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### ► To cite this version:

Fanny Vogelweith, Yannick Moret, Karine Monceau, Denis Thiéry, Jérôme Moreau. The relative abundance of hemocyte types in a polyphagous moth larva depends on diet.. *Journal of Insect Physiology*, 2016, 88, pp.33-39. 10.1016/j.jinsphys.2016.02.010 . hal-01283005

**HAL Id: hal-01283005**

**<https://hal.science/hal-01283005>**

Submitted on 24 Feb 2024

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1       The relative abundance of hemocyte types in a polyphagous moth  
2  
3   larva depends on diet

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21  
22 **Word count:** 5476 words (summary + text + references + figure caption) + 1 table + 3  
23 figures.

24

25 **Abstract**

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

42

43 **Key words:** ecological immunity, *Eupoecilia ambiguella*, grape varieties, hemocyte  
44 differentiation, Lepidoptera, Tortricidae.

45

## 46 **Introduction**

47           In insect immunity, core processes such as coagulation, nodulation, phagocytosis and  
48 encapsulation are strongly associated with hemocytes (Lavine and Strand, 2002). They  
49 recognize and destroy invading pathogens and apoptotic cells during phagocytosis, and  
50 aggregate large numbers of bacteria during the nodulation process. They also bind to larger  
51 targets including eggs of parasitoids, nematodes and protozoa by forming a multilayer capsule  
52 around the intruder (so-called encapsulation; Marmaras and Lampropoulou, 2009).  
53 Hemocytes circulate freely in the hemocoel, and are produced either by hematopoietic organs  
54 or through cell division (Gillespie et al., 1997; Lavine and Strand, 2002; Strand, 2008; Tan et  
55 al., 2013). They differentiate into cell types that have various functions in pathogen defense  
56 (Lavine and Strand, 2002), and have been classified according to their morphology, function  
57 and certain molecular biomarkers. In Lepidopteran caterpillars, including *Bombyx mori*, five  
58 types of hemocytes have been described (Ling et al., 2005; Strand, 2008; Tan et al., 2013):  
59 prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids. Prohemocytes  
60 are the precursor of the other types; they are produced and reside primarily in hematopoietic  
61 organs, and thus rarely circulate in the hemocoel (Lanot et al., 2001). Granulocytes (GR) are  
62 usually the most abundant type in the hemolymph; they adhere strongly to the surface of  
63 foreign bodies, function as phagocytes, and are involved in encapsulation (Strand, 2008).  
64 Plasmatocytes (PL) are polymorphic in shape; they spread asymmetrically on foreign  
65 surfaces, and participate in capsule-formation during the encapsulation reaction (Strand,  
66 2008). In contrast, spherulocytes (SP) are non-adhesive hemocytes and their role in insect  
67 immunity is still unknown, although it has been suggested that they are involved in the  
68 transport of cuticular components (Lavine and Strand, 2002). Oenocytoids (OE) are non-  
69 adhesive hemocytes that contain the main components of the phenoloxidase (PO) cascade

70 (Lavine and Strand, 2002). The proportion of each hemocyte type can vary according to  
71 numerous factors including nutrition.

72 Nutrition is now recognized as a critical factor in immune defense and resistance to  
73 pathogens (Lazzaro and Little, 2009; Ponton et al., 2011; Vogelweith et al., 2013a).  
74 Experimental studies of insects have demonstrated that food deprivation affects immune  
75 responsiveness (Ayres and Schneider, 2009; Kapari et al., 2006; Siva-Jothy and Thompson,  
76 2002; Yang et al., 2008) and changes in the expression of several immunity genes (Pletcher et  
77 al., 2002). Immune effectors can also be affected by changes in food composition (Babin et  
78 al., 2010; Cotter et al., 2011; Povey et al., 2009; Vogelweith et al., 2011). For example,  
79 Shikano et al. (2010) showed that *Trichoplusia ni* larvae reared on broccoli had more  
80 hemocytes than those reared on cucumber. Surprisingly, the influence of diet on the  
81 concentration of the different hemocyte types remains unclear. This issue is relevant since  
82 hemocyte types have different roles in immunity, and nutrition-dependent variation in their  
83 relative abundance might thus affect the immune response of phytophagous larvae. Indeed,  
84 Klowden (2002) suggested that GR are also involved in nutrient transport. Thus, it could be  
85 hypothesized that food deprivation or poor quality food induces a nutritive stress, reducing the  
86 proportion of GR hemocytes and affecting the encapsulation process.

87 Tortricid moths, including *Lobesia botrana* and *Eupoecilia ambiguella*, are the most  
88 harmful pests of grapes in Europe and North America. The larvae are polyphagous, and can  
89 develop on almost any grape variety and more than 25 other host plants (Thiery and Moreau,  
90 2005). Natural populations of tortricid moths are the target of numerous natural enemies and  
91 face variable changes in attacks by natural enemies, in both time and space (Moreau et al.,  
92 2010). For example, biological control methods use both entomopathogenic microorganisms  
93 and parasitoids to control moth populations. However, the success of the attacks depends on  
94 the ability of the pest to defend itself mainly via its immune system. Particularly, hemocytes

95 have been shown to be an important parameter of the insect immune system because of their  
96 involvement in encapsulation (Eslin and Prevost, 1998; Carton et al., 2008). However, not all  
97 hemocyte types have the same role in immunity (Lavine and Strand, 2002) and not all types  
98 fight against the same enemies. *E. ambiguella* can grow on different host plants that affect its  
99 immune parameters (Vogelweith et al., 2011). For this reason, we considered it important to  
100 characterize the hemocyte types and investigate how their relative abundance varies among  
101 host plants to measure the effect of host plant on hemocyte type. This would be important if  
102 larvae can display differential resistance to natural enemies depending on their relative  
103 investment in the proportion of each hemocyte type.

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

110

## 111 **Materials and methods**

### 112 *a) Insect rearing*

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

120 11.5 × 7cm) on a semi-artificial diet (described in Vogelweith et al., 2011) at a density of ca.  
121 100 individuals per 300 ml of diet.

122

### 123 ***b) Artificial diets***

124 Four experimental diets were prepared following the method described by Vogelweith et al.  
125 (2011). Specifically, a rearing diet without added berries was used as the control, and three  
126 test diets were prepared using berries from a different grape variety, respectively:  
127 ‘Chardonnay’, ‘Chasselas’ and ‘Gewürztraminer’. During the last week of July 2009,  
128 insecticide-free bunches of berries for the diets were collected at the pre-veraison stage from  
129 the gene collection of grape plants ‘Domaine de la Grande Ferrade’, INRA-Bordeaux  
130 Aquitaine; this stage corresponds to the grape phenology on which the second annual  
131 generation of *E. ambiguella* occurs.

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

137

### 138 ***c) Collection of hemolymph and immune challenge***

139 Hemocytes were extracted from 5<sup>th</sup> instar larvae using the method described by Vogelweith et  
140 al. (2011; 2013a). Briefly, the larvae were anesthetized on ice for 20 min, and 1 µl of  
141 hemolymph was then collected and flushed into a micro-centrifuge tube containing 20 µl of  
142 sodium cacodylate/CaCl<sub>2</sub> buffer (0.01M sodium cacodylate, 0.005M CaCl<sub>2</sub>; pH 6.5). This  
143 sample was used to measure the total concentration of hemocytes, and to characterize the  
144 different hemocyte types as well as their relative concentration (see section d. and results).

145 Following this first hemolymph collection, larvae were immune challenged in the posterior  
146 part of the ventral side of the abdomen with a sterile needle dipped in a concentrated  
147 suspension of heat-killed *Arthrobacter globiformis* (ca.  $10^9$  cells ml<sup>-1</sup>). This bacterium is  
148 commonly used in the protocol testing of antimicrobial activity (Sadd and Schmid-Hempel,  
149 2007; Vogelweith et al., 2011, 2013a, 2015), as it is very sensitive to antimicrobial peptides of  
150 insects (Dubuffet et al., 2015). After the immune challenge, larvae were kept individually in  
151 micro-centrifuge tubes for 24 h under standard conditions before a second sample of  
152 hemolymph was collected (Vogelweith et al., 2011, 2013b, 2014). The delay between  
153 collecting the two hemolymph samples was determined based on preliminary experiments  
154 showing that the concentration of hemocytes stabilizes 24 hours after the immune challenge at  
155 the earliest (Supplementary material 1). This second sample of hemolymph allowed the  
156 concentration of each hemocyte type and the total concentration of hemocytes after an  
157 immune challenge to be measured. All hemolymph samples were assessed immediately to  
158 avoid coagulation and desiccation of the hemocytes.

159 We noted that the immune challenge induced mortality (5-10%) equally distributed among  
160 diets. We tested 23 larvae reared on Chardonnay, 23 on Chasselas, 28 on Gewürztraminer and  
161 22 on the Control diet.

162

#### 163 ***d) Hemocyte concentration and characterization***

164 Hemocyte concentration was estimated using an improved Neubauer hemocytometer counting  
165 chamber and phase contrast microscopy (magnification 400×). Every sample of hemolymph  
166 was counted twice, once for the total number of hemocytes and once for the characterization  
167 and number of each hemocyte type.

168 Hemocyte characterization was performed using the hemolymph sample that had been taken  
169 before the immune challenge. Hemocyte types were characterized based on their previously



170 reported morphological differences (Beaulaton, 1979; Falleiros et al., 2003; Ribeiro and  
171 Brehelin, 2006). Accordingly, we considered the cell shape (round, oval, deformed) and size  
172 (big, small), the size of the nucleus and its position in the cell (in the middle or offset of the  
173 cell), and the cytoplasm compounds (presence/absence of granules and spherules, transparent  
174 or opaque).

175

#### 176 *e) Larval body size*

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

182

#### 183 *f) Data analysis*

184 The effect of diets on the total number of hemocytes and on the concentration of each  
185 hemocyte type before the immune challenge was tested using Kruskal-Wallis tests because  
186 the data did not meet the assumptions of normality and homoscedasticity for parametric tests  
187 (tested using with Shapiro-Wilk and Levene tests respectively). Kruskal-Wallis multiple  
188 comparisons post hoc tests (MC) were used when needed to test differences among diets. The  
189 effect of both diet and immune challenge on the total concentration of hemocytes and the  
190 concentration of each hemocyte type (arcsine square root transformed) were analyzed using a  
191 linear mixed effects model that included individual identity as a random factor within the diet  
192 effect. The statistical significance of the effects of diet and immune challenge were tested  
193 using F-ratio statistics. When the data did not meet the conditions of normality and

194 homoscedasticity, linear mixed effect models based on rank transformation were used instead  
195 of the classical non-parametric Friedman tests (Baguley, 2012).

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

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

203

### 204 **3. Results**

#### 205 *a) Hemocyte types and morphology*

206 Fifth instar larvae of *E. ambiguella* contained four types of hemocytes that were easily  
207 differentiated: granulocytes (GR), plasmatocytes (PL), oenocytoids (OE) and spherulocytes  
208 (SP) (Fig. 1). The presence of granules makes GR easy to differentiate from the other types;  
209 the GR cells appear as round cells with a small and round nucleus (Fig. 1a, e). PL were also  
210 round cells of variable size (Fig. 1c, f), in which the nucleus was either round or bi-lobed, and  
211 was relatively large in the cell. SP were also easy to recognize because of their inclusions and  
212 spherules, which occupied most of the cytoplasm and deformed the cell and the nucleus (Fig.  
213 1d, h). OE had a dense and opaque cytoplasm with a small overhanging nucleus (Fig. 1c, g).

214

#### 215 *b) Diet effect on the total concentration of hemocytes before the immune challenge*

216 The total concentration of hemocytes was influenced by the larval diet (Wilcoxon test,  $\chi^2_3 =$   
217 11.08;  $p = 0.01$ ) (Fig. 2, before challenge). Larvae reared on Gewürztraminer had more  
218 hemocytes than larvae reared on the control diet. There was no difference between larvae

219 reared on Chardonnay and Chasselas, or between any other combinations of diets (Fig. 2,  
220 before immune challenge).

221

222 ***c) Diet effect on each hemocytes type before the immune challenge***

223 Irrespective of the diet, GR were the most abundant cells in the hemolymph with an average  
224 number of 13603 hemocytes/ $\mu\text{l}$  (95% CI = [11906; 15300], accounting for 67% of the  
225 hemocytes (Fig. 3, before immune challenge). PL were the second most abundant hemocytes  
226 with an average number of 5955 hemocytes/ $\mu\text{l}$  (95% CI = [5115; 6795], accounting for 29%.  
227 SP (mean = 518 hemocytes/ $\mu\text{l}$ ; 95% CI = [384; 652]) and OE (mean = 136 hemocytes/ $\mu\text{l}$  ;  
228 95% CI = [102; 170]) were much less abundant, accounting for 3% and 1% of the hemocytes,  
229 respectively.

230 Larval diet affected the concentrations of GR ( $\chi^2_3 = 12.83$ ;  $p = 0.005$ ), PL ( $\chi^2_3 = 8.86$ ;  $p =$   
231  $0.03$ ) and SP ( $\chi^2_3 = 23.64$ ;  $p < 0.0001$ ) but not that of OE ( $\chi^2_3 = 1.05$ ;  $p = 0.79$ ) (Fig. 3, before  
232 immune challenge). The concentration of GR was lower in larvae reared on the control diet  
233 than in those reared on the Gewürztraminer and Chardonnay diets (Fig. 3a, before immune  
234 challenge). Larvae reared on the control diet also had less PL than those reared on  
235 Gewürztraminer (Fig. 3b, before immune challenge). Except for larvae reared on  
236 Gewürztraminer and control diets which did not differ, all larvae reared on the other diets had  
237 different concentration of SP (Fig. 3c, before immune challenge).

238

239 ***d) Diet and immune challenge effect on the total concentration of hemocytes and on***  
240 ***the concentration of each hemocyte types***

241 The total concentration of hemocytes after the immune challenge was only affected by that  
242 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  
243 interaction between challenge and diet ( $F_{3,110} = 2.62$  ;  $p = 0.06$ ). Irrespective of the diet, the

244 total concentration of hemocytes increased after the immune challenge (Fig. 2, before and  
245 after immune challenge).

246 The immune challenge affected the concentration of all hemocyte types, either alone, or in an  
247 interaction with the diet (Table 1; Fig. 3). Specifically, the concentration of GR in the  
248 hemolymph of larvae increased irrespective of the diet after the immune challenge (MC:  $p <$   
249  $0.0001$ ; Fig. 3a). In contrast, the changes in the concentration of PL, SP and OE caused by the  
250 immune challenge depended on the diet (Table 1; Fig. 3b,c,d). The concentration of PL  
251 decreased after the immune challenge in larvae reared on Gewürztraminer (MC:  $p = 0.01$ ) and  
252 increased in larvae reared on the control diet (MC:  $p = 0.04$ ) (Fig. 3b). The concentration of  
253 PL did, however, not change in larvae reared on Chardonnay and Chasselas (MC:  $p = 0.96$   
254 and  $p = 0.73$ , respectively). The concentration of OE and SP increased after the immune  
255 challenge in larvae reared on the control diet (MC, OE:  $p = 0.006$ ; SP:  $p = 0.0006$ ; Fig 3c,d),  
256 but did not change in larvae reared on Chasselas, Chardonnay or Gewürztraminer (MC, OE,  
257 Chardonnay:  $p = 0.16$ ; Chasselas:  $p = 0.85$ ; Gewürztraminer:  $p = 0.79$  and SP, Chardonnay:  $p$   
258  $= 0.79$ ; Chasselas:  $p = 0.43$ ; Gewürztraminer:  $p = 0.79$ , Fig. 3c,d).

259

#### 260 **4. Discussion**

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

266 Consistent with other studies in lepidopteran species (Falleiros et al., 2003; Lavine and  
267 Strand, 2002; Ling et al., 2005; Ribeiro and Brehelin, 2006), we identified granulocytes,  
268 oenocytoids, plasmatocytes and spherulocytes hemocytes based on their morphological

269 features. However, we did not detect prohemocytes (PR), although these have been reported  
270 in larvae of other lepidopteran species (Falleiros et al., 2003). We tested only mature larvae at  
271 the end of the larval stage. Falleiros et al. (2003) reported that the proportion of PR decreases  
272 with larval age until it reaches zero, which may explain why we found no PR in 5<sup>th</sup> instar  
273 larvae of *E. ambiguella*.

274 As in other species of Lepidoptera, including *Diatraea saccharalis* (Falleiros et al.,  
275 2003), *Euclea delphinii* and *Lithacodes fasciola* (Stoepler et al., 2013), GR and PL were the  
276 most abundant circulating hemocytes in *E. ambiguella*. GR are involved in the recognition of  
277 foreign bodies, phagocytosis, encapsulation and nodulation (Lavine and Strand, 2002;  
278 Nakahara et al., 2010; Ribeiro and Brehelin, 2006; Strand and Pech, 1995). However, their  
279 role in immune processes also depends on the insect species examined (Falleiros et al., 2003).  
280 The high concentration of PL may possibly be due to their involvement in numerous  
281 processes including encapsulation and shaping of the melanotic capsule (Lavine and Strand,  
282 2002; Nakahara et al., 2010; Ribeiro and Brehelin, 2006; Strand and Pech, 1995), and their  
283 production of soluble pattern recognition and antimicrobial proteins (Nakahara et al., 2010).  
284 GR and PL act together in defense against macro-pathogens (e.g. parasitoid eggs) during  
285 encapsulation (Lavine and Strand, 2002; Nakahara et al., 2010; Ribeiro and Brehelin, 2006;  
286 Strand and Pech, 1995). Both the concentrations of GR and PL are higher than those of OE  
287 and SP, certainly because they can differentiate in OE and SP (Beaulaton, 1979; Han et al.,  
288 1998).

289 GR and PL concentrations were both higher in larvae reared on Gewürztraminer than  
290 larvae from others diets and lower in larvae from the control diet than the others diets. The  
291 control diet was the most favorable for larvae survival (Vogelweith et al., 2011). However,  
292 larvae reared on Gewürztraminer could have a higher basal level of hemocytes due to the  
293 compounds such as phenolic substances found in grape varieties rather than microbes growing

294 in berries (Conde et al., 2007; Vogelweith et al., 2015). Indeed, in a previous study, we found  
295 that controlling microbe abundance in diets did not explain variation in the immune function  
296 whereas the presence of berry extracts did (Vogelweith et al., 2015). This suggests that  
297 variation in immune defenses of *E. ambiguella* among grape varieties was caused by  
298 nutritional differences among host plants rather than microbe abundance.

299 In agreement with the results of Kim and Kim (2010), we found that GR concentration  
300 increased after the immune challenge in all diets, thus confirming their important and multiple  
301 roles in insect immunity. Additional GR could have two origins. First, GR could be produced  
302 by hematopoietic organs and/or derived from other hemocytes already in circulation  
303 (Nakahara et al., 2003; Nardi, 2004). Second, the immune challenge may induce the  
304 mobilization of already existing sessile GR attached to different parts of the insect body  
305 (Markus et al., 2009; King and Hillyer 2013). In our data, we are unable to distinguish  
306 between these hypotheses. Only complementary studies such as the one by Markus et al.  
307 (2009) on *Drosophila melanogaster* could explain the origin of these additional hemocytes.  
308 The mechanism by which the concentration of hemocyte is increased upon challenge may  
309 have its importance because different mechanisms might be associated with different costs.  
310 Indeed, the costs associated with the production of new hemocytes would likely be higher  
311 than the costs of the mobilization of already existing hemocytes.

312 PL only increased in the control diet and decreased in Gewürztraminer. However, both GR  
313 and PL act together in pathogen elimination (Lavine and Strand, 2002). As mentioned earlier,  
314 our control diet is expected to be more nutritive than those made of grapes (Vogelweith et al.,  
315 2011). Larvae reared on the control diet were probably in much better condition which might  
316 have enable them to rapidly produce/recruit more hemocytes in response to the immune  
317 challenge. Larvae reared on Gewürztraminer exhibited high basal levels of PL that decreased  
318 after the immune challenge to reach that of larvae reared on the control diet. Such depletion

319 might be caused by the intensive use of PL during the immune response that could not be  
320 renewed.

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

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

343 biological control programs focused on the use of parasitoids or entomopathogenic  
344 microorganisms.

345

### 346 **Acknowledgments**

347 We thank Lionel Delbac and Bastien Quaglietti for their technical assistance, Juliette  
348 Poidatz for drawings, and Maximilian Körner and Jos Kramer for proof-readings. This study  
349 was supported by the CNRS and grants from the ANR (ANR-07-JCJC-0134 and ANR-08-  
350 JCJC-0006). The fourth author's research is part of the Labex Cote research programme.  
351 The authors declare no competing financial interests.

352

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481

482 **Figure legends**

483

484 Figure 1. Photographs and annotated drawings of the hemocyte types: granulocytes (GR; a,e),  
485 plasmatocytes (PL; b,f), oenocytoids (OE; c,g), spherulocytes (SP; d,h). In the annotated  
486 drawing 'C' indicates the cytoplasm, 'G' indicates the granules, 'M' indicates the membrane,  
487 'N' indicates the nuclei and 'S' indicates the spherules.

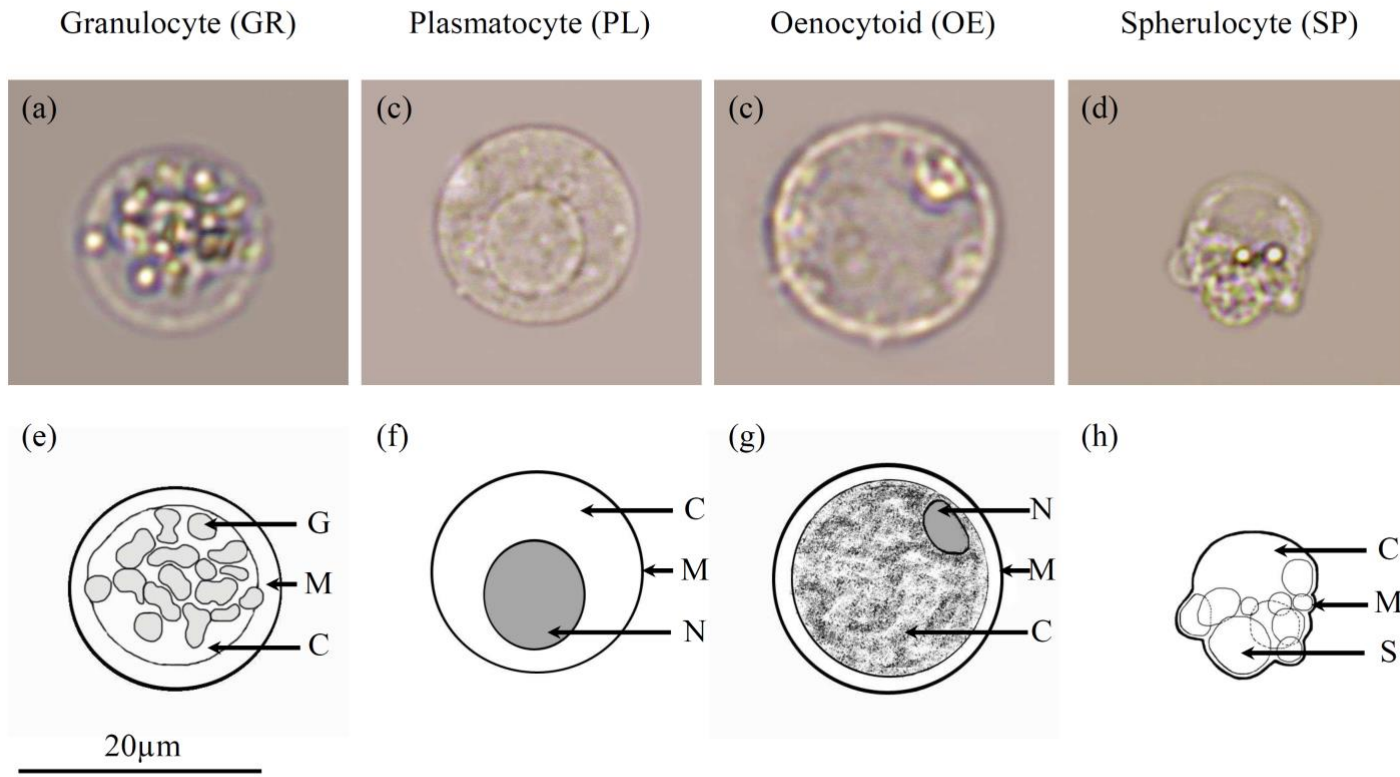
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489 Figure 2. Variation among diets in the total concentration of hemocytes prior to and  
490 following an immune challenge. Means and variation are given for larvae reared on diets  
491 containing Chardonnay (crosses; dotted lines), Chasselas (black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

494

495 Figure 3. Variation in the concentration of granulocytes (a), plasmatocytes (b), spherulocytes  
496 (c) and oenocytoids (d) ( $\pm$  standard error) prior to and following an immune challenge  
497 between the diets. Means and variation are given for larvae reared on diets containing  
498 Chardonnay (crosses; dotted lines), Chasselas (black circles; solid lines) or Gewürztraminer  
499 (black squares; dotted-dashed lines) as well as for larvae reared on the control diet (black  
500 triangles; dashed lines), respectively.

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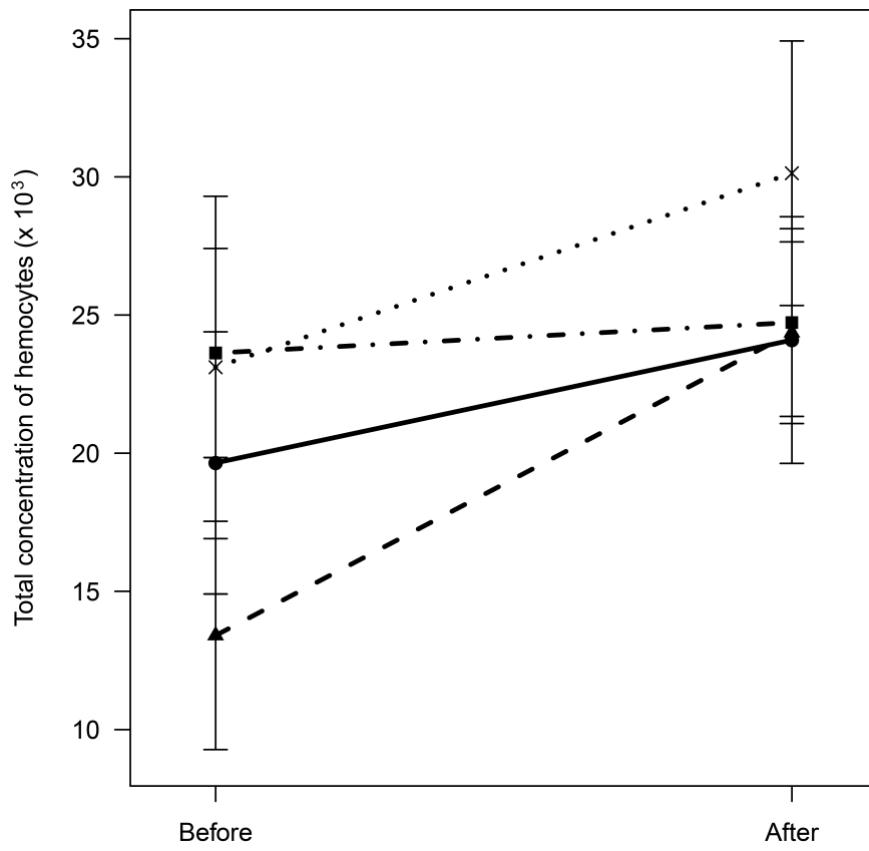
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Figure 1  
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Figure 2

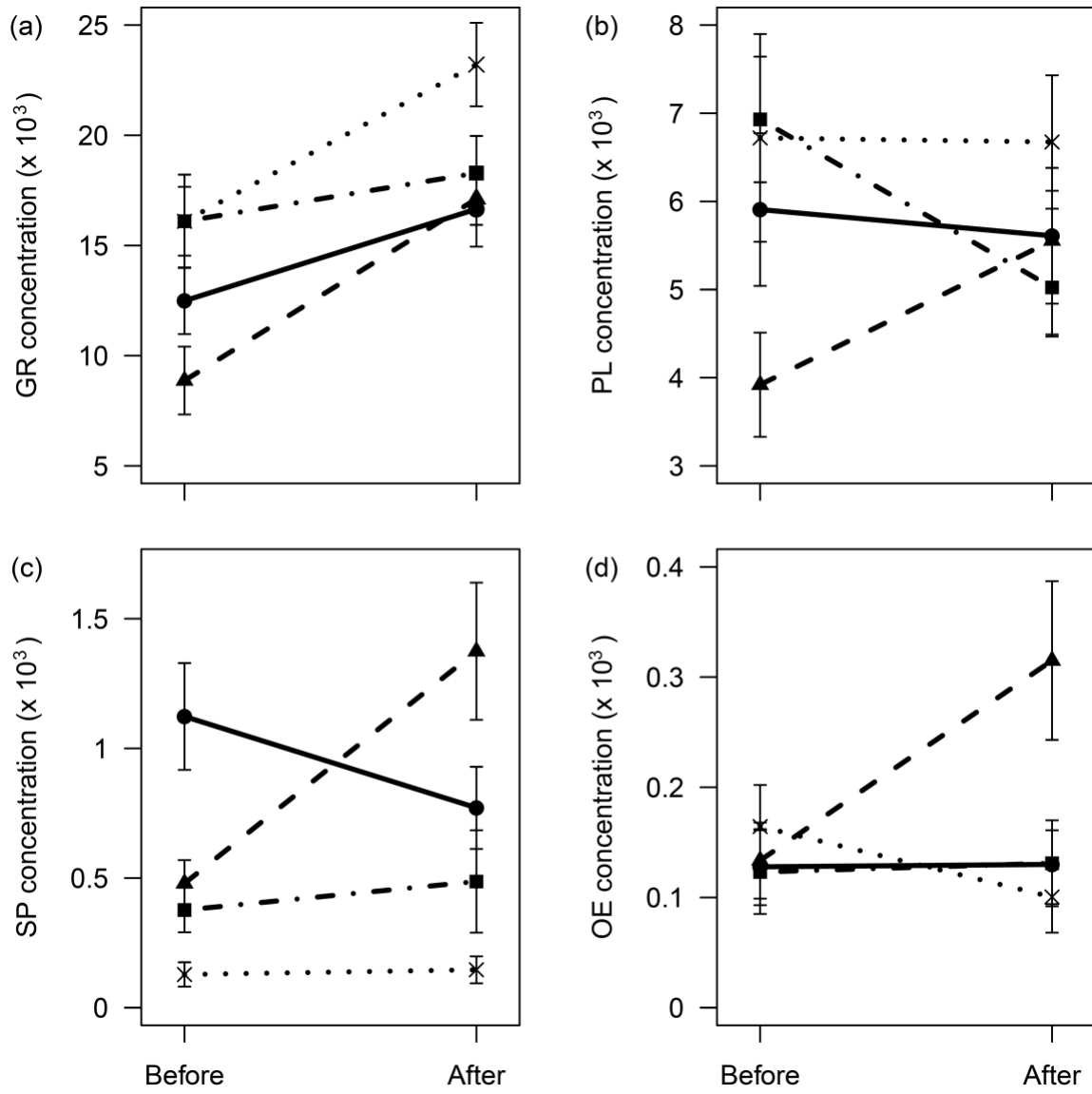
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Figure 3

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

Hemocyte type		$\chi^2$	Df	p-value
Granulocytes	Diet	<b>4.45</b>	<b>3; 94</b>	<b>0.006</b>
	Challenge	<b>29.90</b>	<b>1; 92</b>	<b>&lt; 0.0001</b>
	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	<b>3.59</b>	<b>3; 92</b>	<b>0.02</b>
Spherulocytes	Diet	<b>19.66</b>	<b>3; 94</b>	<b>&lt; 0.0001</b>
	Challenge	1.48	1; 92	0.23
	Diet * Challenge	<b>3.72</b>	<b>3; 92</b>	<b>0.01</b>
Oenocytoids	Diet	1.81	3; 94	0.15
	Challenge	0.73	1; 92	0.40
	Diet * Challenge	<b>2.92</b>	<b>3; 92</b>	<b>0.04</b>