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No evidence of an immune adjustment in response to a parasitoid threat in *Lobesia botrana* larvae

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ABSTRACT

Immune function is a key determinant of an organism's fitness, and natural insect populations are highly variable for this trait, mainly due to environmental heterogeneity and pathogen diversity. We previously reported a positive correlation between infection prevalence by parasitoids and host immunity in natural populations of the vineyard pest *Lobesia botrana*. Here, we tested whether this correlation reflects a plastic adjustment of host immunity in response to the local presence of parasites. To this end, we measured immunity of non-parasitized *L. botrana* larvae exposed, respectively, to one of the two most common species of parasitoids in vineyards, over 6 days. Larvae were able to sense the parasitoid through visual, chemical, or mechanical cues, but contact larvae-parasitoid were excluded. Contrary to our hypothesis, we found that *L. botrana* larvae did not increase their immune defenses in the presence of parasitoids, despite their ability to sense a potential threat. Our results therefore suggest that the positive correlation between infection prevalence by parasitoids and *L. botrana* immunity among natural populations may result from micro-evolutionary changes resulting from long-term local selection pressures imposed by parasitoids in wild populations rather than plastic adjustments of immunity.

1. Introduction

Animals live in dynamic environments and face variation in resource availability, climate and risk of infection over their whole lifespan. Thus, to survive and reproduce successfully, these organisms must allocate resources among competing physiological systems, such as immunity and growth (van der Most et al., 2011), to maximize fitness in changing environments. Immunity is one of the major physiological mechanisms regulating host survival (Lochmiller and Deerenberg, 2000). In insects, an important part of this defense relies on non-specific and constitutive mechanisms that involve the coordinated action of hemocytes and the phenoloxidase (PO) system (Siva-Jothy et al., 2005). Hemocytes are immune cells circulating in the hemolymph involved in the recognition and encapsulation of pathogens (Lavine and Strand, 2002). Conversely, PO mostly mediates the melanization of foreign objects and operates through the activation of the prophenoloxidase (PPO) cascade, its inactive precursor typically stored in the hemolymph and the hemocytes (Cerenius and Söderhäll, 2004).

Insect immunity is associated with inherent costs because it requires energy to build up, maintain and use (Armitage et al., 2003), and thus reduces an individual's ability to invest into other physiological systems. Moreover, the activation of insect immunity produces chemical substances that are harmful for the producer and induce cumulative damage in its body (González-Santoyo and Córdoba-Aguilar, 2012; Nappi and Ottaviani, 2000). For this reason, individuals are expected to flexibly adjust their investment into the immune system to find an optimized balance between their ability to fight off pathogens on the one hand, and saving energy as well as limiting the accumulation of toxic immune components in their body on the other hand. Organisms can indeed be exposed to different threats such as parasites and parasitoids, which can vary among and between populations, as reported in *Drosophila melanogaster* (Kraaijeveld and Alphen, 1995; Tinsley et al., 2006; Corby-Harris and Promislow, 2008), *Gammarus pulex* (Cornet et al., 2009) and *Lobesia botrana* (Moreau et al., 2010; Vogelweith et al., 2013a). These different threats induce substantial variation in the investment in immunity within and among wild populations (Corby-Harris and Promislow, 2008; Cornet et al., 2009; Vogelweith et al., 2013a). For example, recent investigations among natural populations of the grapevine moth (*L. botrana*) revealed that levels of immune defenses in larvae were positively correlated to parasitoid infection

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prevalence (Vogelweith et al., 2013a). At a geographical scale, populations with high levels of immune defenses in non-parasitized larvae (high hemocyte concentrations, PPO enzyme activities and antimicrobial peptides) were also those exhibiting the highest rates of infection by parasitoids (Vogelweith et al., 2013a).

Although this confirms that parasitoid pressure strongly affects host immune investment (Bryan-Walker et al., 2007; Corby-Harris and Promislow, 2008), the exact cause of such a positive relationship remains unknown. Two non-mutually exclusive selective pressures could shape and drive such a variation in immune traits. First, parasites can mediate a local selection response in favor of (constitutively expressed) high levels of immune defense among hosts, which would result in positive covariation between parasite prevalence and immune defense (Lindström et al., 2004; Kalbe and Kurtz, 2006; Tschirren and Richner, 2006). Alternatively, such a positive correlation could arise if hosts plastically adjust their immune response to the (locally varying) risk of infection, since the ability to sense the current risk of parasitism through visual, chemical, or mechanical cues has been reported in some insect species (Peacor, 2003; Fievet et al., 2008). For example, when the risk of parasitism increases, insects show prophylactic investment in their immune defense even in the absence of parasites (Barnes and Siva-Jothy, 2000; Wilson and Reeson, 1998). The velvetbean caterpillar (*Anticarsia gemmatalis*) increased investment in primary defense barriers against parasites, such as the midgut, in response to increased conspecific density and an increased risk of infection (Silva et al., 2016). Such a plastic modulation of immune defenses can be rapid as reported in adult bumble-bee workers (*Bombus terrestris*) (Ruiz-González et al., 2009). A plastic investment in immunity is then likely to result in positive covariation between parasite prevalence and immune defense. To understand how parasitoids can modulate insect immunity, it is important to know which of the two above detailed selective pressures shapes investment into insect immunity.

To our knowledge, no study experimentally investigated how insect can plastically modulate their immune defenses in response to parasitoid pressure. Here, we test the hypothesis whether the presence of parasitoids in the local environment induces a plastic increase of the investment in immune defense. To this end, we exposed *L. botrana* larvae to either parasitoids (excluded physical contacts), or no parasitoid for a period of six days and measured immune parameters (hemocyte concentration and PPO activities system) on the last day of exposure. If *L. botrana* larvae indeed flexibly adjust their immunity to the parasitoid threat, we expected an increase of all immune parameters in the presence of parasitoids. In this scenario, the larvae of *L. botrana* would assess cues related to the presence of parasitoids and adjust their investment in immunity to match any increased threat of infection. *L. botrana* appears to be a good candidate because (1) a variation of larval immune parameters with the parasitoid pressure has already been reported among wild populations (Vogelweith et al., 2013a) and (2) the ability of *L. botrana* larvae to sense the current in the risk of parasitism has been previously demonstrated (Vogelweith et al., 2013b).

2. Material and methods

2.1. Model insect

The European grapevine moth *L. botrana* (Lepidoptera: Tortricidae) is currently the major grape pest in Europe and has also spread to American viticulture (Gilligan et al., 2011). This grapevine moth is a polyphagous insect that completes 2–5 generations a year (depending on latitude) on different grape cultivars, where the larvae can do considerable damage (Thiéry, 2008). The larvae used for this experiment come from inbred stock maintained diapause-free at the French National Institute for Agricultural Research (INRA) (Villenave d'Ornon, France) for more than 10 years. This strain is based on a great number of caged adults (several thousand per week) to which wild adults are periodically added. This laboratory strain has conserved some plasticity because considerable variation is found in immune parameters between larvae (Muller et al., 2015). In addition, a very similar pattern in terms of basal immunity, response to an immune challenge and parasitoid escape behavior have been found between inbred stock and wild populations sampled in French vineyards (Vogelweith et al., 2014). We used larvae from the laboratory to ensure that they were not previously exposed to parasitoids because this could have affected their immunity. Larvae were fed with an ad libitum amount of a semi-artificial diet (Vogelweith et al., 2015), and maintained in boxes (18 × 11.5 × 7 cm) under standard laboratory conditions (22 ± 1 °C, 70 ± 10% r.h., and a L16:D8 photoperiod) at a density of approximately 100 individuals per 300 ml of diet.

2.2. Parasitoid populations

To mimic imminent parasitoid attack on the larvae, we used the two most common parasitoids of *L. botrana* larvae: *Campolex capitator* (Hymenoptera: Ichneumonidae) and *Phytomyptera nigrina* (Diptera: Tachinidae) (Moreau et al., 2010). These parasitoids are known to be larval-parasitoid only and parasitize larvae of *L. botrana* from the 2nd to the 4th instar in vineyards in the south of France where they occur in sympatry (Thiéry, 2008). The parasitism rate of these two parasitoids strongly depends on the year and the vineyard considered (Moreau et al., 2010; Vogelweith et al., 2013a). For instance, in 2012, we observed a parasitism rate of 10% by *C. capitator* and 5% by *P. nigra* while in 2011, we found a parasitism rate of 90% by *C. capitator* in the same vineyard (Vogelweith, unpublish data). Both parasitoid species were obtained from parasitized *L. botrana* larvae collected on Grenache in a vineyard close to Perpignan (southern France) in May 2012 using the procedure by Vogelweith et al. (2013a). In brief, the larvae were collected at the end of their development in grapes and kept in large polyethylene boxes (60 × 40 × 21.4 cm) in the laboratory where they were checked daily for pupation. Pupae were placed individually in glass tubes (70 × 9 mm diameter) closed with cotton plugs and stored under standard laboratory conditions (22 ± 1 °C, 70 ± 10% r.h., and L16:D8 photoperiod). The tubes were checked daily for the emergence of either the moth or the parasitoid. All the parasitoids were of the same age and were used two days after their emergence in the experiment.

2.3. Experimental design

Fourth instar larvae from the laboratory culture (see above) were exposed in groups of five to one female parasitoid (either *C. capitator* or *P. nigrina*) to assess the effect of parasitoid presence on larval immune parameters. In a previous experiment using then same protocol (Vogelweith et al., 2013b), we did not find any difference between control groups either exposed to a non-parasitoid fly or nothing. For this reason, we used additional groups of unexposed *L. botrana* larvae (empty cup) as sole control. The groups of larvae were placed in plastic boxes (98 × 98 × 49 mm) containing 50 ml of semi-artificial diet (Vogelweith et al., 2015) (Fig. 1). A plastic cup (30 mm diameter and 30 mm height) containing one female of *C. capitator* or *P. nigrina* (n = 6 parasitoids and n = 30 larvae, respectively), or no parasitoid (n = 5 and n = 20 larvae) was positioned in the middle of each box. The central cup was transparent and pierced with small holes, which allowed for visual, olfactory and vibratory stimulation of *L. botrana* larvae by the parasitoid. The parasitoids were offered a drop of honey as food for the duration of the experiment. The experimental boxes were maintained in standard laboratory conditions (22 ± 1 °C, 70 ± 10% r.h., and L16:D8 photoperiod). Six days later, a sample of hemolymph was collected from each larva for measurements of the hemocyte concentration and the activity of the PO-PPO system as described in Vogelweith et al. (2011). The larvae that reached metamorphosis (5%) did not provide hemolymph and were equally distributed among treatments. Note that we did not directly expose the larvae to the parasitoid because of the very aggressive behavior of *C. capitator*; since

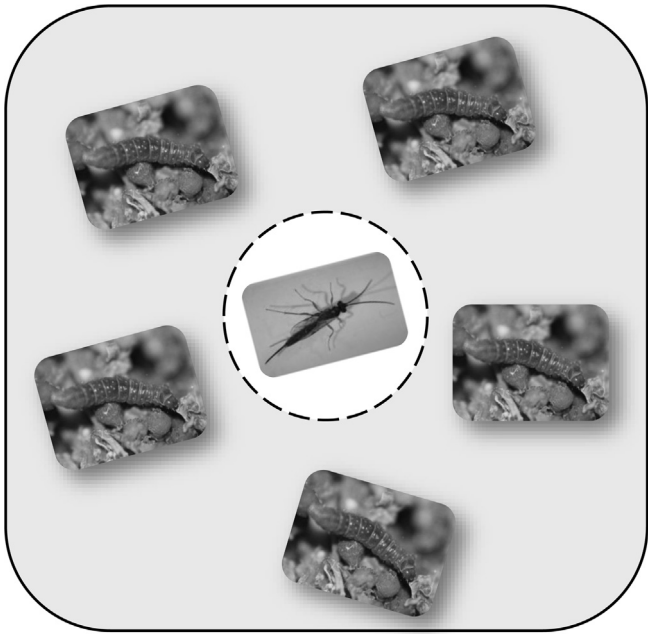

Fig. 1. Diagram of the experimental design. The large square represents the plastic box with the diet (grey) and the larvae. The dotted circle represents the plastic cup with *C. capitator*, *P. nigrina* or no parasitoid inside.

this parasitoid is able to perform more than 10 stings in less than a minute (Vogelweith, unpublished data) inducing consequent injuries to the larvae, possibly affecting immune measurements.

2.4. Hemolymph collection and measurement of the three immune parameters

We measured three key immune parameters on 4th instar larvae well known to reflect parasitoid resistance (Prévost and Eslin, 1998; Carton et al., 2008): the concentration of circulating hemocytes, as well as the PO and total-PO activity, assays established to be sensitive enough to detect rapid changes in immunity (Ruiz-González et al., 2009; Vogelweith et al., 2013c). Each larva was chilled on ice for 20 min, and 1 µl of hemolymph was first extracted with a sterile glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany) from a wound inflicted to the posterior part of the ventral side of the abdomen. This extract was immediately diluted in 25 µl of cold sodium cacodylate/$CaCl_2$ buffer (0.01 M sodium cacodylate, 0.005 M CaCl2; pH 6.5) to measure the two immune parameters. A 10 µl sample of this solution was immediately removed for the counting of hemocytes; the remainder was stored at –27 °C for measurement of the enzymatic activity of the PPO system.

The concentration of hemocytes was measured immediately after hemolymph extraction. This measurement was done using a Neubauer Improved Haemocytometer under phase contrast microscopy (magnification × 400), as described in Vogelweith et al. (2011). The activity of the PPO system was estimated by measuring the enzymatic activity of naturally activated PO enzymes (PO activity), and the activity of the proenzyme together with that of the activated PO (total-PO activity). These measurements were based on a spectrophotometric assay described by Vogelweith et al. (2011, 2013a, 2014).

2.5. Body size of larvae

After the collection of hemolymph, larval body size was estimated by measuring the distance between the most distant lateral sides of the head capsule margins (HC width) (Delbac et al., 2010; Vogelweith et al., 2013c) using a Nikon SMZ-10A stereoscopic microscope and a VTO 232 video analysis system (Linkam Scientific Instruments).

2.6. Statistical analyses

All statistical analyses were conducted using the software R v3.1.2 loaded with the packages *car, lme4* and *MASS*. The hypothesis of a plastic adjustment of immune parameters (hemocyte concentration, PO and total-PO activities) to the parasitoid presence was analyzed using three linear mixed models (LMMs), in which the parasitoid presence, parasitoid species and the body size of the larvae were entered as explanatory variables, whereas the ID of experimental box was used as a random effect. Note that to fulfill homoscedasticity and Gaussian distribution, these models were computed using log + 1-transformed hemocyte concentration, PO and total-PO activities. We used Pearson's rank correlation coefficient with a confidence interval of 95% (C.I.) for the relationship between immune parameters and larval body size (Extract Model Fitted Values). When the C.I. included 0, the correlation was not significant.

3. Results

The presence of parasitoid and parasitoid species had no effect on hemocyte concentration ($F_2 = 0.48$; $p = 0.786$), PO ($F_2 = 0.85$; $p = 0.651$) and total-PO activities ($F_2 = 2.90$; $p = 0.235$) (Fig. 2). However, all immune parameters decreased with larval body size (Hemocyte concentration: $F_1 = 7.08$, $p = 0.008$, and rho = −0.96, C.I. 95% = [−0.98; −0.95]; PO activity: $F_1 = 8.59$, $p = 0.003$, and rho = −0.91, C.I. 95% = [−0.94; −0.86]; Total-PO activity: $F_1 = 5.27$, $p = 0.021$, and rho = −0.73, C.I. 95% = [−0.82; −0.61]).

4. Discussion

This study aimed to explain the positive relationship between immune parameters and parasitoid prevalence previously observed in populations of *L. botrana* larvae inhabiting different French vineyards (Vogelweith et al., 2013a). In particular, we tested the hypothesis that the presence of parasitoids in the local environment can induce a plastic increase of the investment of *L. botrana* larvae into their immune defense. Our results show that the presence/absence of a parasitoid or the species of the parasitoid present in the environment did not affect immunity of *L. botrana* larvae, although our assays previously allowed us to measure reliable changes in immunity due to environmental variation and our protocol maximized the parasitoid perception by the larvae (Vogelweith et al., 2013b) compared to what presumably occurs under natural conditions. However, our results confirm the negative relationships between larval body size and immunity previously found and discussed in this species (Vogelweith et al., 2013a). To our knowledge, our study is the first to test variation of investment into the larval immune system in response to the presence of parasitoids.

Many animals are able to anticipate potential exposure to parasitoids and/or predators by sensing cues such as kairomones, inducing the alteration of several of their traits (e.g., avoidance behavior, morphological changes, and changes in oviposition site) to reduce the cost of infection/predation (Dicke and Grostal, 2001; Sloggett and Weisser, 2002). For example, Sloggett and Wisser (2002) have shown that the pea aphid (*Acyrthosiphon pisum*) produces winged offspring that leave the host plant to flee colonies infected by parasitoids. Our experimental setting prevented any contact of the moth larvae with the parasitoids, and therefore excluded any direct effect of parasitoid infection, but allowed the larvae to assess different (acoustics, chemicals and visual) cues. We are confident that our experimental setup allowed the detection of the parasitoids by the larvae because, using the same protocol, we previously found that larvae of *L. botrana* accelerated their development in the presence of these parasitoids (Vogelweith et al., 2013b). Such a plastic alteration of the larval growth rate leading to earlier metamorphosis is believed to be adaptive, as it reduces the temporal exposure of the larvae to the parasitoid. Altogether, these results

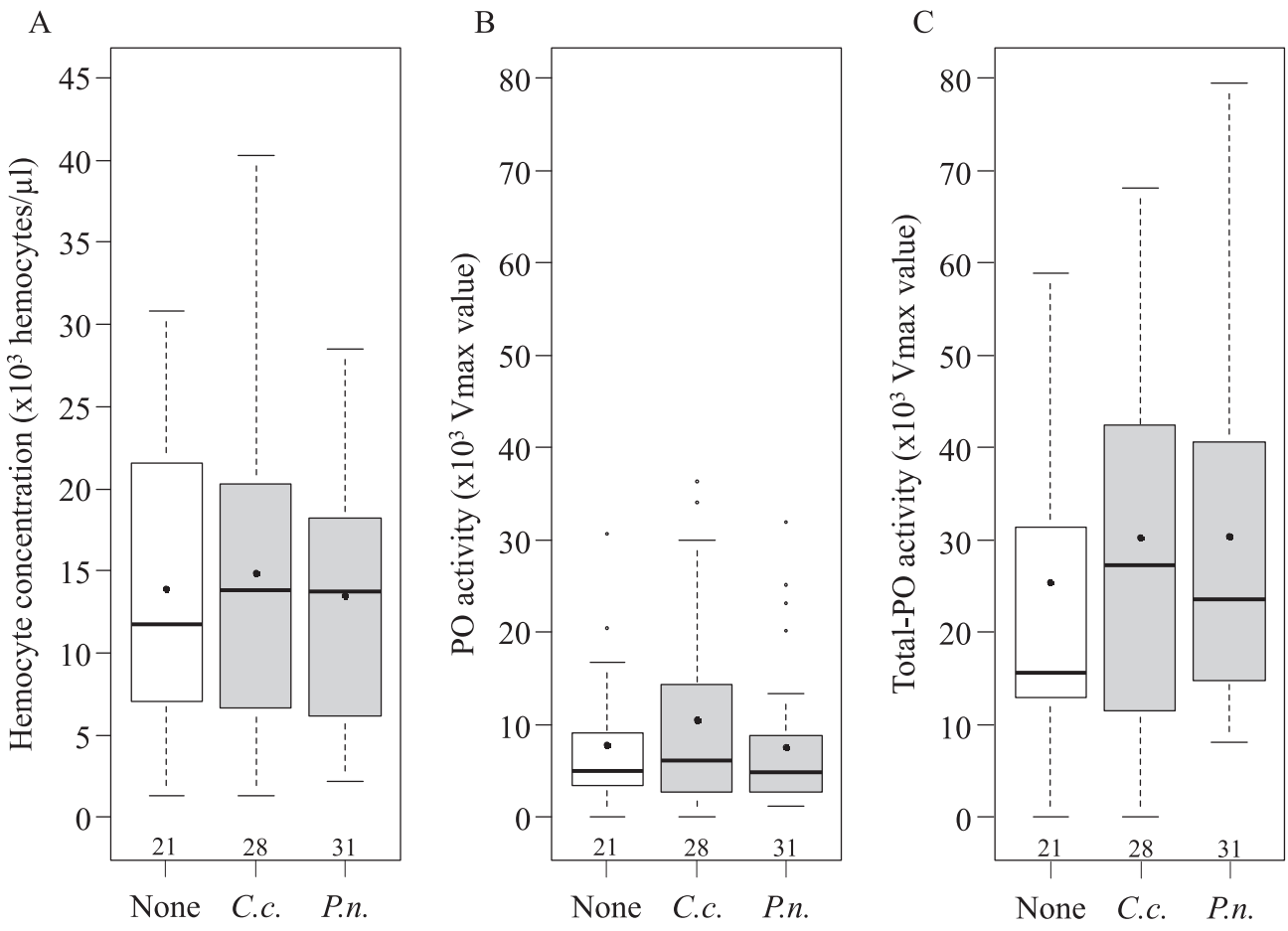

Fig. 2. Immune response of *L. botrana* larvae to the presence of a parasitoid. (A) Hemocyte concentration ($\times 10^3$ hemocytes/µl), (B) PO and (C) total-PO activities ($\times 10^3$ V_{max} value) in the hemolymph of *L. botrana* larvae after six days in the absence (white boxplot) or in the presence of the parasitoids (grey boxplot): *C. capitator* (*C.c.*) or *P. nigrina* (*P.n.*). The edges of the rectangles represent the first and third quartiles, the central features are the medians, the dashed lines are the maxima and minima, and the black circles are the arithmetic means. Numbers above the x-axis represent the number of larvae measured in the experiment.

suggest that developmental acceleration appears to be the better option to escape parasitoid infection as compare to enhancing immunity.

Overall, our results allow us to reject the hypothesis of a plastic change in immunity to explain the positive relationship between larval immunity and parasitism pressure in *L. botrana*. This suggests that the immunity of *L. botrana* larvae might be shaped by – and adapted to – local conditions in response to the local selective pressure imposed by parasites, as demonstrated in others species (Bryan-Walker et al., 2007; Kortet et al., 2007). Because immunity is costly to express (Kraaijeveld and Godfray, 1997), natural selection should favor optimized investments into immune defenses matching the local prevalence and severity of parasitic infections (Kalbe and Kurtz, 2006). Consequently, on a micro-evolutionary scale, natural selection should favor reduced levels of immune defense in populations subjected to low parasite pressure (Kalbe and Kurtz, 2006; Lindström et al., 2004), whereas on macro-evolutionary scale, immunity should protect species from parasitism has already been found in 16 species of Lepidoptera (Smilanich et al., 2009). Further studies should investigate whether local parasitism pressure leads to population-specific micro-evolutionary changes in the immune defense of *L. botrana* larvae. The monitoring and measurements of both larval immunity and parasitism in different vineyards over several years could help to investigate this second hypothesis.

Conflict of interest

The authors declare no competing financial interests.

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