Contents lists available at ScienceDirect

Journal of Insect Physiology

The relative abundance of hemocyte types in a polyphagous moth larva depends on diet

Fanny Vogelweith [a,*], Yannick Moret [b], Karine Monceau [c], Denis Thiéry [d,e], Jérôme Moreau [b]

[a] *Johannes Gutenberg-Universität Mainz, Institut für Zoologie, Abt. Evolutionsbiologie, Johannes-von-Müller-Weg 6, 55128 Mainz, Germany*
[b] *Université Bourgogne-Franche Comté, Équipe Écologie Évolutive, UMR 6282 Biogéosciences, 6 Bd Gabriel, F-21000 Dijon, France*
[c] *Centre d'Etudes Biologiques de Chizé, UMR 7372, CNRS – Université de la Rochelle, 79360 Beauvoir-sur-Niort, France*
[d] *INRA, UMR 1065 Save ISVV, B.P.81, F-33883 Villenave d'Ornon Cedex, France*
[e] *Université de Bordeaux, Bordeaux Sciences Agro, INRA, UMR 1065 Save ISVV, B.P.81, F-33883 Villenave d'Ornon Cedex, France*

ARTICLE INFO

ABSTRACT

Hemocytes are crucial cells of the insect immune system because of their involvement in multiple immune responses including coagulation, phagocytosis and encapsulation. There are various types of hemocytes, each having a particular role in immunity, such that variation in their relative abundance affects the outcome of the immune response. This study aims to characterize these various types of hemocytes in larvae of the grapevine pest insect *Eupoecilia ambiguella*, and to assess variation in their concentration as a function of larval diet and immune challenge. Four types of hemocytes were found in the hemolymph of 5th instar larvae: granulocytes, oenocytoids, plasmatocytes and spherulocytes. We found that the total concentration of hemocytes and the concentration of each hemocyte type varied among diets and in response to the immune challenge. Irrespective of the diet, the concentration of granulocytes increased following a bacterial immune challenge, while the concentration of plasmatocytes and spherulocytes differentially varied between larval diets. The concentration of oenocytoids did not vary among diets before the immune challenge but varied between larval diets in response to the challenge. These results suggest that the resistance of insect larvae to different natural enemies critically depends on the effect of larval diet on the larvae's investment into the different types of hemocytes.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

In insect immunity, core processes such as coagulation, nodulation, phagocytosis and encapsulation are strongly associated with hemocytes (Lavine and Strand, 2002). They recognize and destroy invading pathogens and apoptotic cells during phagocytosis, and aggregate large numbers of bacteria during the nodulation process. They also bind to larger targets including eggs of parasitoids, nematodes and protozoa by forming a multilayer capsule around the intruder (so-called encapsulation; Marmaras and Lampropoulou, 2009). Hemocytes circulate freely in the hemocoel, and are produced either by hematopoietic organs or through cell division (Gillespie et al., 1997; Lavine and Strand, 2002; Strand, 2008; Tan et al., 2013). They differentiate into cell types that have various functions in pathogen defense (Lavine and Strand, 2002), and have been classified according to their morphology, function and certain molecular biomarkers. In Lepidopteran caterpillars, including *Bombyx mori*, five types of hemocytes have been described (Ling et al., 2005; Strand, 2008; Tan et al., 2013): prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids. Prohemocytes are the precursor of the other types; they are produced and reside primarily in hematopoietic organs, and thus rarely circulate in the hemocoel (Lanot et al., 2001). Granulocytes (GR) are usually the most abundant type in the hemolymph; they adhere strongly to the surface of foreign bodies, function as phagocytes, and are involved in encapsulation (Strand, 2008). Plasmatocytes (PL) are polymorphic in shape; they spread asymmetrically on foreign surfaces, and participate in capsule-formation during the encapsulation reaction (Strand, 2008). In contrast, spherulocytes (SP) are non-adhesive hemocytes and their role in insect immunity is still unknown, although it has been suggested that they are involved in the transport of cuticular components (Lavine and Strand, 2002). Oenocytoids (OE) are non-adhesive hemocytes that contain the main components of the phenoloxidase (PO) cascade (Lavine and Strand, 2002). The proportion of each hemocyte type can vary according to numerous factors including nutrition.

* Corresponding author.
 E-mail address: fanny.vogelweith@gmail.com (F. Vogelweith).

Nutrition is now recognized as a critical factor in immune defense and resistance to pathogens (Lazzaro and Little, 2009; Ponton et al., 2011; Vogelweith et al., 2013a). Experimental studies of insects have demonstrated that food deprivation affects immune responsiveness (Ayres and Schneider, 2009; Kapari et al., 2006; Siva-Jothy and Thompson, 2002; Yang et al., 2008) and changes in the expression of several immunity genes (Pletcher et al., 2002). Immune effectors can also be affected by changes in food composition (Babin et al., 2010; Cotter et al., 2011; Povey et al., 2009; Vogelweith et al., 2011). For example, Shikano et al. (2010) showed that *Trichoplusia ni* larvae reared on broccoli had more hemocytes than those reared on cucumber. Surprisingly, the influence of diet on the concentration of the different hemocyte types remains unclear. This issue is relevant since hemocyte types have different roles in immunity, and nutrition-dependent variation in their relative abundance might thus affect the immune response of phytophagous larvae. Indeed, Klowden (2002) suggested that GR are also involved in nutrient transport. Thus, it could be hypothesized that food deprivation or poor quality food induces a nutritive stress, reducing the proportion of GR hemocytes and affecting the encapsulation process.

Tortricid moths, including *Lobesia botrana* and *Eupoecilia ambiguella*, are the most harmful pests of grapes in Europe and North America. The larvae are polyphagous, and can develop on almost any grape variety and more than 25 other host plants (Thiery and Moreau, 2005). Natural populations of tortricid moths are the target of numerous natural enemies and face variable changes in attacks by natural enemies, in both time and space (Moreau et al., 2010). For example, biological control methods use both entomopathogenic microorganisms and parasitoids to control moth populations. However, the success of the attacks depends on the ability of the pest to defend itself mainly via its immune system. Particularly, hemocytes have been shown to be an important parameter of the insect immune system because of their involvement in encapsulation (Eslin and Prevost, 1998; Carton et al., 2008). However, not all hemocyte types have the same role in immunity (Lavine and Strand, 2002) and not all types fight against the same enemies. *E. ambiguella* can grow on different host plants that affect its immune parameters (Vogelweith et al., 2011). For this reason, we considered it important to characterize the hemocyte types and investigate how their relative abundance varies among host plants to measure the effect of host plant on hemocyte type. This would be important if larvae can display differential resistance to natural enemies depending on their relative investment in the proportion of each hemocyte type.

In this study, we characterized the different types of hemocytes in *E. ambiguella* larvae, and assessed the influence of various artificial diets enriched with different grape varieties by measuring the concentration/abundance of each hemocyte type as a function of the food ingested by the larva. Because hemocytes are also expected to vary in numbers and proportion upon infection, we quantified changes in their concentration in response to a standard immune challenge mimicking a bacterial infection.

2. Materials and methods

2.1. Insect rearing

The insects used in this study came from an inbred stock of the European grape berry moth, *E. ambiguella* (Lepidoptera, Tortricidae) reared at the INRA of Bordeaux (Aquitaine, France) for several years. This stock is based on a great number of caged adults (several thousand per week), to which wild adults are regularly added. We found a very similar pattern in terms of the basal immunity level and parasitoid escape behavior between the inbred stock and wild lines sampled in French vineyards (Vogelweith et al., 2014). Thus, the results obtained for this strain are likely to be relevant to field populations. Larvae were maintained in boxes (18 × 11.5 × 7 cm) on a semi-artificial diet (described in Vogelweith et al., 2011) at a density of ca. 100 individuals per 300 ml of diet.

2.2. Artificial diets

Four experimental diets were prepared following the method described by Vogelweith et al. (2011). Specifically, a rearing diet without added berries was used as the control, and three test diets were prepared using berries from a different grape variety, respectively: 'Chardonnay', 'Chasselas' and 'Gewürztraminer'. During the last week of July 2009, insecticide-free bunches of berries for the diets were collected at the pre-veraison stage from the gene collection of grape plants 'Domaine de la Grande Ferrade', INRA-Bordeaux Aquitaine; this stage corresponds to the grape phenology on which the second annual generation of *E. ambiguella* occurs.

Newly hatched larvae (<24 h) were individually reared in centrifuge tubes containing 1.5 ml of diet, which is sufficient for the larvae to complete development (Moreau et al., 2006a,b; Thiery and Moreau, 2005). The lids of the tubes were pierced with a needle to allow air circulation. Larvae were maintained until the 5th larval instar stage under standard laboratory conditions (22 ± 1 °C, 70 ± 10% r.h., photoperiod: L16:D8).

2.3. Collection of hemolymph and immune challenge

Hemocytes were extracted from 5th instar larvae using the method described by Vogelweith et al. (2011, 2013a). Briefly, the larvae were anesthetized on ice for 20 min, and 1 μl of hemolymph was then collected and flushed into a micro-centrifuge tube containing 20 μl of sodium cacodylate/$CaCl_2$ buffer (0.01 M sodium cacodylate, 0.005 M $CaCl_2$; pH 6.5). This sample was used to measure the total concentration of hemocytes, and to characterize the different hemocyte types as well as their relative concentration (see section d. and results). Following this first hemolymph collection, larvae were immune challenged in the posterior part of the ventral side of the abdomen with a sterile needle dipped in a concentrated suspension of heat-killed *Arthrobacter globiformis* (ca. 10^9 cells ml^{-1}). This bacterium is commonly used in the protocol testing of antimicrobial activity (Sadd and Schmid-Hempel, 2007; Vogelweith et al., 2011, 2013a, 2015), as it is very sensitive to antimicrobial peptides of insects (Dubuffet et al., 2015). After the immune challenge, larvae were kept individually in micro-centrifuge tubes for 24 h under standard conditions before a second sample of hemolymph was collected (Vogelweith et al., 2011, 2013b, 2014). The delay between collecting the two hemolymph samples was determined based on preliminary experiments showing that the concentration of hemocytes stabilizes 24 h after the immune challenge at the earliest (Supplementary material 1). This second sample of hemolymph allowed the concentration of each hemocyte type and the total concentration of hemocytes after an immune challenge to be measured. All hemolymph samples were assessed immediately to avoid coagulation and desiccation of the hemocytes.

We noted that the immune challenge induced mortality (5–10%) equally distributed among diets. We tested 23 larvae reared on Chardonnay, 23 on Chasselas, 28 on Gewürztraminer and 22 on the Control diet.

2.4. Hemocyte concentration and characterization

Hemocyte concentration was estimated using an improved Neubauer hemocytometer counting chamber and phase contrast

microscopy (magnification 400×). Every sample of hemolymph was counted twice, once for the total number of hemocytes and once for the characterization and number of each hemocyte type.

Hemocyte characterization was performed using the hemolymph sample that had been taken before the immune challenge. Hemocyte types were characterized based on their previously reported morphological differences (Beaulaton, 1979; Falleiros et al., 2003; Ribeiro and Brehelin, 2006). Accordingly, we considered the cell shape (round, oval, deformed) and size (big, small), the size of the nucleus and its position in the cell (in the middle or offset of the cell), and the cytoplasm compounds (presence/absence of granules and spherules, transparent or opaque).

2.5. Larval body size

Following the extraction of the first hemolymph sample, larval body size was estimated by measuring the maximum head capsule width (HC width; Delbac et al., 2010) using a Nikon SMZ-10A stereoscopic microscope and a VTO 232 video analysis system (Linkam Scientific Instruments). The HC width is the most reliable measure of body size in most lepidopteran larvae (Delbac et al., 2010; Godin et al., 2002; Panzavolta, 2007; Vogelweith et al., 2013b).

2.6. Data analysis

The effect of diets on the total number of hemocytes and on the concentration of each hemocyte type before the immune challenge was tested using Kruskal–Wallis tests because the data did not meet the assumptions of normality and homoscedasticity for parametric tests (tested using with Shapiro–Wilk and Levene tests respectively). Kruskal–Wallis multiple comparisons post hoc tests (MC) were used when needed to test differences among diets. The effect of both diet and immune challenge on the total concentration of hemocytes and the concentration of each hemocyte type (arcsine square root transformed) were analyzed using a linear mixed effects model that included individual identity as a random factor within the diet effect. The statistical significance of the effects of diet and immune challenge were tested using F-ratio statistics. When the data did not meet the conditions of normality and homoscedasticity, linear mixed effect models based on rank transformation were used instead of the classical non-parametric Friedman tests (Baguley, 2012).

Our initial data analyses revealed that larval body size was neither related to the total concentration of hemocytes nor to the concentration of single hemocyte types, irrespective of the time of measurement (i.e. before or after exposure). We hence removed this variable from all further analyses.

Statistical analyses were performed using the R software (version 3.1.1; R Development Core Team, 2008) complemented with the *lawstat* (Levene test) and *nlme* (linear mixed effects model) packages.

3. Results

3.1. Hemocyte types and morphology

Fifth instar larvae of *E. ambiguella* contained four types of hemocytes that were easily differentiated: granulocytes (GR), plasmatocytes (PL), oenocytoids (OE) and spherulocytes (SP) (Fig. 1). The presence of granules makes GR easy to differentiate from the other types; the GR cells appear as round cells with a small and round nucleus (Fig. 1a, e). PL were also round cells of variable size (Fig. 1b, f), in which the nucleus was either round or bi-lobed, and was relatively large in the cell. SP were also easy to recognize

because of their inclusions and spherules, which occupied most of the cytoplasm and deformed the cell and the nucleus (Fig. 1d, h). OE had a dense and opaque cytoplasm with a small overhanging nucleus (Fig. 1c, g).

3.2. Diet effect on the total concentration of hemocytes before the immune challenge

The total concentration of hemocytes was influenced by the larval diet (Wilcoxon text, $\chi_3^2 = 11.08$; $p = 0.01$) (Fig. 2, before immune challenge). Larvae reared on Gewürztraminer had more hemocytes than larvae reared on the control diet. There was no difference between larvae reared on Chardonnay and Chasselas, or between any other combinations of diets (Fig. 2, before immune challenge).

3.3. Diet effect on each hemocytes type before the immune challenge

Irrespective of the diet, GR were the most abundant cells in the hemolymph with an average number of 13,603 hemocytes/µl (95% CI = [11,906; 15,300], accounting for 67% of the hemocytes (Fig. 3, before immune challenge). PL were the second most abundant hemocytes with an average number of 5955 hemocytes/µl (95% CI = [5115; 6795], accounting for 29%. SP (mean = 518 hemocytes/µl; 95% CI = [384; 652]) and OE (mean = 136 hemocytes/µl; 95% CI = [102; 170]) were much less abundant, accounting for 3% and 1% of the hemocytes, respectively.

Larval diet affected the concentrations of GR ($\chi_3^2 = 12.83$; $p = 0.005$), PL ($\chi_3^2 = 8.86$; $p = 0.03$) and SP ($\chi_3^2 = 23.64$; $p < 0.0001$) but not that of OE ($\chi_3^2 = 1.05$; $p = 0.79$) (Fig. 3, before immune challenge). The concentration of GR was lower in larvae reared on the control diet than in those reared on the Gewürztraminer and Chardonnay diets (Fig. 3a, before immune challenge). Larvae reared on the control diet also had less PL than those reared on Gewürztraminer (Fig. 3b, before immune challenge). Except for larvae reared on Gewürztraminer and control diets which did not differ, all larvae reared on the other diets had different concentration of SP (Fig. 3c, before immune challenge).

3.4. Diet and immune challenge effect on the total concentration of hemocytes and on the concentration of each hemocyte types

The total concentration of hemocytes after the immune challenge was only affected by that challenge ($F_{1,110} = 18.44$; $p < 0.0001$), but not by the larval diet ($F_{3,74} = 1.62$; $p = 0.19$) or an interaction between challenge and diet ($F_{3,110} = 2.62$; $p = 0.06$). Irrespective of the diet, the total concentration of hemocytes increased after the immune challenge (Fig. 2, before and after immune challenge).

The immune challenge affected the concentration of all hemocyte types, either alone, or in an interaction with the diet (Table 1; Fig. 3). Specifically, the concentration of GR in the hemolymph of larvae increased irrespective of the diet after the immune challenge (MC: $p < 0.0001$; Fig. 3a). In contrast, the changes in the concentration of PL, SP and OE caused by the immune challenge depended on the diet (Table 1; Fig. 3b, c, d). The concentration of PL decreased after the immune challenge in larvae reared on Gewürztraminer (MC: $p = 0.01$) and increased in larvae reared on the control diet (MC: $p = 0.04$) (Fig. 3b). The concentration of PL did, however, not change in larvae reared on Chardonnay and Chasselas (MC: $p = 0.96$ and $p = 0.73$, respectively). The concentration of OE and SP increased after the immune challenge in larvae reared on the control diet (MC, OE: $p = 0.006$; SP: $p = 0.0006$; Fig 3c, d), but did not change in larvae reared on Chasselas, Chardonnay or Gewürztraminer (MC, OE, Chardonnay: $p = 0.16$;
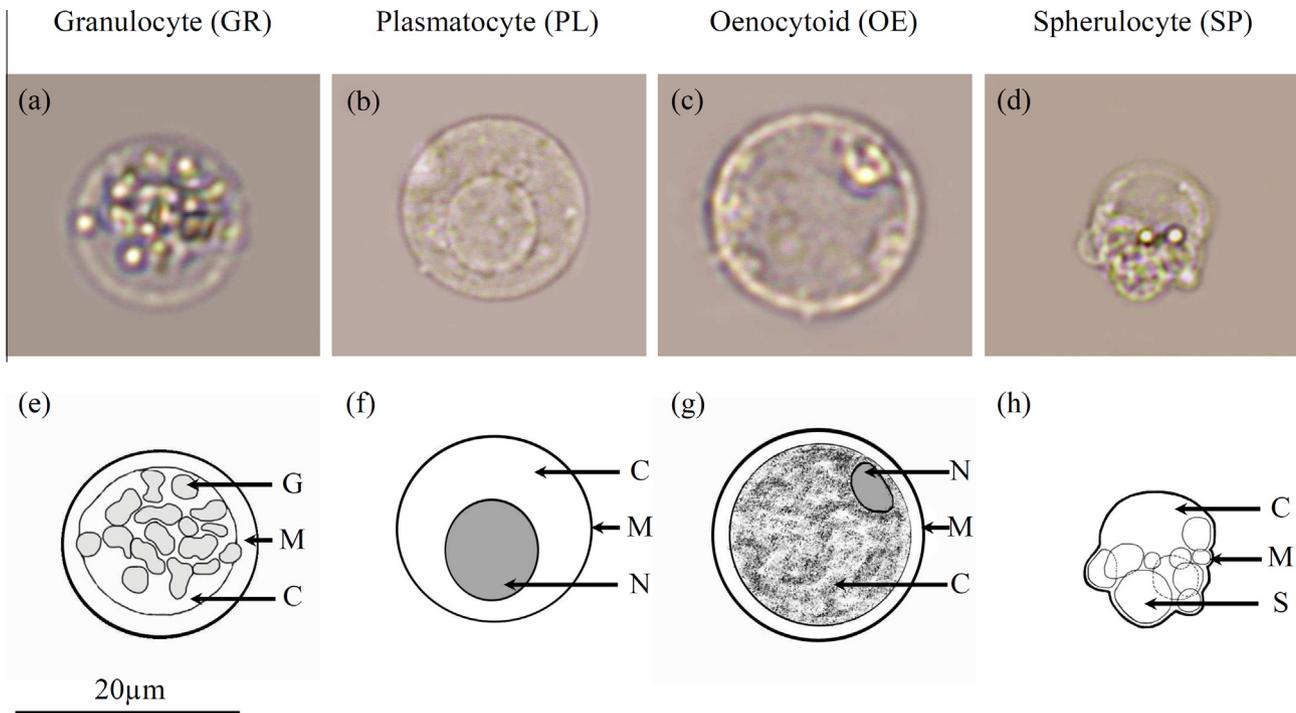
Fig. 1. Photographs and annotated drawings of the hemocyte types: granulocytes (GR; a, e), plasmatocytes (PL; b, f), oenocytoids (OE; c, g), spherulocytes (SP; d, h). In the annotated drawing 'C' indicates the cytoplasm, 'G' indicates the granules, 'M' indicates the membrane, 'N' indicates the nuclei and 'S' indicates the spherules.
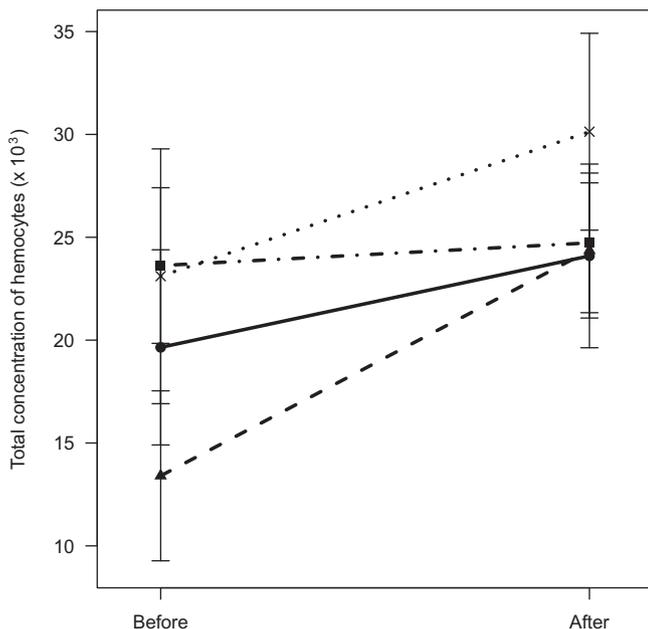

Fig. 2. Variation among diets in the total concentration of hemocytes prior to and following an immune challenge. Means and variation are given for larvae reared on diets containing Chardonnay (crosses; dotted lines), Chasselas (black circles; solid lines) or Gewürztraminer (black squares; dotted-dashed lines) as well as for larvae reared on the control diet (black triangles; dashed lines), respectively.

Chasselas: $p = 0.85$; Gewürztraminer: $p = 0.79$ and SP, Chardonnay: $p = 0.79$; Chasselas: $p = 0.43$; Gewürztraminer: $p = 0.79$, Fig. 3c, d).

4. Discussion

This study aimed to characterize the different types of hemocytes in 5th instar larvae of *E. ambiguella*, and to test the effects of diet and immune challenge on the concentration of each hemocyte type. We showed that the total concentration of hemocytes and the concentration of the four hemocyte types varied among diets and in response to the immune challenge.

Consistent with other studies in lepidopteran species (Falleiros et al., 2003; Lavine and Strand, 2002; Ling et al., 2005; Ribeiro and Brehelin, 2006), we identified granulocytes, oenocytoids, plasmatocytes and spherulocytes hemocytes based on their morphological features. However, we did not detect prohemocytes (PR), although these have been reported in larvae of other lepidopteran species (Falleiros et al., 2003). We tested only mature larvae at the end of the larval stage. Falleiros et al. (2003) reported that the proportion of PR decreases with larval age until it reaches zero, which may explain why we found no PR in 5th instar larvae of *E. ambiguell*a.

As in other species of Lepidoptera, including *Diatraea saccharalis* (Falleiros et al., 2003), *Euclea delphinii* and *Lithacodes fasciola* (Stoepler et al., 2013), GR and PL were the most abundant circulating hemocytes in *E. ambiguella*. GR are involved in the recognition of foreign bodies, phagocytosis, encapsulation and nodulation (Lavine and Strand, 2002; Nakahara et al., 2010; Ribeiro and Brehelin, 2006; Strand and Pech, 1995). However, their role in immune processes also depends on the insect species examined (Falleiros et al., 2003). The high concentration of PL may possibly be due to their involvement in numerous processes including encapsulation and shaping of the melanotic capsule (Lavine and Strand, 2002; Nakahara et al., 2010; Ribeiro and Brehelin, 2006; Strand and Pech, 1995), and their production of soluble pattern recognition and antimicrobial proteins (Nakahara et al., 2010). GR and PL act together in defense against macro-pathogens (e.g. parasitoid eggs) during encapsulation (Lavine and Strand, 2002; Nakahara et al., 2010; Ribeiro and Brehelin, 2006; Strand and Pech, 1995). Both the concentrations of GR and PL are higher than those of OE and SP, certainly because they can differentiate in OE and SP (Beaulaton, 1979; Han et al., 1998).

GR and PL concentrations were both higher in larvae reared on Gewürztraminer than larvae from others diets and lower in larvae
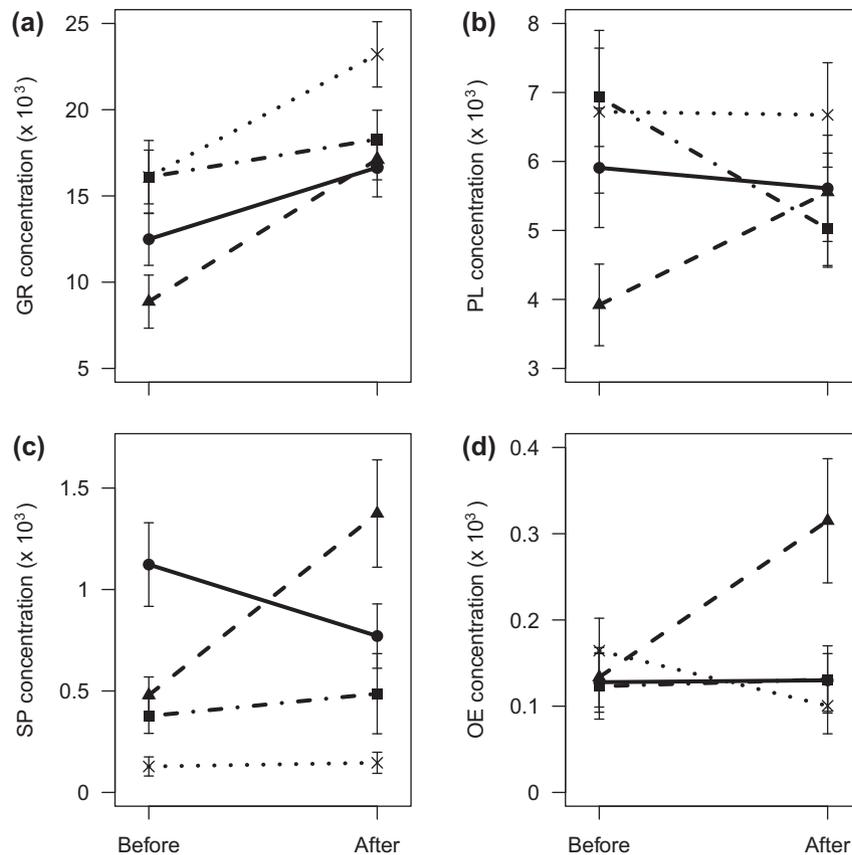
Fig. 3. Variation in the concentration of granulocytes (a), plasmatocytes (b), spherulocytes (c) and oenocytoids (d) (±standard error) prior to and following an immune challenge between the diets. Means and variation are given for larvae reared on diets containing Chardonnay (crosses; dotted lines), Chasselas (black circles; solid lines) or Gewürztraminer (black squares; dotted-dashed lines) as well as for larvae reared on the control diet (black triangles; dashed lines), respectively.

Table 1
Diet and challenge effects on the concentration of each hemocyte type (granulocytes, plasmatocytes, spherulocytes and oenocytoids). Significant tests (p-value $\leqslant 0.05$) are shown in bold.

Hemocyte type		χ^2	Df	p-Value
Granulocytes	Diet	**4.45**	**3; 94**	**0.006**
	Challenge	**29.90**	**1; 92**	**<0.0001**
	Diet * Challenge	2.17	3; 92	0.10
Plasmatocytes	Diet	1.46	3; 94	0.23
	Challenge	0.19	1; 92	0.66
	Diet * Challenge	**3.59**	**3; 92**	**0.02**
Spherulocytes	Diet	**19.66**	**3; 94**	**<0.0001**
	Challenge	1.48	1; 92	0.23
	Diet * Challenge	**3.72**	**3; 92**	**0.01**
Oenocytoids	Diet	1.81	3; 94	0.15
	Challenge	0.73	1; 92	0.40
	Diet * Challenge	**2.92**	**3; 92**	**0.04**

from the control diet than the others diets. The control diet was the most favorable for larvae survival (Vogelweith et al., 2011). However, larvae reared on Gewürztraminer could have a higher basal level of hemocytes due to the compounds such as phenolic substances found in grape varieties rather than microbes growing in berries (Conde et al., 2007; Vogelweith et al., 2015). Indeed, in a previous study, we found that controlling microbe abundance in diets did not explain variation in the immune function whereas the presence of berry extracts did (Vogelweith et al., 2015). This suggests that variation in immune defenses of *E. ambiguella* among grape varieties was caused by nutritional differences among host plants rather than microbe abundance.

In agreement with the results of Kim and Kim (2010), we found that GR concentration increased after the immune challenge in all diets, thus confirming their important and multiple roles in insect immunity. Additional GR could have two origins. First, GR could be produced by hematopoietic organs and/or derived from other hemocytes already in circulation (Nakahara et al., 2003; Nardi, 2004). Second, the immune challenge may induce the mobilization of already existing sessile GR attached to different parts of the insect body (Markus et al., 2009; King and Hillyer 2013). In our data, we are unable to distinguish between these hypotheses. Only complementary studies such as the one by Markus et al. (2009) on *Drosophila melanogaster* could explain the origin of these additional hemocytes. The mechanism by which the concentration of hemocyte is increased upon challenge may have its importance because different mechanisms might be associated with different costs. Indeed, the costs associated with the production of new hemocytes would likely be higher than the costs of the mobilization of already existing hemocytes.

PL only increased in the control diet and decreased in Gewürztraminer. However, both GR and PL act together in pathogen elimination (Lavine and Strand, 2002). As mentioned earlier, our control diet is expected to be more nutritive than those made of grapes (Vogelweith et al., 2011). Larvae reared on the control diet were probably in much better condition which might have enable them to rapidly produce/recruit more hemocytes in response to the immune challenge. Larvae reared on Gewürztraminer exhibited high basal levels of PL that decreased after the immune challenge to reach that of larvae reared on the control diet. Such depletion might be caused by the intensive use of PL during the immune response that could not be renewed.

OE and SP were the least abundant among the hemocytes (<5% of the total). OE are responsible for the synthesis and release of the PO enzyme (Kanost et al., 2004), and may be involved in clotting (Eleftherianos and Revenis, 2011; Lavine and Strand, 2002). The basal OE concentration was not affected by the larval diet, contrary to that of SP. The role of SP is poorly known. Schmitz et al. (2012) have shown that SP are involved in the coagulation process quickly after hemolymph collection. They also suggest that SP participate in energy storage and lipid transport (Schmitz et al., 2012). The effect of diet on SP concentration is the opposite of that on GR concentration. SP are more numerous in larvae reared on Chasselas than in those reared on Chardonnay. In a previous study, we suggested that variation in immune defenses of *E. ambiguella* among grape varieties is caused by nutritional differences among host plants (Vogelweith et al., 2015). Further investigations are needed to determine which compounds of these plant varieties could affect hemocyte types. Following the immune challenge, both OE and SP (such as GR and PL) increased in the control diet. The larvae reared on this diet possess a very low basal level of hemocytes but they are the most able to mobilize or synthesize hemocytes after the immune challenge.

In conclusion, we described four types of hemocyte in *E. ambiguella* at the 5th larval instar stage. The concentration of each type of hemocytes was affected by larval diet and an immune challenge. We suggest that larvae can display differential resistance to natural enemies depending on their relative investment in the proportion of each hemocyte type. This result would however need to be confirmed on larvae reared on different grape varieties in natural populations. We predict that larvae having a high proportion of GR will best resist bacteria and parasitoids. Such a finding would be important in the development of future biological control programs focused on the use of parasitoids or entomopathogenic microorganisms.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2016.02.010.

References

Ayres, J.S., Schneider, D.S., 2009. The role of anorexia in resistance and tolerance to infections in Drosophila. PLoS Biol. 7.

Babin, A., Biard, C., Moret, Y., 2010. Dietary supplementation with carotenoids improves immunity without increasing its cost in a crustacean. Am. Nat. 176, 234–241.

Baguley, T., 2012. Serious Stats: A Guide to Advanced Statistics for the Behavioral Sciences. Palgrave Macmillan, Palgrave.

Beaulaton, J., 1979. Hemocytes and hemocytopoiesis in silkworms. Biochimie 61, 157–164.

Carton, Y., Poirié, M., Nappi, A.J., 2008. Insect immune resistance to parasitoids. Insect Sci. 15, 67–87.

Conde, C., Silva, P., Fontes, N., Dias, A.C.P., Tavares, R.M., Sousa, M.J., Agasse, A., Delrot, S., Geros, H., 2007. Biochemical changes throughout grape berry development and fruit and wine quality. Food 1, 1–22.

Cotter, S.C., Simpson, S.J., Raubenheimer, D., Wilson, K., 2011. Macronutrient balance mediates trade-offs between immune function and life history traits. Funct. Ecol. 25, 186–198.

Delbac, L., Lecharpentier, P., Thiéry, D., 2010. Larval instars determination for the European Grapevine Moth (Lepidoptera: Tortricidae) based on the frequency distribution of head capsule widths. Crop Prot. 29, 623–630.

R Development Core Team, 2008. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. Available online at <http://www.R-project.org> (accessed 5 November 2014).

Dubuffet, A., Zanchi, C., Boutet, G., Moreau, J., Teixeira, M., Moret, Y., 2015. Trans-generational immune priming protects the eggs only against gram-positive bacteria in the mealworm beetle. PLoS Pathog. 11, e1005178.

Eleftherianos, I., Revenis, C., 2011. Role and importance of phenoloxidase in insect hemostasis. J. Innate Immun. 3, 28–33.

Eslin, P., Prevost, G., 1998. Hemocyte load and immune resistance to *Asobara tabida* are correlated in species of the *Drosophila melanogaster* subgroup. J. Insect Physiol. 44, 807–816.

Falleiros, A.M.F., Bombonato, M.T.S., Gregorio, E.A., 2003. Ultrastructural and quantitative studies of hemocytes in the sugarcane borer, *Diatraea saccharalis* (Lepidoptera: Pyralidae). Braz. Arch. Biol. Technol. 46, 287–294.

Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. Annu. Rev. Entomol. 42, 611–643.

Godin, J., Maltais, P., Gaudet, S., 2002. Head capsule width as an instar indicator for larvae of the cranberry fruitworm (Lepidoptera: Pyralidae) in southeastern New Brunswick. J. Econ. Entomol. 95, 1308–1313.

Han, S.-S., Lee, M.-H., Kim, W.-K., Wago, H., Yoe, S.-M., 1998. Hemocytic differentiation in hemopoietic organ of *Bombyx mori* larvae. Zool. Sci. 15, 371–379.

Kanost, M.R., Jiang, H.B., Yu, X.Q., 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol. Rev. 198, 97–105.

Kapari, L., Haukioja, E., Rantala, M.J., Ruuhola, T., 2006. Defoliating insect immune defense interacts with induced plant defense during a population outbreak. Ecology 87, 291–296.

Kim, G.S., Kim, Y., 2010. Up-regulation of circulating hemocyte population in response to bacterial challenge is mediated by octopamine and 5-hydroxytryptamine via Rac1 signal in *Spodoptera exigua*. J. Insect Physiol. 56, 559–566.

King, J.G., Hillyer, J.F., 2013. Spatial and temporal in vivo analysis of circulating and sessile immune cells in mosquitoes: hemocyte mitosis following infection. BMC Biol. 11, 55.

Klowden, M.J., 2002. Physiological Systems in Insects. Elsevier Academic Press, New York.

Lanot, R., Zachary, D., Holder, F., Meister, M., 2001. Postembryonic hematopoiesis in Drosophila. Dev. Biol. 230, 243–257.

Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32, 1295–1309.

Lazzaro, B.P., Little, T.J., 2009. Immunity in a variable world. Philos. Trans. R. Soc. B Biol. Sci. 364, 15–26.

Ling, E., Shirai, K., Kanekatsu, R., Kiguchi, K., 2005. Hemocyte differentiation in the hematopoietic organs of the silkworm, *Bombyx mori*: prohemocytes have the function of phagocytosis. Cell Tissue Res. 320, 535–543.

Markus, R., Laurinyecz, B., Kurucz, E., Honti, V., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., Ando, I., 2009. Sessile hemocytes as a hematopoietic compartment in *Drosophila melanogaster*. PNAS 106 (12), 4805–4809.

Marmaras, V.J., Lampropoulou, M., 2009. Regulators and signalling in insect haemocyte immunity. Cell. Signal. 21, 186–195.

Moreau, J., Arruego, X., Benrey, B., Thiery, D., 2006a. Differences in nutritional quality of parts of *Vitis vinifera* berries affect fitness of the European grapevine moth. Entomol. Exp. Appl. 119.

Moreau, J., Benrey, B., Thiery, D., 2006b. Assessing larval food quality for phytophagous insects: are the facts as simple as they appear? Funct. Ecol. 20, 592–600.

Moreau, J., Villemant, C., Benrey, B., Thiery, D., 2010. Species diversity of larval parasitoids of the European grapevine moth (*Lobesia botrana*, Lepidoptera: Tortricidae): the influence of region and cultivar. Biol. Control 54, 300–306.

Nakahara, Y., Kanamori, Y., Kiuchi, M., Kamimura, M., 2003. In vitro studies of hematopoiesis in the silkworm: cell proliferation in and hemocyte discharge from the hematopoietic organ. J. Insect Physiol. 49, 907–916.

Nakahara, Y., Kanamori, Y., Kiuchi, M., Kamimura, M., 2010. Two hemocyte lineages exist in silkworm larval hematopoietic organ. PLoS One 5.

Nardi, J.B., 2004. Embryonic origins of the two main classes of hemocytes – granular cells and plasmatocytes – in *Manduca sexta*. Dev. Genes Evol. 214, 19–28.

Panzavolta, T., 2007. Instar determination for *Pissodes castaneus* (Coleoptera: Curculionidae) using head capsule widths and lengths. Environ. Entomol. 36, 1054–1058.

Pletcher, S.D., Macdonald, S.J., Marguerie, R., Certa, U., Stearns, S.C., Goldstein, D.B., Partridge, L., 2002. Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. Curr. Biol. 12, 712–723.

Ponton, F., Wilson, K., Cotter, S.C., Raubenheimer, D., Simpson, S.J., 2011. Nutritional immunology: a multi-dimensional approach. PLoS Pathog. 7.

Povey, S., Cotter, S.C., Simpson, S.J., Lee, K.P., Wilson, K., 2009. Can the protein costs of bacterial resistance be offset by altered feeding behaviour? J. Anim. Ecol. 78, 437–446.

Ribeiro, C., Brehelin, M., 2006. Insect haemocytes: What type of cell is that? J. Insect Physiol. 52, 417–429.

Sadd, B.M., Schmid-Hempel, P., 2007. Facultative but persistent transgenerational immunity via the mother's eggs in bumblebees. Curr. Biol. 17, 1046–1047.

Schmitz, A., Anselme, C., Ravallec, M., Rebuf, C., Simon, J.-C., Gatti, J.-L., Poirié, M., 2012. The cellular immune response of the Pea Aphid to foreign intrusion and symbiotic challenge. PLoS One 7, e42114.

Shikano, I., Ericsson, J.D., Cory, J.S., Myers, J.H., 2010. Indirect plant-mediated effects on insect immunity and disease resistance in a tritrophic system. Basic Appl. Ecol. 11, 15–22.

Siva-Jothy, M.T., Thompson, J.J.W., 2002. Short-term nutrient deprivation affects immune function. Physiol. Entomol. 27, 206–212.

Stoepler, T.M., Castillo, J.C., Lill, J.T., Eleftherianos, I., 2013. Hemocyte density increases with developmental stage in an immune-challenged forest caterpillar. PLoS One 8.

Strand, M.R., 2008. The insect cellular immune response. Insect Sci. 15, 1–14.

Strand, M.R., Pech, L.L., 1995. Immunological basis for compatibility in parasitoid host relationships. Annu. Rev. Entomol. 40, 31–56.

Tan, J., Xu, M., Zhang, K., Wang, X., Chen, S.Y., Li, T., Xiang, Z.H., Cui, H.J., 2013. Characterization of hemocytes proliferation in larval silkworm, *Bombyx mori*. J. Insect Physiol. 59, 595–603.

Thiery, D., Moreau, J., 2005. Relative performance of European grapevine moth (*Lobesia botrana*) on grapes and other hosts. Oecologia 143, 548–557.

Vogelweith, F., Thiery, D., Quaglietti, B., Moret, Y., Moreau, J., 2011. Host plant variation plastically impacts different traits of the immune system of a phytophagous insect. Funct. Ecol. 25, 1241–1247.

Vogelweith, F., Dourneau, M., Thiery, D., Moret, Y., Moreau, J., 2013a. Geographical variation in parasitism shapes larval immune function in a phytophagous insect. Naturwissenschaften 100, 1149–1161.

Vogelweith, F., Thiery, D., Moret, Y., Moreau, J., 2013b. Immunocompetence increases with larval body size in a phytophagous moth. Physiol. Entomol. 38, 219–225.

Vogelweith, F., Thiery, D., Moret, Y., Colin, E., Motreuil, S., Moreau, J., 2014. Defense strategies used by two sympatric vineyard moth pests. J. Insect Physiol. 64, 54–61.

Vogelweith, F., Moreau, J., Thiéry, D., Moret, Y., 2015. Food-mediated modulation of immunity in a phytophagous insect: an effect of nutrition rather than parasitic contamination. J. Insect Physiol. 77, 55–61.

Yang, S.Y., Ruuhola, T., Haviola, S., Rantala, M.J., 2008. Effects of host-plant shift on immune and other key life-history traits of an eruptive Geometrid, *Epirrita autumnata* (Borkhausen). Ecol. Entomol. 33, 510–516.