial activity (Table 1, Fig. 3). For instance, for Domaine de Boisviel, 69 % of the larvae sampled from the Arinarnoa variety

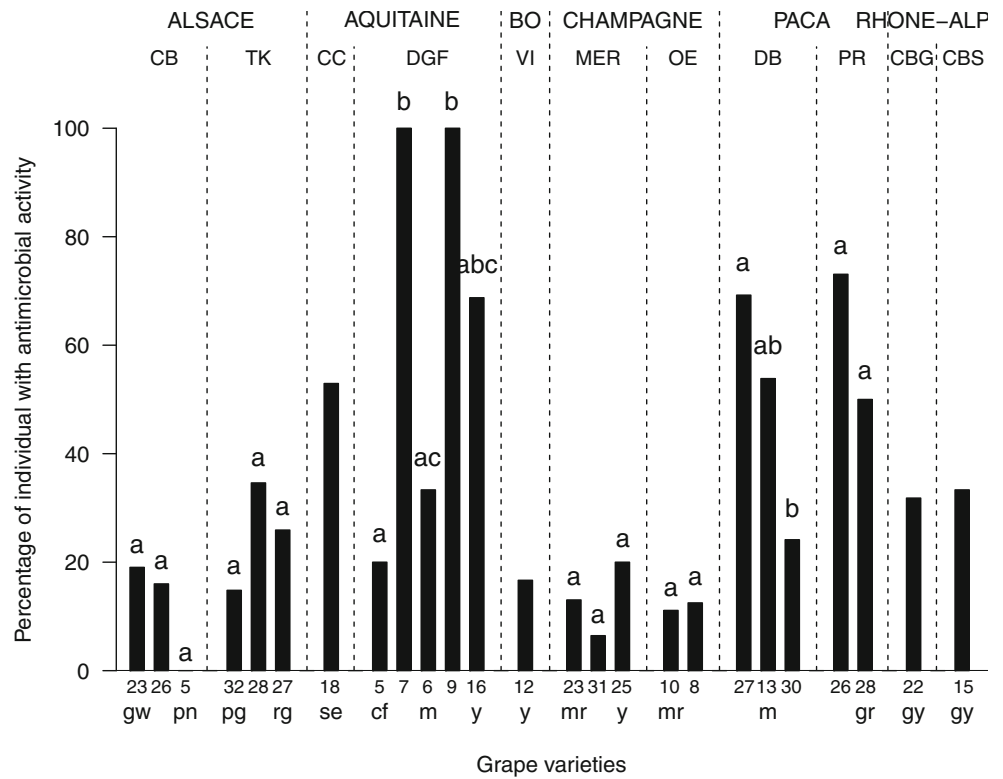
had antimicrobial activity, while only 24 % of those sampled from the Chardonnay variety had this activity. The parasitism was also dependent on the grape variety in these two vineyards (Table 1, Fig. 4). For example, for Domaine de la Grande Ferrade, the larvae collected on the Merlot variety were significantly more parasitized than those collected on the Cabernet Sauvignon and Chardonnay varieties (Fig. 4). For Domaine de Boisviel, the larvae collected on the Arinarnoa and Merlot varieties were more heavily parasitized than those collected on



**Fig. 2** Hemocyte count ( $\times 10^3$  hemocytes  $\mu\text{L}^{-1}$ ) in the hemolymph of larvae from various grape varieties in different vineyards. The edges of the rectangles represent the first and third quartiles, the central features are the medians, the dashed lines are the maxima and minima, and the

black circles are the means. Within a vineyard, grape varieties having the same letter are not significantly different ( $P > 0.05$ ). For each grape variety, the number of larvae tested is shown below the x-axis. See Fig. 1 for definitions of the acronyms

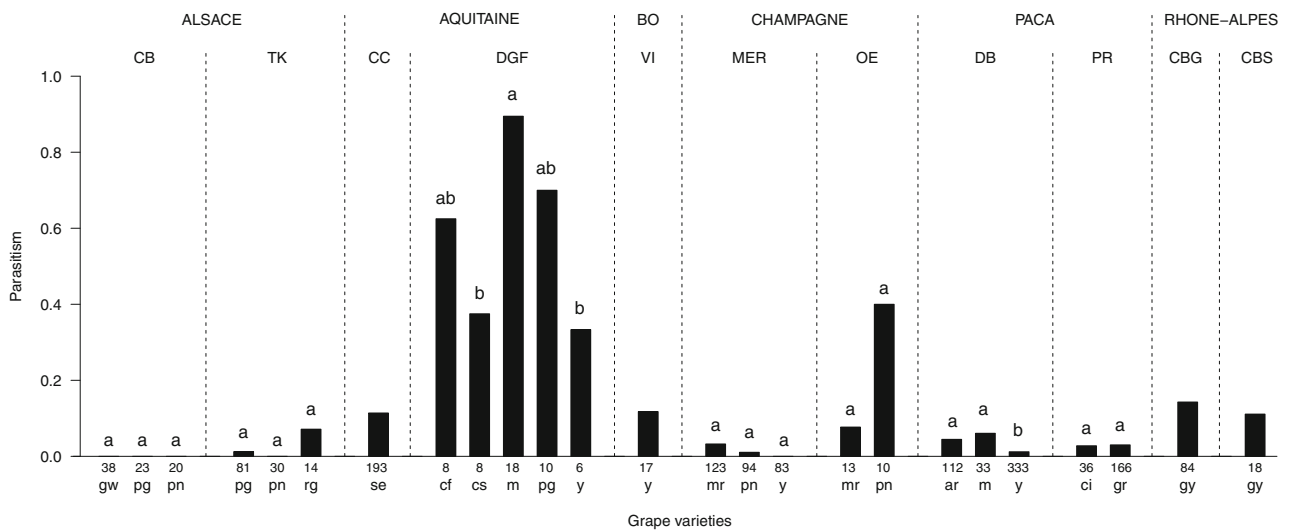
**Fig. 3** Percentage of larvae having hemolymph antimicrobial activity among grape varieties from different vineyards. Within a vineyard, grape varieties having the *same letter* are not significantly different ( $P>0.05$ ). For each grape variety, the number of larvae tested is shown below the *x*-axis. See Fig. 1 for definitions of the acronyms



the Chardonnay variety (Fig. 4). The body size of larvae was affected by grape variety at only one vineyard (Domaine de Boisviel; Table 1), with those collected on the Chardonnay variety being larger ( $0.99\pm 0.04$  mm) than those from the Arinarnoa variety ( $0.96\pm 0.07$  mm).

Larval immune parameters, parasitism, and larval size on the same grape variety among vineyards

The Chardonnay, Gamay, Meunier, Pinot Noir, and Pinot Gris varieties were selected to assess the effect of vineyard. The



**Fig. 4** Parasitism of larvae among grape varieties from different vineyards. Within a vineyard, grape varieties with the *same letter* are not significantly different ( $P>0.05$ ). For each grape variety, the number

of larvae tested is shown below the *x*-axis. See Fig. 1 for definitions of the acronyms

hemocyte count and PO activity of larvae collected from the Chardonnay variety depended on the vineyard from which they were collected (Table 2). For instance, the larvae collected from Domaine de Boisviel had higher hemocyte counts and PO activity than larvae collected from other vineyards. The vineyard of collection also affected the total PO and antimicrobial activity associated with larvae collected on the Chardonnay, Pinot Noir, and Pinot Gris varieties (Table 2). The rate of parasitism of larvae from the Pinot Gris, Pinot Noir, and Chardonnay varieties depended on the vineyard (Table 2), with those collected from the Pinot Gris variety in Domaine de la Grande Ferrade being less parasitized than those from the other vineyards. Larvae sampled from the Pinot Noir variety at Oeuilly were less parasitized than those collected from the other three vineyards. Larvae collected from the Chardonnay variety at Domaine de Boisviel and Mercier were more parasitized than those collected at Domaine de la Grande Ferrade and Villiers. The vineyard of collection also affected the body size of larvae from the Pinot Gris and Chardonnay varieties (Table 2). Larvae collected from the Pinot Gris variety in Alsace (Cave de Beblenheim and Turckheim) were larger than those collected in Domaine de la Grande Ferrade. Larvae sampled from the Chardonnay variety at Domaine de la Grande Ferrade were smaller than those collected from the other vineyards.

#### Relationship between immune parameters, parasitism, body size, and temperature among different larval populations

A strong positive and statistically significant relationship was found between the four immune parameters measured among the populations tested (Table 3). From these immune parameters, we obtained a summary value for the immune system

(PC1), which was strongly positively correlated with the four immune parameters (Table 4).

The summary immune value was positively correlated with the parasitism (Spearman's rank correlation coefficient: between grape varieties:  $\rho=0.71$ ;  $p<0.0001$ ; C.I.=[0.37; 0.90]; Fig. 5a). In addition, the summary immune value was negatively correlated with larval head capsule size (Spearman's rank correlation coefficient: between grape varieties:  $\rho=-0.46$ ;  $p=0.02$ ; Fig. 5b). However, the confidence interval included 0 (C.I.=[-0.78; 0.2]), suggesting that this correlation is rather weak.

Figure 5 suggests that the correlations between parasitism and head capsule size with the summary immune value are driven by samples from the DGF vineyard. When removing this particular vineyard (DGF) from the data analysis, the positive correlation between PC1 and the parasitism remains (Spearman's rank correlation coefficient:  $\rho=0.46$ ;  $p=0.02$ ; C.I.=[0.2; 0.81]). However, the head capsule size was not correlated anymore to the summary value of the immune system (Spearman's rank correlation coefficient:  $\rho=0.02$ ;  $p=0.9$ ; C.I.=[-0.51; 0.59]).

Finally, when considering the vineyards only, the summary immune value was positively correlated with the parasitism (Spearman's rank correlation coefficient between vineyards:  $\rho=0.66$ ;  $p=0.01$ ; C.I.=[0.1; 0.96]) but not with larval head capsule size (Spearman's rank correlation coefficient between vineyards:  $\rho=-0.30$ ;  $p=0.38$ ; C.I.=[-0.82; 0.60]).

Moreover, the summary immune value was also positively correlated with temperature (Spearman's rank correlation coefficient:  $\rho=0.65$ ;  $p<0.0001$ ; C.I.=[0.33; 0.85]); larval populations having high summary values came from the warmest geographical locations. Similar results were found when removing the DGF vineyard (Spearman's rank correlation

**Table 2** Vineyard effect for each grape variety with respect to hemocyte count, PO activity, total PO activity, antimicrobial activity, parasitism, and larval body size. *P* values  $\leq 0.05$  are shown in bold. The letters indicate which vineyards were compared. *A* vineyards CBG and CBS were compared, *B* vineyards MER and OE were compared, *C* vineyards CB, MER, OE and TK were compared, *D* vineyards CB, DGF and TK were

compared, *E* vineyards DB, DGF, MER and VI were compared. The outcome of these multiple statistical tests was validated by the probability *p* ( $p<0.0001$ ) calculation of having *k* ( $k=14$ ) significant tests over *N* ( $N=30$ ) performed tests following  $p=[N!/(N-K)!K!] \times \alpha^K (1-\alpha)^{N-K}$  (Moran 2003)

Grape varieties <sub>df</sub>	Hemocyte count <sup>a</sup>		PO activity <sup>b</sup>		Total PO activity <sup>b</sup>		Antimicrobial activity <sup>c</sup>		Parasitism <sup>c</sup>		Body size <sup>d</sup>	
	$\chi$	<i>P</i>	$\chi$	<i>P</i>	$\chi$	<i>P</i>	$\chi^2$	<i>P</i>	$\chi^2$	<i>P</i>	<i>F</i>	<i>P</i>
Chardonnay <sub>4</sub> <sup>E</sup>	<b>6.223</b>	<b>0.0007</b>	<b>18.15</b>	<b>0.0004</b>	<b>23.46</b>	<b>0.0001</b>	<b>14.25</b>	<b>0.003</b>	<b>44.95</b>	<b>0.0001</b>	<b>13.84</b>	<b>0.0001</b>
Gamay <sub>1</sub> <sup>A</sup>	1.32	0.25	0.92	0.34	0.096	0.76	0.009	0.92	0.13	0.72	0.32	0.57
Meunier <sub>1</sub> <sup>B</sup>	1.51	0.22	0.22	0.64	0.0015	0.97	0.02	0.88	0.65	0.42	0.006	0.94
Pinot Noir <sub>4</sub> <sup>C</sup>	5.95	0.11	5.78	0.12	<b>8.61</b>	<b>0.03</b>	<b>8.73</b>	<b>0.03</b>	<b>66.68</b>	<b>0.0001</b>	0.03	0.99
Pinot Gris <sub>2</sub> <sup>D</sup>	<b>18.76</b>	<b>0.0001</b>	5.61	0.06	<b>9.11</b>	<b>0.01</b>	<b>27.89</b>	<b>0.0001</b>	<b>52.49</b>	<b>0.0001</b>	<b>5.6</b>	<b>0.005</b>

<sup>a</sup> NBGLM

<sup>b</sup> Kruskal–Wallis rank sum test

<sup>c</sup> Pearson's  $\chi^2$  tests

<sup>d</sup> ANOVA



**Table 3** Spearman's rank correlation coefficients (with  $p$  value and C.I.) between four immune system parameters (hemocyte count, PO activity, total PO activity, and antimicrobial activity) in the hemolymph of *L. botrana* populations. Values in bold indicate significant correlations

	Hemocyte count	PO activity	Total PO activity	Antimicrobial activity
Hemocyte count	–	<b>0.81; 0.0001</b> [0.63; 0.89]	<b>0.78; 0.0001</b> [0.54; 0.90]	<b>0.73; 0.0001</b> [0.49; 0.87]
PO activity		–	<b>0.86; 0.0001</b> [0.65; 0.94]	<b>0.75; 0.0001</b> [0.53; 0.88]
Total activity			–	<b>0.55; 0.002</b> [0.21; 0.78]
Antimicrobial activity				–

coefficient:  $\rho=0.66$ ;  $p=0.002$ ; C.I.=[0.31; 0.86]), or when considering vineyards only in the analysis (Spearman's rank correlation coefficient between vineyards:  $\rho=0.78$ ;  $p=0.002$ ; C.I.=[0.29; 0.97]).

## Discussion

In this study, we found evidence for a marked geographical variation in immune defense and the parasitism among natural populations of the grapevine moth, *L. botrana*. All the immune parameters assessed during this study (hemocyte count, PO activity, total PO activity, and antimicrobial activity) were strongly and positively correlated at the population scale. Larval immune system activation among the larval populations was also strongly positively correlated to the parasitism and the local mean temperature. These results are consistent with the hypothesis that parasitoid infection selects for greater investment in the immune system in *L. botrana*. Moreover, larval immune parameters tended to be negatively correlated with larval body size.

Because immunity is costly (Ilmonen et al. 2000; Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000; Sadd and Siva-Jothy 2006; Valtonen et al. 2010), a negative relationship between investments in immune defense and larval body size is expected, revealing a trade-off between these fitness components. Such a relationship was, indeed, found but it was mainly due to samples from one vineyard (DGF) only. This results confirm that the cost of immunity remain difficult to evidence in natural populations (Cornet et al. 2009) despite the fact that previous study has already

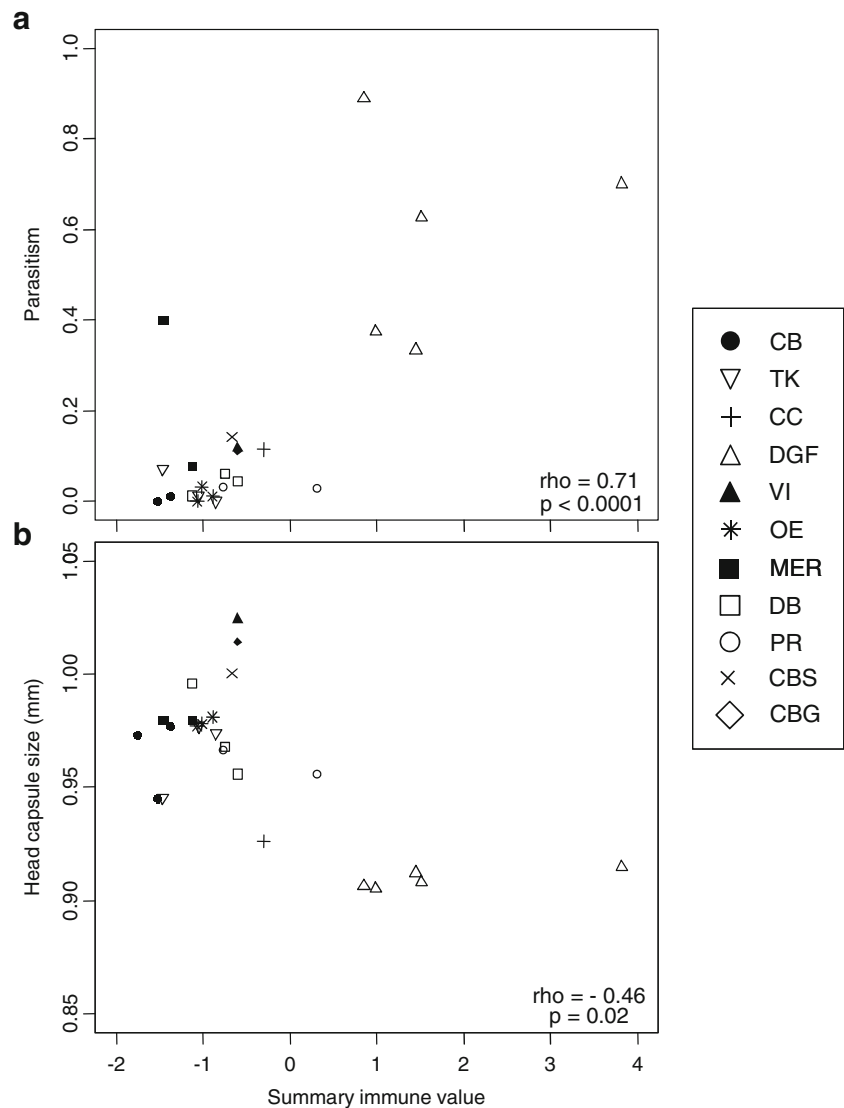
found such a trade-off (Rantala and Roff 2005). Indeed, difference in allocation strategies, in combination with potential differences in genotype quality, may have variable consequences for what is observed at the population level. Hence, despite a negative trade-off between immune defenses and larval growth may exist for every individual of a population, no correlation or even a positive correlation between these competitive fitness components could still be observed at the population level (Reznick et al. 2000; Schmid-Hempel 2011).

The geographical location of the vineyards and the grape variety affected the immune system of *L. botrana* larvae. For several vineyards (Turckheim, Domaine de la Grande Ferrade, Plateau de Roquemartine, and Domaine de Boisviel), the grape variety influenced variations in hemocyte counts and antimicrobial activity in the larvae. This is consistent with previous experimental findings that for the tortricid sibling species, *Eupoecilia ambiguella*, the host plant variety affects the expression of immune defense (Vogelweith et al. 2011). The immune system parameters varied for larvae collected from the Chardonnay, Pinot Gris, and Pinot Noir varieties from different vineyards, indicating that the parameters were affected by the geographical area sampled. The mean local temperature varied along a north–south gradient (north: Champagne-Ardenne, Alsace; south: Aquitaine, Provence-Alpes-Côte d'Azur), which could explain geographical variations within the same grape variety (Chardonnay, Pinot Gris, and Pinot Noir). Indeed, we found a positive relationship between the summary immune defense values and the mean temperature recorded during the 2 months prior to sampling. Empirical evidence suggests that the activity of the insect's immune defense system is dependent on the ambient temperature but that warm temperatures can either decrease or increase the activity of some immune effectors (Linder et al. 2008; Adamo and Lovett 2011). In our study, larvae sampled in the southern area from the Chardonnay, Pinot Gris, and Pinot Noir varieties tended to have a greater number of hemocytes and a higher level of antimicrobial activity than larvae sampled from these varieties in the northern area (Figs. 2–3). Unfortunately, our data did not enable us to establish whether the elevated immune functions observed in populations sampled from southern France directly resulted from warmer temperatures experienced by the larvae. It is

**Table 4** Principal component analysis for four immune system parameters (hemocyte count, PO activity, total PO activity, and antimicrobial activity) in the hemolymph of *L. botrana*. Component loadings describe the relationships between the first principal component (PC1) and the variables from which they were derived

	PC1
Eigenvalue	3.05
Explained variance (%)	76.22
Component loading	
Hemocyte count	0.92
PO activity	0.94
Total PO activity	0.90
Antimicrobial activity	0.79

**Fig. 5** Correlation between summary values for the immune system (obtained from principal component analysis), **a** the parasitism in the various vineyards, and **b** the head capsule size in the various vineyards. For **a** and **b**, the *symbols* represent vineyards



possible that we observed the result of natural selection or an indirect effect mediated by parasitism. Indeed, the populations showing greatest immune investment were those that were exposed to both higher temperatures and greater parasitoid pressure. Laboratory experiments will be needed to confirm the effect of temperature on the immune defense of larvae of *L. botrana* and distinguish between the direct or indirect effect (see Adamo and Lovett 2011 for an example). In addition to the effects of grape variety and geographical location, the presence of parasitoids in the vineyard may be a major factor modulating the immune system of *L. botrana*.

Contrary to a previous study on a related species (Vogelweith et al. 2011), we found a positive correlation between the different immune parameters. Generally, larvae tested in laboratory experiment were from inbred stock only allowing testing the influence of environment. However, in wild populations, genetic variation and its interaction with local environmental factors are not controlled. Similarly to

the observed absence of a significant negative relationship between immune defense and body size of larvae, despite negative trade-offs existing between arms of the immune system for individuals, no correlation or even positive correlations might be observed at the population level (Reznick et al. 2000; Schmid-Hempel 2011). In French and Swiss vineyards, parasitism has been shown to be highly variable among different geographical regions (Moreau et al. 2010). We found a high parasitism on larvae in the Domaine de la Grande Ferrade (DGF), but no parasitoids in Cave de Beblenheim (DB). This marked variation in the parasitism among our sampling areas enabled us to examine how this variation related to immunological investment. We assumed that the immune effectors studied are relevant to the insect's immune functions with respect to a broad range of pathogens (from micro- to macroparasites). We found that the populations having high levels of immune defense (high hemocytes count, PO enzymes, and antimicrobial peptides) were also

those that had the highest level of parasitoid infection. This result provides strong evidence that parasitism shapes host investment in immune defenses as already observed for other parasites in invertebrates (Bryan-Walker et al. 2007; Corby-Harris and Promislow 2008; Kortet et al. 2007). Two nonmutually exclusive hypotheses can be proposed to explain the covariation between the parasitism and the level of investment in immune defense in our study.

The first hypothesis is that the presence of parasitoids in the local environment may induce the host to plastically increase their investment in immune defense. Thus, in this scenario, the larvae of *L. botrana* would assess cues related to the presence of parasitoids and adjust their investment in immunity to match any increased threat of infection. Although the ability to sense changes in the risk of predation or parasitism through visual, chemical, or mechanical cues has been reported for some insect species (Peacor 2003; Fievet et al. 2008), there is currently no evidence suggesting that *L. botrana* has this ability.

The second hypothesis is that parasites are locally expected to mediate a selective response in favor of high levels of immune defense among hosts, which should translate as well in a positive covariation between parasitism and immune investments (Kalbe and Kurtz 2006; Tschirren and Richner 2006; Lindstrom et al. 2004). This hypothesis implies the occurrence of genetic differentiation among populations in terms of investment in immune defense. Populations characterized by higher levels of parasitism face greater selective pressure, thus favoring hosts with high capacity for investment in immune function. This hypothesis assumes that local parasitoid pressure remains constant within populations for several generations. However, the temporal variation in parasitism preceding our study is unknown. The hypothesis of local adaptation may be supported by the low dispersal capacity of adult grapevine moths (Thiery and Moreau 2005). Additional research is needed to clarify the relative contribution of each of the above hypotheses in explaining the patterns of variation observed in our study.

Overall, these findings are consistent with parasitoids having a substantial role as selective forces shaping host immune defenses. Our study highlights major bottom-up and top-down forces on the immune system of this herbivore. Studies of this kind are central to understanding the selective forces on the immune systems of phytophagous populations. However, further research is necessary to clarify the influence of grape variety on the immune system and to establish whether immune capacity is the result of local adaptation or phenotypic plasticity.

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**Conflict of interest** We declare that we have no conflict of interest.

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