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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Immune benefits from alternative host plants could maintain polyphagy in a phytophagous insect

Karen Muller · Fanny Vogelweith · Denis Thiéry · Yannick Moret · Jérôme Moreau

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Abstract The tritrophic interactions hypothesis, integrating bottom-up (plant-herbivore) and top-down (herbivore-natural enemies) effects, predicts that specialist herbivores should outcompete generalists. However, some phytophagous insects have generalist diets, suggesting that maintenance of a diverse diet may confer certain fitness advantages that outweigh diet specialization. In field conditions, the European grapevine moth, *Lobesia botrana*, feeds on diverse locally rare alternative host plants (AHPs) although grapevines are a highly abundant and predictable food source. The laboratory studies presented here show that survival, growth, and constitutive levels of immune defences (concentration of haemocytes and phenoloxidase activity) of *L. botrana* larvae were significantly enhanced when they were fed AHPs rather than grape. These results indicated a strong positive effect of AHPs on life history traits and immune defences of *L. botrana*. Such positive effects of AHPs should be advantageous to the moth under heavy selective pressure by natural enemies and, as a consequence, favour the maintenance of a broad diet preference in this species. We therefore believe that our results account for the role of immunity in the maintenance of polyphagy in phytophagous insects.

Keywords Diet breadth · Herbivory · Immunocompetence · *Lobesia botrana* · Polyphagy

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K. Muller (✉) · F. Vogelweith · Y. Moret · J. Moreau
Equipe Ecologie Evolutive, Université de Bourgogne, UMR CNRS 6282 Biogéosciences, 6 Boulevard Gabriel, 21000 Dijon, France
e-mail: karen.muller@u-bourgogne.fr

F. Vogelweith
e-mail: fanny.vogelweith@gmail.com

Y. Moret
e-mail: yannick.moret@u-bourgogne.fr

J. Moreau
e-mail: jerome.moreau@u-bourgogne.fr

F. Vogelweith · D. Thiéry
Institut des Science de la Vigne et du Vin, INRA UMR 1065 Santé et Agroécologie du Vignoble, Ave E. Bourleaux, 33883 Villenave d'Ornon Cedex, France
e-mail: thiery@bordeaux.inra.fr

D. Thiéry
Université de Bordeaux, INRA UMR 1065, Save, Bordeaux Sciences Agro, Ave E. Bourleaux, 33883 Villenave d'Ornon Cedex, France

Introduction

Most phytophagous insects are specialists, in that they feed on a single host plant species (Schoonhoven et al. 2005). While ecological conditions promoting the evolution of such a widespread specialization are still not fully understood (Singer et al. 2004; Smilanich et al. 2009; Mooney et al. 2012), it is generally assumed that specialist phytophagous insects should have a selective advantage compare to generalist ones (Mooney et al. 2012). Studies on the evolution of diet breadth of insect herbivores (monophagy vs. polyphagy) initially had a bitrophic perspective, in that they only considered the interaction of host plants and phytophagous insects (Ehrlich and Raven 1964). From this perspective, specialization can be driven by several factors such as habitat predictability (Wiklund 1974), host-finding capability (Futuyma and Moreno 1988), and physiological constraints due to host plant chemistry (Ehrlich and Raven 1964). Thus, consideration of the bitrophic insect-plant relationship leads to the prediction that herbivore diet

breadth will be wider when host plants are rare and less predictable, and narrower when host plants are abundant and seasonally stable (Logarzo et al. 2011).

However, this narrow bitrophic view has been criticized (Bernays and Graham 1988; Singer and Stireman 2005). It is now well established that phytophagous insects are under pressure from host plants and natural enemies, such as predators, parasitoids, and pathogens. Thus, the ability to escape from or defend against these organisms must also be considered in order to completely understand the evolution of insect diet breadth (Singer et al. 2004; Mason et al. 2011; Forister et al. 2012). Mooney et al. (2012) recently proposed the integrative tritrophic interactions hypothesis which consolidates and integrates bottom-up (plant-herbivore) and top-down (predators-herbivores) effects and explains the coexistence of specialist and generalist herbivores. They showed that specialists outperform generalists under diverse combinations of natural enemies and host-plant-quality effects. The tritrophic view has led to the enemy free space hypothesis (Jeffries and Lawton 1984), which states that specialists are better adapted than generalists when using their host plants for protection against natural enemies (Bernays and Graham 1988). Numerous mechanisms may contribute to the reduced predation vulnerability, including superior use of 'biochemical crypsis' (De Moraes and Mescher 2004), superior ability to sequester defensive secondary metabolites (Vencl et al. 2005) or superior insect immune defence.

Insect immune defence, which relies on constitutive and inducible mechanisms involving cellular and humoral components, is thought to be associated with fitness because it protects against a large range of parasites (Schmid-Hempel 2003; Diamond and Kingsolver 2011). Haemocytes are immune cells that circulate in the haemolymph and are recruited during phagocytosis of microbes and encapsulation of eukaryotic parasites such as nematodes and parasitoid eggs. They are certainly the most important functional elements involved in the recognition and encapsulation of pathogens (Lavine and Strand 2002). This latter process often involves melanization of the foreign object through activation of the prophenoloxidase (PPO) cascade, a widespread general response to parasite invasion (Cerenius and Söderhäll 2004). Indeed, the melanin synthesis is catalysed by the phenoloxidase (PO) enzyme, which is produced from its inactive precursor, the PPO stored in the haemolymph and haemocytes. Both of these components of constitutive immune defences (haemocytes and PO cascade activity) are of primary importance to combat larval parasitoids (Eslin and Prevost 1996; Kacsoh and Schlenke 2012), which are a major source of mortality in holometabolous phytophagous insects (Hawkins et al. 1997). The induced response is an additional line of defence that mainly consists of production of a variety of antimicrobial peptides (Iwanaga and Lee 2005) usually within a few hours of infection; the resulting antibacterial activity of the haemolymph can last for several days (Haine et al. 2008).

Many phytophagous species evolve towards monophagy due to an optimization of the use of their host plants. Polyphagy will be maintained by natural selection when rare host plants offer fitness advantages relative to abundant hosts, so as to offset selection for specialization on the abundant host. Identification of the specific ecological conditions that provide additional fitness is a current challenge for ecologists. It has been already shown that larval performance (growth, size and survival) and adult fitness (Thiéry and Moreau 2005; Rodrigues and Freitas 2013) were enhanced when growing on rare host plants, but only few studies have attempted to integrate parameters of immunological defences to explain these different adaptations to host plants (but see Yang et al. 2008). Indeed, herbivore resistance to infections mostly depends on the immune system (Zuk and Stoehr 2002). In addition, several recent studies showed that the immune systems of phytophagous insects were highly dependent on host plant quality (Ojala et al. 2005; Haviola et al. 2007; Smilanich et al. 2009; Diamond and Kingsolver 2011; Vogelweith et al. 2011). Therefore, variation in the immune response as it relates to host plant quality coupled with differential predation success has the potential to influence the evolution of diet breadth (Smilanich et al. 2009; Lampert 2012).

The European grapevine moth, *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae), is the major grapevine pest in the Palearctic region (Bovey 1966). Its adaptation to grapes is considered to be relatively recent, because intense damage of vineyards was only reported at the beginning of the twentieth century (Thiéry 2005). Despite the abundance of vineyards across Europe, this insect is a generalist feeder, feeding on flowers and fruits of various plant species (Thiéry 2005). Females oviposit on various plants, including rosemary (*Rosmarinus officinalis*) (Lamiales: Lamiaceae) and *Daphne gnidium* (Malvales: Thymelaeaceae). Furthermore, larvae can complete their development on more than 35 species, belonging to 26 phylogenetically distant families, ranging from eudicotyledons to monocotyledons. This means that *L. botrana* is a 'true generalist' species, feeding on more than 20 families of plants (Bernays and Graham 1988). In addition, *L. botrana* is an ideal species with which to test the role of the immune system in the maintenance of polyphagy, because previous research indicated that different grape cultivars have different effects on the immune system (Vogelweith et al. 2013) and life history traits (Moreau et al. 2006; Thiéry et al. 2014). Given the abundance and predictability of grapes as a food source, *L. botrana* is a good species for examination of the ability of generalist populations or individuals to evolve specialization on particular host plants, because theoretical studies suggest that specialized phytophagous

species generally use predictable, abundant, and easily found plants (reviews by Jaenike 1990; Mayhew 1997; but see Futuyma 1976; Beccaloni and Symons 2000). In other words, *L. botrana* could have evolved towards specialization on grapes, but the fact that it remains polyphagous allows us to examine the factors that underlie the maintenance of polyphagy.

An initial study showed that alternative host plants (AHP; i.e., species of host plants other than the principal host on which larvae are able to complete their entire life cycle) enhanced larval performance and adult reproductive success of *L. botrana* (Thiéry and Moreau 2005). In this study, larvae fed wild host plants exhibited lower mortality, shorter development time, greater pupal weight, better growth rate, longer life span and higher fecundity, longer egg-laying duration and better mating success. This suggests that the use of AHPs may be maintained in the host range because it greatly improves fitness. In the present study, we tested the effect of different host plants on the immune systems of *L. botrana* larvae. Based on our previous study (Thiéry and Moreau 2005), we hypothesize that the immunocompetence of larvae would be higher when they are raised on host plants other than grape. Thus, we compared the basal levels of immune defence of larvae (haemocyte count, activity of PPO cascade, antimicrobial activity) that were raised on different AHPs with those raised on two different cultivars of grape.

Materials and methods

Study organisms

An inbred strain of *L. botrana* (INRA Bordeaux) was used in this study. This culture is based on a great number of caged adults (several thousand per week) to which wild adults are periodically added. This laboratory strain has conserved genetic variability because considerable variation is found in immune parameters among larvae (Vogelweith et al. 2011). The stock colony was maintained without diapause on an artificial diet [1,000 mL water, 15 g agar, 84.63 g maize flour, 41.25 g wheat germ, 45.48 g yeast, 6.03 g ascorbic acid, 3.35 g Wesson salts, 0.32 mL Scala, 5 mL ethanol (95 %), 2.65 g benzoic acid, 2.76 g Nipagin; as described by Thiéry and Moreau (2005)], and maintained at 22 ± 1 °C, 60 ± 10 % relative humidity, and under a 16-h light:8-h dark photoperiod. All tests were performed in a climate-controlled chamber.

Plants, larval diets and general procedure

We tested six different plants that were all suitable hosts for first-generation *L. botrana*, which mostly appeared during May. The plant hosts included berries of Chardonnay (CHA) and Gewürztraminer (GEW) grape cultivars that were collected from young grape bunches (*Vitis vinifera*) (Vitales: Vitaceae) (VIT) at the beginning of the growing season, stage 27 (Eichhorn and Lorenz 1977) in an experimental vineyard close to Bordeaux (INRA Bordeaux-Aquitaine). The four other hosts were flowers and berries of yarrow (*Achillea millefolium*) (Asterales: Asteraceae) (ACH) collected in Dijon (Burgundy), berries of strawberry tree (*Arbutus unedo*) (Ericales: Ericaceae) (ARB) collected in Martillac close to Bordeaux, flowers and berries of flax-leaved daphne (*Daphne gnidium*) (Malvales: Thymelaeaceae) (DAPH) picked in a sand dune of Soulac, and flowers of tansy (β-thujone chemotype) (*Tanacetum vulgare*) (Asterales: Asteraceae) (TAN) collected in our INRA research centre (see above). Depending on the AHP, the first- generation *L. botrana* larvae grow either on the flower or the fruit in natura. For each of these plants, the corresponding parts were used to make the artificial diet. All plant materials were deep frozen (−18 °C) immediately after collection and then freeze dried.

The influence of host plant species on larval performance and immunity was tested by feeding larvae ad libitum with equivalent amounts of food. Larvae were raised individually in centrifuge tubes filled with 1.5 mL of one of the seven diets made of agar with plant material added from the different species for the six diets. The last diet, the control diet, is highly nutritious and has the same composition as the semi-artificial diet. The six other diets were made with the same composition of the artificial diet but contained only 20 % of the previous amounts of maize flour and wheat germ. The 80 % missing diet was substituted with freeze-dried host plant material from different species ground into fine powder. This procedure has been successfully employed in previous experiments and presents at least three main advantages: (1) larvae are fed in isolation, thus biases due to competition and subsequent food deprivation are prevented; (2) differences in bunch compactness, which has an effect on larval feeding behaviour, are prevented; (3) incidences of infections by fungi, which naturally grow on harvested bunches and can modify larval fitness, are prevented (Savopoulou-Soultani and Tzanakakis 1988; Mondy and Corio-Costet 2000; Moreau et al. 2006).

Each centrifuge tube was filled with the diet and a newly hatched larva (younger than 24 h) was transferred into the tube with a fine brush. Two hundred individual larvae were put on each of the seven diets for a total of 1,400 larvae. The lids of the centrifuge tubes were pierced to allow air circulation. Larval development was monitored weekly. After 2 weeks, larvae were checked every second day to determine when they reached the fifth larval instar. Immune parameters and larval size were measured at this time.

Haemolymph collection

A sample of haemolymph was collected from each larva for the quantification of immune parameters (Vogelweith et al. 2011). Each larva was chilled on ice for 15 min before collection of a 3-µL sample. A perforation in the posterior part of the ventral side of the abdomen was made with an insulin syringe (Terumo), and haemolymph was collected in a sterile glass capillary (Hirschmann Laborgeräte). One microlitre was placed into a microcentrifuge tube containing 25 µL of buffer (0.01 M sodium cacodylate, 0.005 M $CaCl_2$; pH 6.5). Ten microlitres of this solution was immediately used for the determination of haemocyte count and the remainder was stored at −27 °C for subsequent determination of PO-PPO activity. The other 2 µL from the capillary was placed into a 0.5 µL *N*-phenylthiourea (Sigma P7629) coated microcentrifuge tube that contained 3 µL of the same buffer, a treatment that inhibits PO-PPO activity. This solution was stored at −27 °C for the subsequent determination of antimicrobial activity.

Immune parameters

Haemocyte count

The haemocyte count was measured by transfer of 10 µL of the haemolymph solution into a Neubauer improved haemocytometer and examination by phase contrast microscopy at 400× (Nikon Eclipse E200).

PO and total PO activities

Enzymatic activities of the PO-PPO system were measured with a spectrophotometric assay, as described by Cornet et al. (2009). PO activity was quantified without further activation. Total activity was measured following activation of PPO into PO with chymotrypsin. After thawing and centrifugation (6,500 r.p.m., 15 min, 4 °C), the activity of naturally activated PO enzymes was quantified by adding 5 µL of the sample to a solution consisting of 140 µL distilled water and 20 µL phosphate buffered saline (8.74 g NaCl, 1.78 g $Na_2HPO_4{}^2H_2O$, 1 L distilled water; pH 6.5). Total enzymatic activity (PO and PPO activities) was measured after activation with a chymotrypsin solution (Sigma C7762; 0.007 mg/mL distilled water). Then, 20 µL of an L-3,4-dihydroxyphenylalanine (L-DOPA) solution (Sigma D9628; 4 mg/mL distilled water) was added into each well of a 96-well microplate (655161; Greiner Bio-one). The conversion of L-DOPA to dopachrome was allowed to proceed for 40 min at 30 °C in a microplate reader (Versamax; Molecular Devices). Readings were taken every 15 s at 490 nm and the results were analysed using SOFT-MaxPro 4.0 (Molecular Devices, Sunnyvale, CA). Enzyme activity was measured as the slope (V_{max}) of the reaction curve during the linear phase of the reaction and reported as activity per 1 µL of haemolymph.

Antimicrobial activity

Antimicrobial activity in the haemolymph was measured using a zone of inhibition assay (Moret and Schmid-Hempel 2000). Petri dishes (9-cm diameter; Sterlin) were prepared by addition of 6 mL of a suspension of *Arthrobacter globiformis* (10^5 cells/mL) in 6 mL of a sterile broth medium (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, 1000 mL distilled water, pH 7.5) with 1 % bacto-agar. There were 14 wells per plate and a Pasteur pipette fitted with a ball pump was used to add 2 µL of the extract solution to each well. A positive control (Tetracycline; Sigma T3383) was included on each plate. The plates were incubated for 48 h at 30 °C. Inhibition of *A. globiformis* growth was quantified as the presence/absence of a clear circular zone around each well.

Head capsule size

The body size of each larva was evaluated by measuring its head capsule size. In particular, the distance between the most distant lateral sides of the head capsule was measured with a stereoscopic microscope (Nikon SMA-10A) coupled with a video analysis system (Linkam Scientific Instruments). This estimate of larval body size is commonly used in Lepidoptera and is well acknowledged to categorize instars and body sizes even though weight variation may exist within a single category of size (Godin et al. 2002; Panzavolta 2007; Delbac et al. 2010). In *L. botrana*, body length and weight measurements were not used because sizing and weighing living larvae is extremely difficult, especially in the case of older larvae, which move vigorously and are thus difficult to handle. In fact, measurement of head capsule width of dead larvae is the most reliable measurement of size in *L. botrana*.

Statistical analysis

All statistical tests were performed using R Software version 2.15.0 (R Development Core Team 2011). The responses of larvae to different host plants were assessed by: (1) measuring the effect of each of the seven different diets; and (2) measuring the effect of diet with a priori pooling into three diet groups—the AHP (ARB, DAPH, TAN, and ACH) group, the VIT (CHA and GEW) group, and the control group.

1. Survival of larvae until the last larval stage in the seven diet groups was compared using Pearson's χ^2-test. The

effect of diet on head size of larvae was tested with a one-way ANOVA followed by Tukey's test. Log transformation of haemocyte count and square root transformation of basal PO and total PO activities were used to normalize the data before subsequent analysis. These parameters were analysed according to diet using a one-way ANOVA followed by Tukey's test. The presence of antimicrobial activity in the haemolymph was tested with Pearson's χ^2-test.

2. For each variable in the analysis, effect sizes were calculated to provide a standardised measure of differences between the three diet groups. We used Cohen's *d*-statistic to compare differences in group means standardised by their pooled SD. For each mean *d*-statistic, we determined the 95 % bootstrap confidence interval (CI) (Nakagawa and Cuthill 2007). Effect sizes were considered to be significantly different from zero if the CI did not bracket the zero value. A greater *d*-value indicates a larger effect.

Correlations of immune parameters (concentration of haemocytes, PO and total activities) were assessed using Spearman's test on raw data. For each coefficient ρ, the 95 % bootstrap CI was determined. Anti-microbial activity was not considered here because it is a non-continuous variable.

Results

Growth performance: larval survival and head size

The percentage of larvae that survived to the fifth instar was different for larvae fed the different diets (Pearson $\chi^2_6 = 292.61$; $p < 0.0001$). In particular, survival on the control diet was about 75 %, and was significantly higher than survival on AHP diet or the VIT diet (Table 1).

Larval head capsule width (a proxy for overall size) was also different for larvae fed the different diets ($F_{6,199} = 10.61$; $p < 0.0001$) (Table 1), but there were no differences among the four non-grape species. The last instars of larvae reared on AHP were much larger than those reared on VIT cultivars, but had the same size as larvae reared on the control diet (Fig. 3).

Immune parameters

Analysis of larval haemocyte concentration indicated positive correlations with PO activity [Spearman, $\rho = 0.40$ (95 %CI = 0.33; 0.64); $p < 0.0001$] and total PO activity [Spearman, $\rho = 0.66$ (95 %CI = 0.50; 0.77); $p < 0.0001$]. These two activities were also positively correlated with each other [Spearman, $\rho = 0.60$ (95 %CI = 0.53; 0.72); $p < 0.0001$].

Table 1 Effect of diet on performance [percent survival and head capsule size (mm; mean ± SEM)] of *Lobesia botrana* larvae

Larval diet	Larval performance	
	Survival ($n = 200$ for each diet) (%)	Head capsule size
Yarrow	19.999 ab	1.041 ± 0.010 a (28)
Strawberry tree	31.000 c	1.016 ± 0.009 a (50)
Daphne	19.436 ab	1.014 ± 0.009 a (24)
Tansy	26.679 bc	1.022 ± 0.008 a (32)
Chardonnay	14.549 a	0.9671 ± 0.008 b (20)
Gewürztraminer	13.995 a	0.9415 ± 0.010 b (20)
Control	75.147 d	1.025 ± 0.007 a (31)

Diets with the *same letter* were not significantly different ($p > 0.05$) based on Pearson's χ^2-test (survival) and a one-way ANOVA followed by a Tukey's test (head capsule size). The number of larvae fed each diet for head capsule size is given *in parentheses*

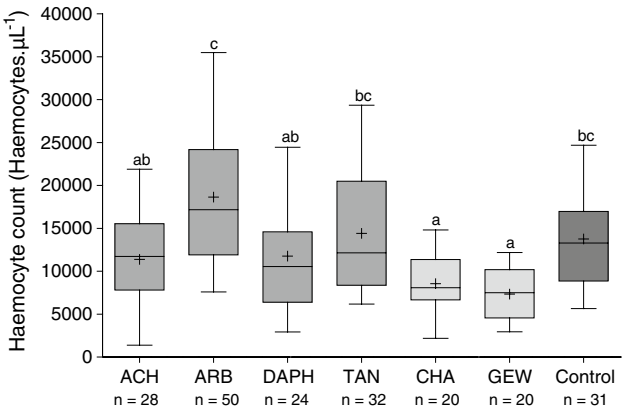

Fig. 1 Effect of diet on haemocyte concentration of fifth-instar larvae of *Lobesia botrana*. *Top of each box* indicates the first quartile, *bottom of each box* the third quartile, *central line* the median, *cross* the mean, *lines above the rectangle* the maximum, *lines below the rectangle* the minimum. Comparisons with Tukey's test were performed on the seven diets, six supplemented with plants [*Achillea millefolium* (*ACH*), *Arbutus unedo* (*ARB*), *Daphne gnidium* (*DAPH*), *Tanacetum vulgare* (*TAN*), Chardonnay (*CHA*), Gewürztraminer (*GEW*)] and the control diet. Boxes with the *same letter* are not significantly different ($p > 0.05$). The number of larvae fed each diet appears below the *x*-axis

The haemocyte concentration of haemolymph was different in larvae fed the seven different diets ($F_{6,198} = 11.31$; $P < 0.0001$) (Fig. 1). In particular, larvae fed the two different grape cultivars had the lowest concentrations of haemocytes (Fig. 1). There was also variability among the different host plants. Larvae fed ARB contained nearly 20,000 haemocytes/µL, whereas larvae fed ACH or DAPH had fewer than 15,000 haemocytes/µL (Fig. 1). Larvae reared

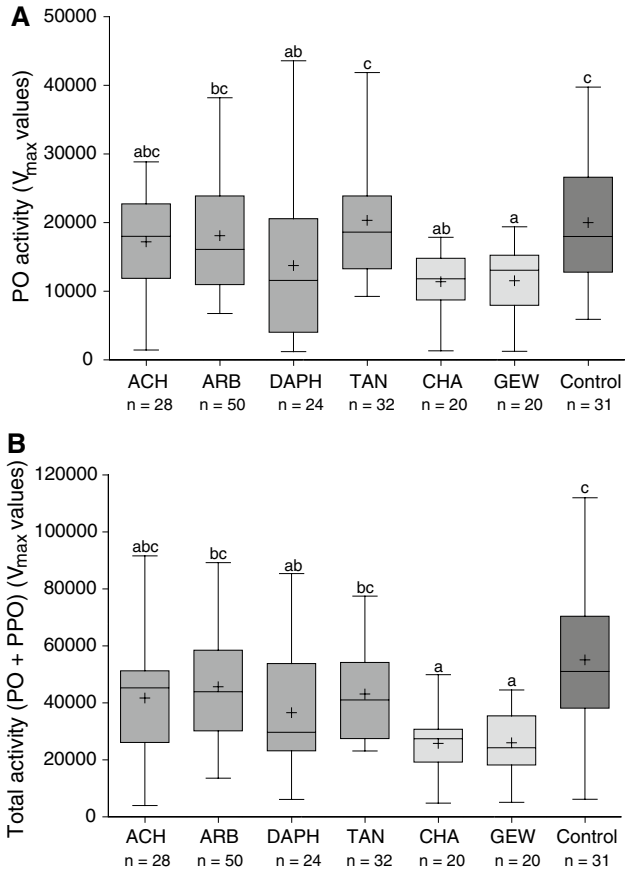

Fig. 2 Effect of diet on phenoloxidase (*PO*) and prophenoloxidase (*PPO*) activity (V_{max}) in the haemolymph of fifth-instar larvae of *L. botrana*. *Top of each box* indicates the first quartile, *bottom of each box* the third quartile, *central line* the median, *cross* the mean, *line above the box* the maximum, *lines below the box* the minimum. Comparisons with Tukey's test were performed on the seven diets, six supplemented with plants and the control diet. Boxes with the *same letter* are not significantly different ($p > 0.05$). The number of larvae fed each diet appears below the *x*-axis. For other abbreviations, see Fig. 1

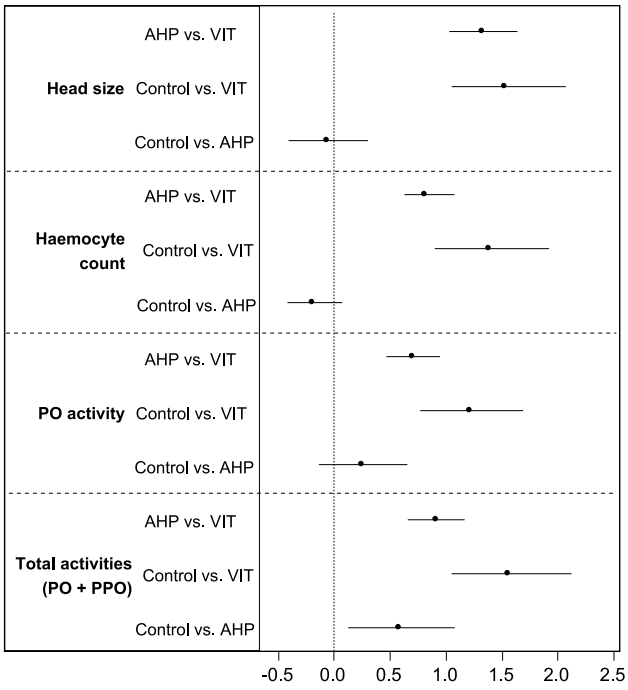

Fig. 3 Cohen's *d* and 95 % confidence intervals (CI) for head capsule size and larval immune parameters [haemocyte count, PO and total (PO + PPO) activities] in larvae fed alternative host plants (*AHP*s), *Vitis vinifera* (*VIT*), or the control diet. Effect sizes were considered to be significantly different from zero if the CI did not bracket the zero value. For other abbreviations, see Figs. 1 and 2

on AHP had more haemocytes than those reared on VIT, but had the same haemocyte count as larvae reared on the control diet (Fig. 3).

Diet also had a significant effect on PO activity ($F_{6,198} = 5.48$, $p < 0.0001$) and total PO activity ($F_{6,198} = 7.05$, $p < 0.0001$) (Fig. 2). There was variability among the different host plant alternatives, and larvae fed CHA and the GEW had the lowest PO and total activities. As previously, activities of both enzymes were higher for larvae reared on AHPs than those reared on VIT cultivars (Fig. 3). PO activity did not differ for larvae fed AHPs and the control diet, but total activity was higher in larvae fed a control diet than the AHP diet (Fig. 3).

Except for the haemolymph of larvae that were fed ACH, which had no evident antimicrobial effect, haemolymph antimicrobial activity ranged from 12 to 20 % for larvae fed the different diets (data not shown). There were no significant differences among the seven experimental diets ($\chi^2_6 = 7.159$, $p = 0.3064$).

Discussion

The results of the present study support the hypothesis that AHPs are more beneficial than VIT cultivars for the general performance of *L. botrana* larvae, including basal levels of immune defences. In particular, larval performance (survival and growth estimated by head capsule width after molting to the fifth instar) was better when larvae fed on several AHPs rather than VIT cultivars. In addition, the basal activity of the larval immune system (haemocyte count and activities of PO and total PO) was greater when larvae fed on AHPs rather than VIT cultivars. However, we found no difference in the antimicrobial activity of haemolymph of larvae fed AHPs or VIT cultivars. Finally, the development and immunocompetence (except for PO activity), of larvae fed AHPs were similar to those of larvae fed the control diet, which is highly nutritious.

We found that mortality was lower and size was greater when larvae were fed AHPs rather than VIT cultivars. These results are consistent with the results of Thiéry and Moreau (2005) who studied the same species of moth used

in this study. Thiéry and Moreau (2005) found that larvae fed wild host plants had lower mortality and reduced development time, and also had greater pupal weight, growth rate, female longevity, female fecundity, egg-laying duration, and mating success. Taken together, the results of these two studies suggest that AHPs support higher fitness for *L. botrana* larvae than VIT cultivars. This conclusion is reinforced by the fact that larval performance was similar on AHP diets and the control diet, which provides the best source of nutrients. Interestingly, the two grape cultivars used in our study (CHA and GEW) appeared to be of reduced food quality for *L. botrana* larvae. This may be explained by the presence of diverse secondary metabolites in grapes (Conde et al. 2007) that could be toxic to larvae during their early developmental stages (Moreau et al. 2006). For example, high concentrations of condensed and hydrolysable tannins negatively affect growth and development in some Lepidoptera species (Reese et al. 1982; Manuwoto and Scriber 1986). Grape berries contain abundant polyphenols, especially tannins (Hegarty et al. 1991). The lack of certain essential nutrients, such as amino acids or proteins, in grape berries may also explain the poor performance of larvae that fed on VIT cultivars because proteins are essential for larval growth and development (De Bruyn et al. 2002).

Our finding that AHPs provide better immunocompetence to *L. botrana* larvae is consistent with a study in the moth, *Epirrita autumnata* (Lepidoptera: Geometridae). In this latter species, immunity is similar or better in individuals fed AHPs than the main host plant, the mountain birch (*Betula pubescens*) (Fagales: Betulaceae) (Yang et al. 2008). Our findings indicate a strong effect of host plant on the innate immune system. In agreement, recent studies have reported that plant nutritive quality (levels of sugars, proteins, and fatty acids), amount of water, and multiple defence compounds are often positively correlated with immune activity, such as encapsulation rate (Ojala et al. 2005; Haviola et al. 2007) and haemocyte count (Shikano et al. 2010) or PO activity (Klemola et al. 2007). Previous research indicated that host plants with low levels of tyrosine (a major substrate for PO) may adversely affect the ability of phytophagous insects to resist parasitoid attacks through encapsulation and egg melanization (Renault et al. 2002). Hence, it is possible that AHPs may have contained more tyrosine than the two grapevine cultivars. Another possible explanation is that secondary metabolites in our two grape cultivars could have negatively affected the larval immune system due to a direct toxic effect or by forcing larvae to devote energy to detoxification rather than immune function (Smilanich et al. 2009; Lampert 2012 and references therein). For example, in *E. autumnata*, there is a negative relationship between the amount of hydrolysable tannins in the foliage consumed by larvae and immune response (measured as the melanotic encapsulation rate) (Haviola et al. 2007). Moreover, high PO activity is an indication of good overall health of phytophagous insects (González-Santoyo and Córdoba-Aguilar 2012).

Our results showing enhanced basal levels of immune defence (haemocyte count and PPO activity) on AHPs add to those of Thiéry and Moreau (2005) on general fitness (better growth rate and reproductive rate on AHPs) and support the idea that *L. botrana* is poorly adapted to cultivated grapevines compared to various AHPs. Indeed, if AHPs are likely to provide a competitive advantage to *L. botrana* in terms of growth and reproduction, this advantage might be strengthened in the presence of natural enemies such as parasites and parasitoids. The parasitism rate in *L. botrana* can reach 50 % in some vineyard plots (Xuéreb and Thiéry 2006), and even if abundance and diversity of larval parasitoids may vary between years and geographic regions (Moreau et al. 2010), parasitism pressure coupled with exposure to entomopathogenic fungi appears to be a rather constant trend among most European vineyards. This means that the positive effects of AHPs in terms of survival and growth combined with immunocompetence should be advantageous to the moth under heavy selective pressure by various parasites.

Our laboratory results lead to the hypothesis that the feeding upon AHPs by phytophagous insects in nature may be maintained because dietary diversity increases insect fitness. This may be explicated as follows. Recently mated females must find a host plant for oviposition, and the availability of suitable host plants is an important factor in the choice of oviposition site (West and Cunningham 2002). For example, a study demonstrated that fall webworm females (*Hyphantria cunea*) (Lepidoptera: Arctiidae) are under selection to reduce the time needed to find oviposition sites, and that they choose the most abundant host in their environment (Mason et al. 2011). In the case of *L. botrana*, grapevines are a highly predictable and abundant resource, so females should be able to easily and quickly find a suitable grape host. In this context, we hypothesized that only a small number of mated females would lay eggs on AHPs, and indeed this was the case (Thiéry and Moreau 2005). However, larvae developing on AHPs had better survival, greater growth rate, and shorter development time than those developing on VIT cultivars. Moreover, larvae from AHPs also have stronger immune responses against parasitoid attacks, the highest biotic source of mortality among phytophagous insects (Hawkins et al. 1997). Development time appears to be an important component of fitness in the field because slow development increases the duration of exposure to predators and parasites (Benrey and Denno 1997). Furthermore, Smilanich et al. (2009) argued that the immune response of lepidopteran larvae is one of the most important defence systems against parasitoids.

Thus, larvae that feed on AHPs could have a selective advantage because a functional immune system is essential for the encapsulation of parasitoid eggs (Eslin and Prevost 1996). Finally, adult females of *L. botrana* have a greater reproductive success in terms of longevity and fecundity when grown on AHPs than Vitaceae (Thiéry and Moreau 2005). Under the strong selective pressure exerted by parasitoids in this system, even if AHPs are rare in nature, the fitness benefits and especially the immune benefits they provide to *L. botrana* may be sufficient to prevent the formation of host-specific sub-populations that specialize on grapes. This hypothesis requires testing in the field.

This study highlights the importance of knowing the effect of host plants on the pest immune system to customize biological control programs and improve their success. One interesting application could, for example, rely on adapting a number of parasitoids released as a function of larval host-plant characteristics. It now becomes clear that extending our knowledge of the specificity of the interactions between host plants and the immune system of phytophagous insects in the context of tritrophic systems should provide important insights that should help us to propose solutions for the successful biological control of pests.

In summary, this study demonstrated a strong positive effect of AHPs on the life history traits and immune defences of the European grapevine moth, *L. botrana*. This species may have a diverse diet because such a diet improves immune responses and important life history characteristics. Therefore, this study emphasizes the importance of viewing insect-plant relationships from a tritrophic perspective. Plant chemistry and natural enemies, which can each elicit immune responses, could also interact with each other to influence the diet breadth of herbivores. Indeed, AHPs, which are rarely used by *L. botrana* in the field, could confer many advantages to larvae when the selective pressure exerted by natural enemies is considered. We suggest that additional studies should further examine the role of the immune system in the maintenance of polyphagy in phytophagous insects.

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