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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 5th 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 5th 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 5th 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/CaCl₂ buffer (0.01M sodium cacodylate, 0.005M CaCl₂; 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⁻¹). 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/ μl (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/ μl (95% CI = [5115; 6795], accounting for 29%.
227 SP (mean = 518 hemocytes/ μl ; 95% CI = [384; 652]) and OE (mean = 136 hemocytes/ μl ;
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 5th 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 5th 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 5th 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

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352

353 **References**

- 354 Ayres, J.S., Schneider, D.S., 2009. The Role of Anorexia in Resistance and Tolerance to
355 Infections in *Drosophila*. *Plos Biology* 7.
- 356 Babin, A., Biard, C., Moret, Y., 2010. Dietary Supplementation with Carotenoids Improves
357 Immunity without Increasing Its Cost in a Crustacean. *American Naturalist* 176, 234-241.
- 358 Baguley, T., 2012. *Serious stats: A guide to advanced statistics for the behavioral sciences*.
359 Palgrave Macmillan, Palgrave.
- 360 Beaulaton, J., 1979. Hemocytes and hemocytogenesis in silkworms. *Biochimie* 61, 157-164.
- 361 Carton, Y., Poirié, M., Nappi, A.J., 2008. Insect immune resistance to parasitoids. *Insect*
362 *Science* 15, 67-87.
- 363 Conde, C., Silva, P., Fontes, N., Dias, A.C.P., Tavares, R.M., Sousa, M.J., Agasse, A., Delrot,
364 S., Geros, H., 2007. Biochemical changes throughout grape berry development and fruit
365 and wine quality. *Food* 1, 1-22.

366 Cotter, S.C., Simpson, S.J., Raubenheimer, D., Wilson, K., 2011. Macronutrient balance
367 mediates trade-offs between immune function and life history traits. *Functional Ecology*
368 25, 186-198.

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

372 Dubuffet, A., Zanchi, C., Boutet, G., Moreau, J., Teixeira, M., Moret, Y., 2015. Trans-
373 generational Immune Priming Protects the Eggs Only against Gram-Positive Bacteria in
374 the Mealworm Beetle. *PLoS Pathogens* 11, e1005178.

375 Eleftherianos, I., Revenis, C., 2011. Role and Importance of Phenoloxidase in Insect
376 Hemostasis. *Journal of Innate Immunity* 3, 28-33.

377 Eslin, P., Prevost, G., 1998. Hemocyte load and immune resistance to *Asobara tabida* are
378 correlated in species of the *Drosophila melanogaster* subgroup. *Journal of Insect Physiology*
379 44, 807-816.

380 Falleiros, A.M.F., Bombonato, M.T.S., Gregorio, E.A., 2003. Ultrastructural and quantitative
381 studies of hemocytes in the sugarcane borer, *Diatraea saccharalis* (Lepidoptera:
382 Pyralidae). *Brazilian Archives of Biology and Technology* 46, 287-294.

383 Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity.
384 *Annual Review of Entomology* 42, 611-643.

385 Godin, J., Maltais, P., Gaudet, S., 2002. Head capsule width as an instar indicator for larvae of
386 thecranberry fruitworm (Lepidoptera: Pyralidae) in southeastern New Brunswick. *Journal*
387 *of Economic Entomology* 95, 1308-1313.

388 Han, S.-S., Lee, M.-H., Kim, W.-K., Wago, H., Yoe, S.-M., 1998. Hemocytic differentiation
389 in hemopoietic organ of *Bombyx mori* larvae. *Zoological Science* 15, 371-379.

390 Kanost, M.R., Jiang, H.B., Yu, X.Q., 2004. Innate immune responses of a lepidopteran insect,
391 *Manduca sexta*. Immunological Reviews 198, 97-105.

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

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

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

399 Klowden, M. J., 2002. Physiological systems in insects. Elsevier Academic Press, New York.

400 Lanot, R., Zachary, D., Holder, F., Meister, M., 2001. Postembryonic hematopoiesis in
401 *Drosophila*. Developmental Biology 230, 243-257.

402 Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect
403 Biochemistry and Molecular Biology 32, 1295-1309.

404 Lazzaro, B.P., Little, T.J., 2009. Immunity in a variable world. Philosophical Transactions of
405 the Royal Society B-Biological Sciences 364, 15-26.

406 Ling, E., Shirai, K., Kanekatsu, R., Kiguchi, K., 2005. Hemocyte differentiation in the
407 hematopoietic organs of the silkworm, *Bombyx mori*: prohemocytes have the function of
408 phagocytosis. Cell and Tissue Research 320, 535-543.

409 Marmaras, V.J., Lampropoulou, M., 2009. Regulators and signalling in insect haemocyte
410 immunity. Cellular Signalling 21, 186-195.

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

414 Moreau, J., Arruego, X., Benrey, B., Thiery, D., 2006a. Differences in nutritional quality of
415 parts of *Vitis vinifera* berries affect fitness of the European grapevine moth. *Entomologia*
416 *Experimentalis Et Applicata* 119.

417 Moreau, J., Benrey, B., Thiery, D., 2006b. Assessing larval food quality for phytophagous
418 insects: are the facts as simple as they appear? *Functional Ecology* 20, 592-600.

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

422 Nakahara, Y., Kanamori, Y., Kiuchi, M., Kamimura, M., 2003. In vitro studies of
423 hematopoiesis in the silkworm: cell proliferation in and hemocyte discharge from the
424 hematopoietic organ. *Journal of Insect Physiology* 49, 907-916.

425 Nakahara, Y., Kanamori, Y., Kiuchi, M., Kamimura, M., 2010. Two Hemocyte Lineages
426 Exist in Silkworm Larval Hematopoietic Organ. *Plos One* 5.

427 Nardi, J.B., 2004. Embryonic origins of the two main classes of hemocytes - granular cells
428 and plasmatocytes - in *Manduca sexta*. *Development Genes and Evolution* 214, 19-28.

429 Panzavolta, T., 2007. Instar determination for *Pissodes castaneus* (Coleoptera: Curculionidae)
430 using head capsule widths and lengths. *Environmental Entomology* 36, 1054-1058.

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

434 Ponton, F., Wilson, K., Cotter, S.C., Raubenheimer, D., Simpson, S.J., 2011. Nutritional
435 Immunology: A Multi-Dimensional Approach. *PLoS Pathog.* 7.

436 Povey, S., Cotter, S.C., Simpson, S.J., Lee, K.P., Wilson, K., 2009. Can the protein costs of
437 bacterial resistance be offset by altered feeding behaviour? *Journal of Animal Ecology* 78,
438 437-446.

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

442 Ribeiro, C., Brehelin, M., 2006. Insect haemocytes: What type of cell is that? *Journal of*
443 *Insect Physiology* 52, 417-429.

444 Sadd, B.M. and Schmid-Hempel, P., 2007. Facultative but persistent transgenerational
445 immunity via the mother's eggs in bumblebees. *Current Biology* 17, 1046-1047.

446 Schmitz, A., Anselme, C., Ravallec, M., Rebuf, C., Simon, J.-C., Gatti, J.-L., Poirié, M.,
447 2012. The cellular immune response of the Pea Aphid to foreign intrusion and symbiotic
448 challenge. *Plos One* 7, e42114.

449 Shikano, I., Ericsson, J.D., Cory, J.S., Myers, J.H., 2010. Indirect plant-mediated effects on
450 insect immunity and disease resistance in a tritrophic system. *Basic and Applied Ecology*
451 11, 15-22.

452 Siva-Jothy, M.T., Thompson, J.J.W., 2002. Short-term nutrient deprivation affects immune
453 function. *Physiological Entomology* 27, 206-212.

454 Stoepler, T.M., Castillo, J.C., Lill, J.T., Eleftherianos, I., 2013. Hemocyte Density Increases
455 with Developmental Stage in an Immune-Challenged Forest Caterpillar. *Plos One* 8.

456 Strand, M.R., 2008. The insect cellular immune response. *Insect Science* 15, 1-14.

457 Strand, M.R., Pech, L.L., 1995. Immunological basis for compatibility in parasitoid host
458 relationships. *Annual Review of Entomology* 40, 31-56.

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

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

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

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

470 Vogelweith, F., Thiery, D., Moret, Y., Colin, E., Motreuil, S., Moreau, J., 2014. Defense
471 strategies used by two sympatric vineyard moth pests. *Journal of Insect Physiology* 64, 54-
472 61.

473 Vogelweith, F., Thiery, D., Moret, Y., Moreau, J., 2013b. Immunocompetence increases with
474 larval body size in a phytophagous moth. *Physiological Entomology* 38, 219-225.

475 Vogelweith, F., Thiery, D., Quaglietti, B., Moret, Y., Moreau, J., 2011. Host plant variation
476 plastically impacts different traits of the immune system of a phytophagous insect.
477 *Functional Ecology* 25, 1241-1247.

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

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.

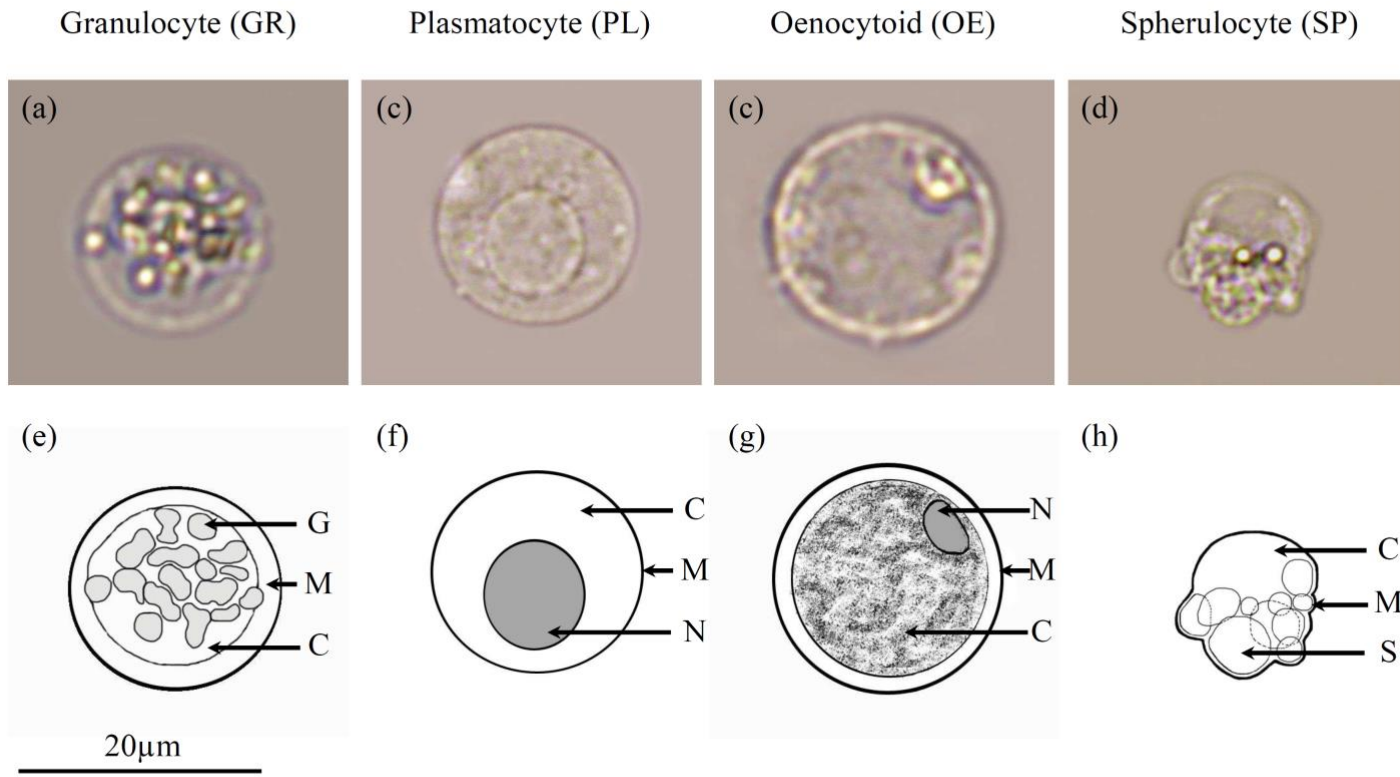
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489 Figure 2. Variation among diets in the total concentration of hemocytes prior to and
490 following an immune challenge. Means and variation are given for larvae reared on diets
491 containing Chardonnay (crosses; dotted lines), Chasselas (black 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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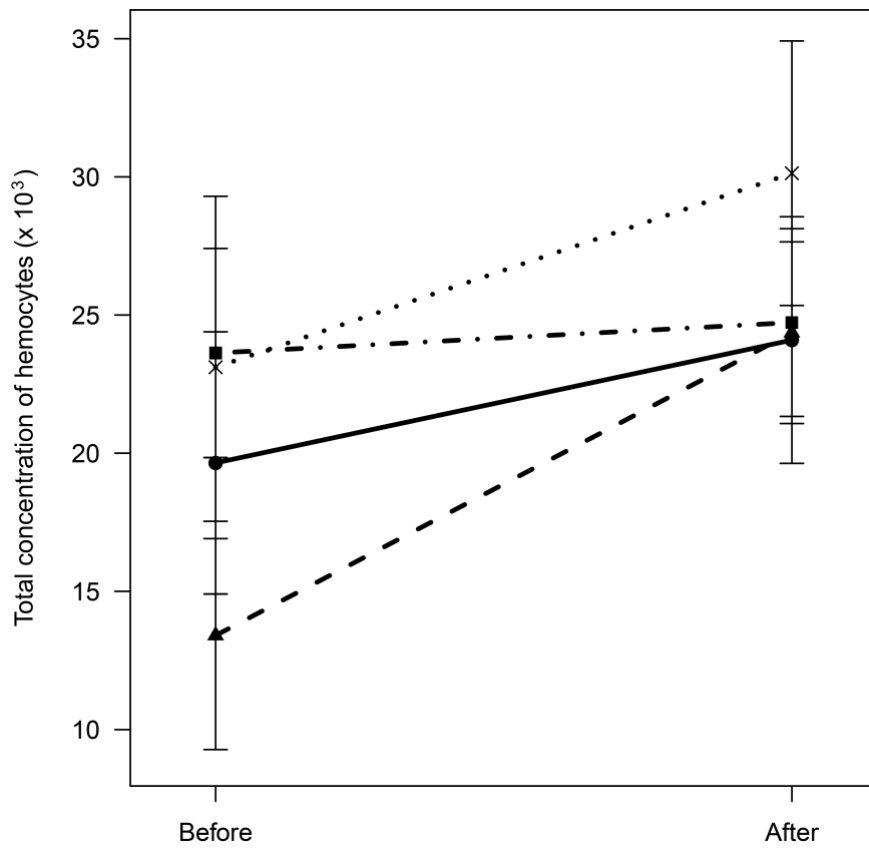
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Figure 1
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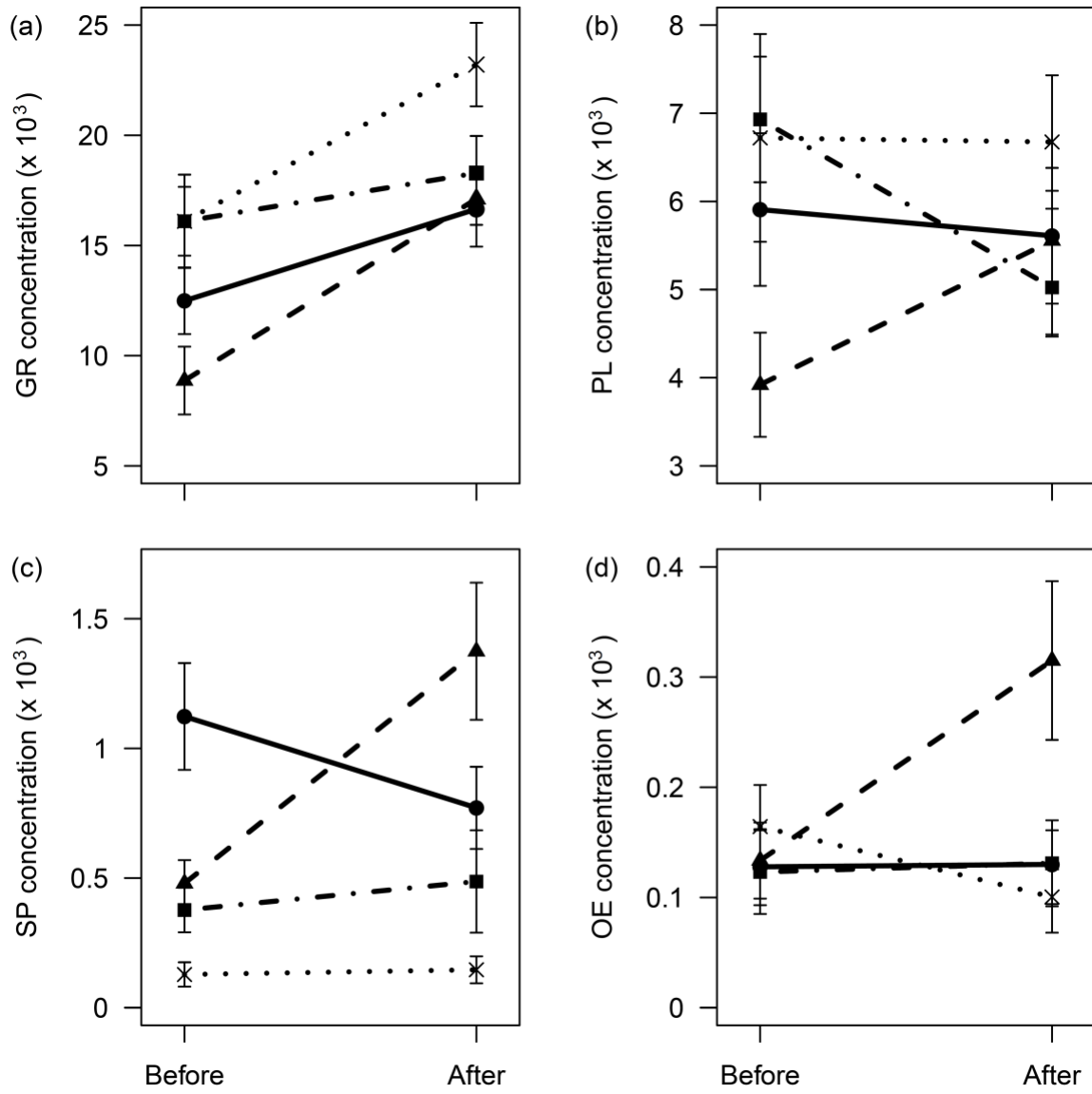
Figure 2

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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 ≤ 0.05) are shown in
 520 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