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

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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.

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.

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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 ($18 \times 11.5 \times 7$ 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