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Food-mediated modulation of immunity in a phytophagous insect: An effect of nutrition rather than parasitic contamination

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ABSTRACT

Inherent to the cost of immunity, the immune system itself can exhibit tradeoffs between its arms. Phytophagous insects face a wide range of microbial and eukaryotic parasites, each activating different immune pathways that could compromise the activity of the others. Feeding larvae are primarily exposed to microbes, which growth is controlled by antibiotic secondary metabolites produced by the host plant. The resulting variation in abundance of microbes on plants is expected to differentially stimulate the insect antimicrobial immune defenses. Under the above tradeoff hypothesis, stimulation of the insect antimicrobial defenses is expected to compromise immune activity against eukaryote parasites. In the European grape berry moth, *Eupoecilia ambiguella*, immune effectors directed towards microbes are negatively correlated to those directed towards eukaryotic parasites among host plants. Here, we hypothesize this relationship is caused by a variable control of the microbial community among host plants by their antibiotic metabolites. To test this hypothesis, we first quantified antimicrobial activity in berries of several grape varieties. We then measured immune defenses of *E. ambiguella* larvae raised on artificial diets in which we mimicked levels of antimicrobial activity of grape berries using tetracycline to control the abundance of growing microbes. Another group of larvae was raised on artificial diets made of berry extracts only to control for the effect of nutrition. We found that controlling microbe abundance with tetracycline in diets did not explain variation in the immune function whereas the presence of berry extracts did. This suggests that variation in immune defenses of *E. ambiguella* among grape varieties is caused by nutritional difference among host plants rather than microbe abundance. Further study of the effects of berry compounds on larval immune parameters will be needed to explain the observed tradeoff among immune system components.

1. Introduction

Immune defense is an important trait in biology with profound implications for the fitness of organisms. Because immunity is costly to maintain and use, its activation can affect other important physiological processes, and vice versa (Armitage et al., 2003). In addition, as the immune system is multifaceted, tradeoffs can also occur between pathways of the immune system itself. Organisms are therefore expected to invest in defense based on the risk of infection, or allocate defense in response to the most fitness-threatening parasite (Graham, 2001; Moret, 2003).

The invertebrate immune system involves humoral antimicrobial peptides used to combat microbial infection (Imler and Bulet, 2005), and the phenoloxidase–prophenoloxidase (PO–PPO) system, which is a component of the oxidative and melanization defenses used against eukaryotic parasites (Cerenius and Soderhall, 2004). Negative correlations between these two pathways of the immune system have been observed in bumblebees (Moret and Schmid-Hempel, 2009), the cabbage looper *Trichoplusia ni* (Freitak et al., 2007) and the European grape berry moth *Eupoecilia ambiguella* (Vogelweith et al., 2011). Changes in relative expression among immune effectors may result from selection across host generations, but might also result from plastic modulations of the immune system within individuals either in response to external cues predicting the presence of parasites (Wilson and Reeson, 1998) or upon infection. In the latter case, as hosts are facing a wide range of different parasite types, each

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activating different immune effectors, the infection by one parasite type may compromise the activity of other immune effectors that are competent against other parasite types (Freitak et al., 2007; Moret and Schmid-Hempel, 2009; Vogelweith et al., 2011).

As many invertebrates, phytophagous insects are exposed to a large range of microbial and eukaryotic parasites. Feeding larvae are primarily exposed to variable abundance and diversity of microbes on host plant structures (Renouf et al., 2005). Consumption of microbes is known to affect the expression of immune effectors in insects (Brown et al., 2003; Freitak et al., 2007). Hence, the relative expression of immune effectors might be affected by the ability of the host plant to control the microbial community on its surfaces. Some secondary metabolites of host plants have antibiotic activity that controls microbial development in the plant (Visser, 2011). Variation in plant antibiotic chemicals can differentially affect microbial growth on host plants, and feeding larvae would then be subject to variable amount of microbes, activating the antimicrobial immune response of phytophagous insect larvae. Assuming tradeoffs between the inducible productions antimicrobial peptides and the constitutive activity of the phenoloxidase (Moret and Schmid-Hempel, 2009), variation in the antibiotic activity of plants should indirectly affect how phytophagous insects allocate resource between these two immune pathways.

Besides being sources of microbial infection, host plants are also sources of nutrients of variable quantity and quality for phytophagous insects. Nutrition appears to be an important modulator of the host immune response. For instance, food deprivation leads to reduced immune responsiveness (Siva-Jothy and Thompson, 2002), lower resistance to pathogenic infection (Feder et al., 1997), and sometimes greater tolerance to pathogenic infection (Ayres and Schneider, 2009). Furthermore, variation in the intake of macronutrients in the food, including proteins and carbohydrates, could markedly affect immune traits (Cotter et al., 2011; Povey et al., 2009). Other dietary substances, including carotenoids, have also been found to influence the immune response of both vertebrates (Chew and Park, 2004) and invertebrates (Babin et al., 2010).

Therefore among phytophagous insects, natural variation in immune defense and resistance to pathogens could be partly host-plant dependent. On the one hand, expression of immune defense might be indirectly affected by the host plant antibiotic activity, controlling microbial pathogens consumed by larvae. On the other hand, changes in immune defense could directly result from variation in the nutritional quality of the host plant. Testing the relative importance of these non-exclusive factors should therefore improve our understanding of how host plants affect the immune response of phytophagous insects, and ultimately their fitness.

E. ambiguella (Lepidoptera: Tortricidae) is one of the two major moth pests of several grape vine cultivars in Europe. Antimicrobial activity in the hemolymph of *E. ambiguella* larvae was found negatively associated with the concentration of circulating hemocytes, and the activity of the PO–PPO system among vine cultivars (Vogelweith et al., 2011). This pattern of covariation between immune defenses is consistently observed in *Lobesia botrana* collected on grapes in the field (Vogelweith et al., 2014) and in *E. ambiguella* fed on diets made of grape extracts (Vogelweith et al., 2011). The grape surface is a phyllosphere habitat containing yeasts, bacteria and fungi (Boe, 2005), and varies with factors including climatic conditions and viticultural practices (Barata et al., 2012). To control these microbes, grape berries produce several antimicrobial peptides constitutively or in response to infection (Visser, 2011). Grape varieties vary in their susceptibility to microbial infection. For instance, Chardonnay vines in South Africa have been shown to be highly susceptible to crown gall (Burr et al., 1998), whereas the Pinot Noir variety appears to be protected through the production of a berry-specific defensin that has antifungal activity (de Beer and Vivier, 2008).

This study tested whether microbe abundance regulated by the antibiotic activity of grape berries affects the relative expression of the different immune effectors in the phytophagous insect, *E. ambiguella*. To this purpose, we first measured antimicrobial activity from berries of different grape variety and estimated it as a concentration of tetracycline, an antibiotic substance with a large spectrum of antimicrobial activity. Then, we raised larvae on six artificial diets that varied in their concentration of tetracycline to mimic natural levels of antimicrobial activity found in the different grape varieties. One of these diets did not contained tetracycline but was supplemented with bacteria. These diets enabled to assess the influence of host plant microbial control on the phytophagous insect immune system, independently of the plant nutritional quality. We also raised larvae on 2 artificial diets that did not contain tetracycline but berry extracts, which were used to test the direct effect of grape berries and variable plant nutritional quality. At the end of the larval stage, we measured constitutive (concentration of hemocytes, and PO and total-PO activities) and induced (antimicrobial activity) immune defenses. We previously found that induced immune defenses in the hemolymph of *E. ambiguella* larvae were negatively associated with constitutive ones among vine cultivars (Vogelweith et al., 2011). If the presence of microbes in the diet is important, we expect that diets containing tetracycline that control microbe growth will favor the maintenance of high levels of PPO activity in the hemolymph of larvae. Conversely, the absence of tetracycline should favor the development of microbes in the diet, which should stimulate antimicrobial activity in the hemolymph, at the expense of the PO–PPO system.

2. Materials and methods

2.1. Insect model

The insects used in this study were from an inbred stock maintained for several years at the INRA-Bordeaux Aquitaine (France). The stock culture is based on a large number of caged adults (several thousand per week) to which wild adults are periodically added. This laboratory strain has conserved genetic variability as considerable variation is found in immune parameters among larvae (Vogelweith et al., 2011). Larvae were maintained in boxes ($18 \times 11.5 \times 7$ cm) under standard laboratory conditions (22 ± 1 °C; $70 \pm 10\%$ rh; photoperiod: L16:D8), and provided with an *ad libitum* supply of a semi-artificial diet (1000 ml water, 15 g agar, 84.63 g maize flour, 41.25 g wheat germ, 45.48 g yeast, 6.03 g ascorbic acid, 0.32 g Scala®, 3.35 g mineral salt, 5 ml ethanol, 2.65 g benzoic acid and 2.76 g Nipagine) (Thiery and Moreau, 2005). The density of individuals was 100 per box (300 ml of diet).

2.2. Experimental design

2.2.1. Antibiotic activity in grape berries

The first part of this study involved measures of the levels of antimicrobial activity in berries from six grape varieties, and equating them to standard tetracycline concentrations. During the last week of July 2010 (which corresponds to the grape stage on which the second annual generation of *E. ambiguella* occurs), bunches of berries at the pre-veraison stage were collected from the 'gene collection of grape plants', Domaine de la Grande Ferrade (INRA-Bordeaux Aquitaine). To avoid a potential plant effect, we took 10 bunches per grape variety on different plant and at different place in the plant. All grape varieties were cultivated using similar practices and without insecticide application, in a small vineyard having homogeneous soil characteristics and

consistent climatic conditions. We collected and frozen (−30 °C) berries of Chardonnay, Chasselas, Gewurztraminer, Merlot, Pinot Noir and Riesling varieties.

Berries were deseeded and grinded together to produce berry juice (skin + pulp) for measuring antibiotic activity, which was recorded as the zone of inhibition on agar plates inoculated with *Arthrobacter globiformis* (Pasteur Institute CIP 105365), as described previously in Vogelweith et al. (2011, 2013a). Briefly, an overnight culture of *A. globiformis* was inoculated onto 1% agar plates. Wells were made into the agar using a sterilized glass Pasteur pipette fitted with a bulb-pump. 2 µl sample of each berry juice was poured in each well, and the plates were incubated at 30 °C for 48 h. Then, antibiotic activity of each sample could be measured as a clear bacterial growth of inhibition around the well. The diameter of the inhibition zone was measured and compared with those produced by standard dilutions of a tetracycline solution, from 0.005 to 0.05 mg mL^{-1}, to enable conversion of the sample antibiotic activity to an equivalent tetracycline concentration (see Section 3.1; Figs. 1 and 2).

For practical reasons, two grape varieties having contrasting levels of antibiotic activity, Chardonnay (C) and Pinot Noir (PN) (see results), were used as references for preparation of the experimental diets to which larvae of *E. ambiguella* were exposed.

2.2.2. Larval diets

A total of eight experimental diets were prepared (Table 1) and divided on two groups.

(1) *Six diets without berry extracts* (Table 1; white lines). We made six diets based on the recipe used for the rearing diet described above (see Section 2.1). We used this rearing diet as Control (Ctl). Two diets were enriched with tetracycline to mimic the levels of antibiotic activity found in extracts of Chardonnay berries, named thereafter T[C], and Pinot Noir berries, named T[PN] (Table 1). Two diets, named thereafter T[0.02] and T[0.04] were prepared as positive controls for the effect of the antibiotic chemical (Table 1). The latter diet (named B) was mimicking food highly contaminated with microbes and consisted of the control diet inoculated with bacteria (10^5 cells mL^{-1}) previously collected on this diet after one week, and cultured in broth medium (48 h at 28 °C) (Table 1).

(2) *Two diets with berry extracts* (Table 1; gray lines). We made two diets with berries of Chardonnay (C) and Pinot Noir (PN) to represent the range of nutritional quality and levels of antibiotic activity. The recipe of these diets was similar to that described above, but included 16.23 g of maize flour, 8.25 g of wheat germ, and 30 g of freeze-dried deseeded berry powder (Vogelweith et al., 2011) made from the respective grape variety.

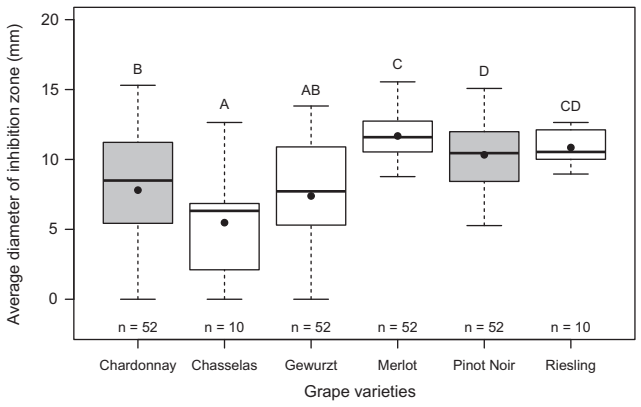

Fig. 1. Average zone of inhibition (diameter, mm) for berries of various grape varieties. The edges of the rectangles represent the first and third quartiles, the central features are the medians, the dashed lines are the maxima and minima, and the black circles are the means. The same letter indicates a nonsignificant difference ($P > 0.05$). For each grape variety the number of larvae tested is shown below the *x*-axis. Grape varieties in gray are those selected for further experiment.

A total of 1128 newly hatched larvae (age < 24 h) were randomly allocated to the eight experimental diets. Larvae were reared individually in centrifuge tubes filled with 1.5 mL of diet, which was sufficient for the larvae to complete development (Thiery and Moreau, 2005). The lids of the tubes were pierced with a needle, enabling air circulation. The larvae were maintained and checked every second day for survival until they reached the 5th instar stage, at which time a hemolymph sample was collected to measure the concentration of hemocytes, the antibacterial peptide activity and the activity of the PPO system. The body size of each larva was also estimated by measuring the distance between the most distant lateral sides of the head capsule margins (HC width) (Delbac et al., 2010) using a Nikon SMZ-10A stereoscopic microscope and VTO 232 video analysis system (Linkam Scientific Instruments). The HC width measurement is the most reliable measurement of body size in most Lepidoptera larvae (Godin et al., 2002; Panzavolta, 2007; Vogelweith et al., 2013b).

2.2.3. Bacterial growth on the diets

To make sure berry extracts and tetracycline in diets really inhibited microbial growth as expected from the antimicrobial activity measured in berries, we assessed microbial growth on the diets at the end of the larval development period on sub-sample. For each diet this involved adding 500 mg of the diet to 1 mL of phosphate buffered saline (PBS) in a micro-centrifuge tube. After incubation for 24 h, 100 µL samples of the solution were spread in triplicate onto 6 mL agar medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, 20 g agar, 1 L of distilled water; pH 7.0) in Petri dishes. The plates were incubated at 30 °C for 48 h, and the number of colony-forming units (CFU) was recorded. The mean of these 3 CFU values was used as data point.

2.2.4. Immune parameters

To assess the investment of larvae in constitutive and induced pathways, we measured three keys immune parameters: the concentration of hemocytes and the activity of the PPO system (constitutive pathway) and the antimicrobial activity of hemolymph (induced pathway).

Individual larvae at the 5th larval instar stage were chilled on ice for 20 min, and a 3 µL sample of hemolymph was collected from each larva using a sterile glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany) from a wound made in the posterior part of the ventral side of the abdomen. A 1 µL hemolymph sample was transferred to a micro-centrifuge 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 hemocyte counting; the remainder was stored at −27 °C for later measurement of the enzymatic activity of the PPO system. The remaining hemolymph in the capillary (1 µL) was flushed into a micro-centrifuge tube internally coated with n-phenylthiourea (Sigma P7629, Sigma–Aldrich, St Louis, MO, USA) and containing 2 µL of cold sodium cacodylate/$CaCl_2$ buffer. The tube was stored at −27 °C for later antibacterial activity measurement.

The concentration of hemocytes was determined from counts made using an improved Neubauer hemocytometer and phase contrast microscopy (400× magnification). The activity of the PPO system was estimated by measurement of the enzymatic activity of naturally activated PO enzymes (PO activity), and the activity of the proenzyme together with that of the PO activity

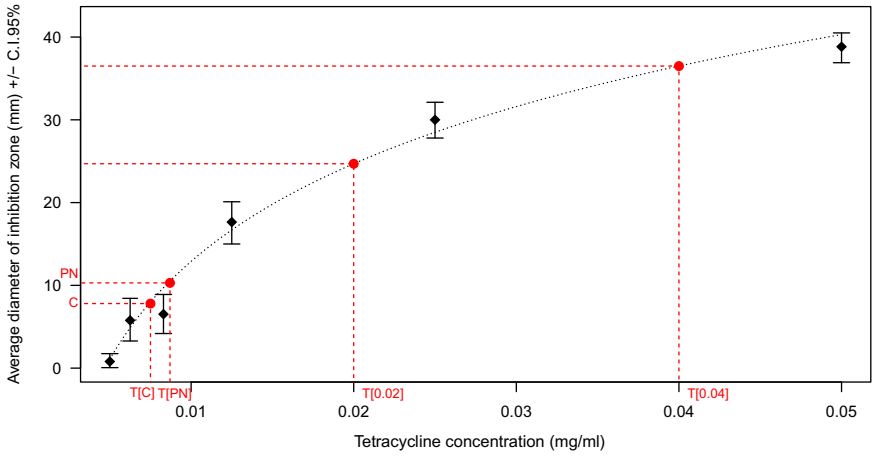

Fig. 2. Average zone of inhibition (diameter, mm ± C.I.) for various concentrations of tetracycline (mg mL^{-1}) (black diamond). The red circles represent the various concentrations used in the experiment and the name of the associated diet. The dotted line represents the tendency curve ($y = 1.7026 \ln(x) + 9.1031$). 'C' is the average antimicrobial activity of Chardonnay berries and T[C] is its tetracycline equivalent. 'PN' is the average antimicrobial activity of Pinot Noir berries and T[PN] is its tetracycline equivalent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Components (berry extract, tetracycline or bacteria) added to the various diets used in the experiment. White lines represent diets without added berry extract and gray lines represent diets with berry extract added.

Diet name	Berry extract	Tetracycline (mg mL^{-1})	Bacteria (cells mL^{-1})
Ctl	–	–	–
T[C]	–	0.0025	–
T[PN]	–	0.005	–
T[0.02]	–	0.02	–
T[0.04]	–	0.04	–
B	–	–	10^5
C	Chardonnay	–	–
PN	Pinot Noir	–	–

(total-PO activity). The measurements were based on a spectrophotometric assay described by Vogelweith et al. (2011, 2013a, 2014).

Antimicrobial activity in the hemolymph was recorded as the zone of inhibition in the assay described above (see Section 2.2.1). The plates were inoculated with the assay organism, hemolymph samples were thawed on ice, 2 μL of each sample was placed in a well, the plates were incubated at 28 °C for 48 h, and the diameters of inhibition zones were measured.

2.3. Statistics

Diets without and with grape berry extract were analyzed separately, as the basic recipes differed (see descriptions above). The effect of grape variety or diet on the antibiotic activity of berries, bacterial growth, PO and total-PO activity, antimicrobial activity of larvae and synthetic value of the immune system was tested using the Kruskal–Wallis rank sum test because the data were not normally distributed. The statistical significance of grape variety was assessed using likelihood ratio-based Chi-square test associated with a pairwise Wilcoxon multiple comparison test. Larval survival across experimental diets was analyzed using a Pearson's χ^2 test. We assessed the effect of experimental diets on the concentration of hemocyte using a Negative Binomial generalized linear model (GLM). The statistical significance of each term was assessed using likelihood ratio-based Chi-square test associated with a pairwise Wilcoxon multiple comparison test. A Negative Binomial GLM accounts for overdispersion bias associated with count data, and so it was preferred over a classical Poisson model (Sileshi, 2006). Larval body size was normally distributed, and was assessed using analysis of variance (ANOVA) and the statistical significance of each parameter was assessed by an F-statistic associated with the Tukey HSD test. To investigate the relationships among the four immune parameters measured we used Spearman's rank correlation coefficient with a confidence interval (C.I.) of 95%, because the data did not satisfied the condition for using parametric tests. 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 statistical analyses were performed using R software (version 3.1.1, R Development Core Team, 2008).

3. Results

3.1. Antimicrobial activity of grape berries

The antimicrobial activity of berries was variable among grape varieties ($\chi_5 = 53.84$; $p < 0.0001$; Fig. 1). Merlot berries had the highest level of antibiotic activity, and Chasselas berries had the lowest. Post-hoc tests revealed two groups of grape varieties with respect to antibiotic activity: the Chardonnay, Chasselas and Gewurztraminer varieties, with a mean diameter of growth inhibition of 7 mm, and the Merlot, Pinot Noir and Riesling varieties, with a mean diameter of growth inhibition of 11 mm (Fig. 1). We selected one grape variety from each group (Chardonnay and Pinot Noir, respectively) for further study. Based on comparison with serially diluted tetracycline, the Chardonnay (C) berries contained antibiotic activity equivalent to a tetracycline concentration of 7.53×10^3 mg mL^{-1}, while for Pinot Noir (PN) the equivalent tetracycline concentration was 8.74×10^3 mg mL^{-1} (Fig. 2).

3.2. Bacterial growth on diets

Bacterial growth on diets was significantly affected by the diet ($\chi^2_4 = 19.95$; $p < 0.0001$). The number of CFU in the control diet (T: mean = 32.66 CFU; CI 95% = [23.95; 41.36]) is significantly more important than in the other diets (T[C]: mean = 2.19 CFU, CI 95% = [0.94; 8.60]; T[PN]: mean = 0.71 CFU, CI 95% = [0; 4.59]; C: mean = 2.75 CFU, CI 95% = [0; 8.53]; PN: mean = 0.57 CFU, CI 95% = [0; 2.59]).

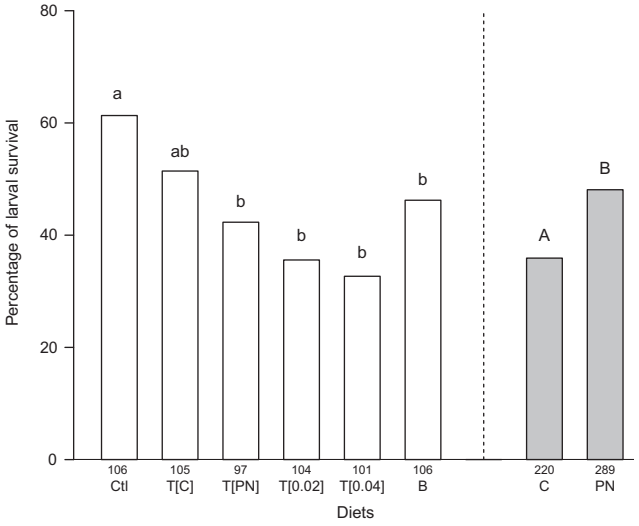

Fig. 3. Percentage of larval survival from hatching to the 5th instar stage as a function of diet. Diets with the same letter are not significantly different ($P > 0.05$). Lowercase letters refer to the diets without berry extract added and capital letters refer to diets with berry extract added. The number of larvae tested on each diet appears below the *x*-axis. See Section 2 for acronyms. White bars represent diets without added berry extract and gray bars represent diets with berry extract added.

3.3. Survival and larval body size

Larval survival was significantly affected by the addition of tetracycline or bacteria in the diet ($\chi^2_5 = 24.30$; $p = 0.0005$). The larvae on the control diet (Ctl) had the highest survival, whereas the addition of tetracycline or bacteria increased mortality (Fig. 3). No significant difference in larval survival was found between diets added with tetracycline and those with added bacteria (Fig. 3).

Larvae reared on the diet containing PN berries had higher survival than those reared on the diet containing C berries ($\chi^2_1 = 7.58$; $p = 0.006$; Fig. 3).

Larval body size was affected neither by the diet without added berry extract ($F_{5,\ 228} = 1.03$; $p = 0.40$) nor by the diet with berry extract added ($F_{1,\ 100} = 3.87$; $p = 0.052$).

3.4. Larval immune parameters

Among diets without berry extracts, larval concentration of hemocytes ($\chi^2_5 = 10.42$; $p = 0.10$; Table 2), PO activity ($\chi^2_5 = 8.51$; $p = 0.20$; Table 2), total-PO activity ($\chi^2_5 = 8.11$; $p = 0.23$; Table 2) and antimicrobial activity ($\chi^2_5 = 5.71$; $p = 0.46$; Table 2) were not affected by diets.

Among the diets containing berry extracts, antimicrobial activity was greater in larvae reared on the C diet than on the PN diet ($\chi^2_1 = 8.87$; $p = 0.003$; Table 2). However, constitutive defenses were similar on the C and PN diets, including hemocyte concentration ($\chi^2_1 = 0.31$; $p = 0.58$; Table 2), PO activity ($\chi^2_1 = 0.55$; $p = 0.46$; Table 2) and total-PO activity ($\chi^2_1 = 0.81$; $p = 0.37$; Table 2).

For both types of diet (with and without berry extracts), most of the immune defense components were correlated. Hemocyte concentration, the PO and total-PO activities were positively correlated, and these parameters were all negatively correlated with antimicrobial activity (Table 3).

These correlations highlighted the tradeoff between induced (antimicrobial activity) and constitutive (concentration of hemocytes and the activity of the PO-PPO system) defenses (Tables 3 and 4). The synthetic value of the immune system (PC1) did not vary among the diets without berry extracts ($\chi^2_5 = 9.73$; $p = 0.14$), but differed significantly among those containing berry extracts ($\chi^2_1 = 4.72$; $p = 0.03$; Fig. 4). Larvae reared on the C diet exhibited higher levels of induced defense and lower levels of constitutive defense than did the larvae reared on the PN diet.

4. Discussion

Our study investigated whether microbe abundance regulated by the antibiotic activity of grape berries affects the balance between components of the immune system in a phytophagous insect (*E. ambiguella*). Immune defense of *E. ambiguella* has previously been found to be influenced by grape variety, which partly mediate physiological tradeoffs between constitutive and induced immune defense mechanisms (Vogelweith et al., 2011). Such a modulation of the immune system in *E. ambiguella* was hypothesized to result from two nonexclusive causes. First, the balance between the components of the immune system of *E. ambiguella*

Table 2
Mean (±s.d.) of hemocyte concentration (hemocytes μL^{-1}), PO and total-PO activities (milli-units min^{-1}) and antimicrobial activity (mm) in the hemolymph of the 5th instar larvae according to the different diets. White lines represent diets without added berry extract and gray lines represent diets with berry extract added.

Diets	Hemocyte concentration	PO activity	Total-PO activity	Antimicrobial activity
Ctl	13,445 ± 1242	1941.25 ± 677.30	2161.95 ± 588.55	3.13 ± 1.25
T[C]	10,906 ± 1267	758.66 ± 367.63	942.88 ± 403.37	7.81 ± 2.20
T[PN]	9372 ± 1030	728.15 ± 367.37	1614.75 ± 852.02	6.04 ± 1.81
T[0.02]	9665 ± 955	1006.35 ± 613.94	1468.37 ± 1065.07	9.84 ± 2.53
T[0.04]	9700 ± 1304	587.17 ± 190.90	453.09 ± 170.65	7.99 ± 2.09
B	10,006 ± 1157	350.39 ± 67.95	17253.06 ± 16803.23	7.92 ± 1.96
C	13,773 ± 1963	720 ± 223	1619 ± 842	7.91 ± 1.71
PN	14,838 ± 1055	1345 ± 446	2482 ± 904	2.91 ± 0.78

Table 3
Spearman rank correlation coefficients and 95% C.I. among four immune system parameters (hemocyte concentration, PO activity, total-PO activity, antimicrobial activity) in the hemolymph of *E. ambiguella*. The values in white (above the diagonal) are those for the diets without berry extract added, and the values in gray (below the diagonal) are those for the diets with berry extract added. Values in bold indicate significant correlations.

	Hemocyte concentration	PO activity	Total-PO activity	Antimicrobial activity
Hemocyte concentration	–	**0.25; [0.10; 0.38]**	**0.35; [0.21; 0.47]**	**−0.24; [−0.37; −0.11]**
PO activity	0.07; [−0.13; 0.27]	–	**0.65; [0.53; 0.75]**	0.07; [−0.20; 0.07]
Total-PO activity	**0.21; [0.003; 0.40]**	**0.82; [0.69; 0.92]**	–	**−0.28; [−0.40; −0.16]**
Antimicrobial activity	**−0.28; [−0.46; −0.08]**	−0.09; [−0.28; 0.10]	**−0.27; [−0.41; −0.06]**	–

larvae could indirectly result from the presence of antibiotic compounds that differentially regulate the growth of microbes in the berries. Berries having lower levels of antibiotic activity are expected to contain more microbes, which should favor investment in specific antimicrobial immune defenses among phytophagous insects. Conversely, berries having high levels of antibiotic activity are expected to harbor few microbes, which should favor investment in constitutive defenses among phytophagous insect. Second, berries from different grapes varieties are likely to differ in their nutritional quality for the phytophagous insect; the composition of macronutrients is known to modulate investment among components of the immune system of insects (Ponton et al., 2011, 2013). Our study focused mainly on the first cause, but we controlled for the potential effect of nutrition by using diets to which berry extracts were added.

Neither the addition of tetracycline to the diet, mimicking antibiotic activity in the berries, nor the inoculation with bacteria, mimicking high microbial contamination of the food, affected the immune system of *E. ambiguella* larvae. However, the addition of tetracycline in the diet effectively inhibited bacterial growth in the diets. Conversely, the addition of grape berries to the diet resulted in changes in the expression of constitutive and induced defenses of the moth immune system. These results suggest that the presence of variable amounts of antibiotic activity or bacteria in the diet does not explain the modulation of the immune system previously reported in larvae (Vogelweith et al., 2011). Our results also contrast with those of Freitak et al. (2007), who found that microbial contamination of the food with *Escherichia coli* enhanced antimicrobial defenses in the hemolymph at the expense of the PPO system. One explanation for the immune system modulation is that *E. ambiguella* is less sensitive to microbial infection of the gut. Alternatively, because different microbes induce different immune responses in insects (Lemaitre et al., 1997), it is possible that the naturally-occurring bacteria growing on the diet were not sufficiently immunogenic. From these results it appears unlikely that variation in the presence of antibiotic substances in grape berries is responsible for variation in the immune function of *E. ambiguella*.

In contrast, the immune defenses of *E. ambiguella* were modulated by the presence of berry extracts in the diet. This suggests that substances other than antibiotics in the berries affected the larval immune system. Phenolic compounds are the most abundant secondary metabolites in plants, and in grape berries. These compounds are mainly present in the skin (which is the part of the berry first attacked by larvae) and the seeds of berries (Conde et al., 2007). The concentration of phenolic compounds in the berries is highly dependent on grape variety, but also on viticultural practices and environmental factors (Conde et al., 2007; Zhu et al., 2012). For instance, Zhu et al. (2012) showed that flavonol concentration was higher in Syrah, Cabernet Sauvignon and Merlot berries than in Gamay and PN berries. Given these variations within a single compound group (flavonols), it is likely that other berry compounds are highly variable among grape variety as well. In a recent study of the moth *Manduca sexta*, del Campo et al. (2013) found that larvae fed on diet supplemented with chlorogenic acid (an ester of caffeic acid) had a higher number of circulating hemocytes and greater resistance to bacterial infection than larvae fed on unsupplemented diet. Caffeic acid is involved in biosynthesis of phenolic compounds in grape berries (Conde et al., 2007), and is known to be an effective antimicrobial agent (Almeida et al., 2006). Such phenolic compounds may explain the tradeoff observed between larval immune defense components.

Variation in the presence of antibiotic substances in grape berries of different varieties did not explain variation in the immune function of *E. ambiguella*. The immune defenses of *E. ambiguella* were modulated by the presence of berry extracts in the diet, indicating that the effect on the immune system of the larvae resulted from substances present in the berries. Further study of the effects

Table 4
Principal component analysis for four immune system parameters (concentration of hemocytes, PO activity, total-PO activity, antimicrobial activity) in the hemolymph of *E. ambiguella*. The component loadings describe the relationships between the first principal component (PC1) and the variables from which they were derived.

	Diets without berry extract added	Diets with added berry extract
Eigenvalue	1.37	1.32
Explained variance (%)	34.37	33.04
Component loading		
Concentration of hemocytes	0.63	0.78
PO activity	0.45	0.36
Total-PO activity	0.65	0.48
Antimicrobial activity	−0.67	−0.72

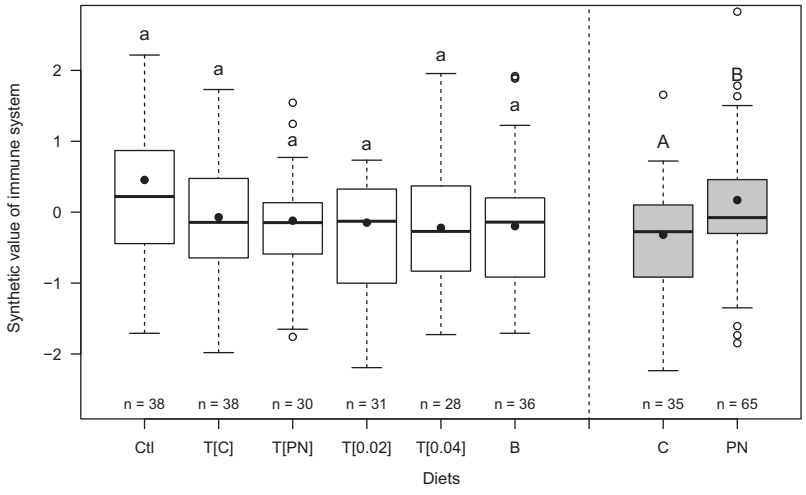

Fig. 4. Synthetic value of the immune system of 5th instar larvae, as a function of the diet. The edges of the rectangles represent the first and third quartiles; the central features are the medians; and the maxima and minima are represented by dashed lines. Diets with the same letter are not significantly different ($P > 0.05$). Lowercase letters refer to the diets without berry extract added and capital letters refer to diets with berry extract added. The number of larvae tested on each diet appears below the *x*-axis. See Section 2 for acronyms. White boxplot represent diets without added berry extract and gray boxplot represent diets with berry extract added.

of berry compounds on larval immune parameters will be needed to explain the observed tradeoff among immune system components.

Conflicts of interest

The authors declare no competing financial interests.

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