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Variation in immune defence in relation to developmental pathway in the green-veined white butterfly, *Pieris napi*

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ABSTRACT

Question: Is immune defence affected by developmental pathway in a bivoltine butterfly?

Hypotheses: Individuals of the pierid butterfly, *Pieris napi*, undergoing direct development (development without diapause) are time and nutrient stressed compared with overwintering individuals. If this is the case, direct developers will have a diminished immune defence system.

Organism: Green-veined white butterfly, *Pieris napi*.

Methods: In a laboratory experiment, we examined phenoloxidase activity in larvae of *P. napi* and their adult encapsulation ability in response to artificial parasites made of nylon monofilaments. We reared larvae on two different food plants, *Alliaria petiolata* and *Armoracia rusticana*.

Results: The developmental pathway (direct or diapause) can have a strong impact on the defence system in *Pieris napi*. Individuals of the direct-developing summer generation had lower phenoloxidase activity and lower encapsulation ability than individuals of the overwintering generation. Larvae reared on the two different food plants showed no difference regarding phenoloxidase activity, but encapsulation ability was higher for individuals reared on *Armoracia rusticana*. Males had higher phenoloxidase activity but lower encapsulation ability than females.

Keywords: diapause, direct development, immune system, phenotypic plasticity, seasonal polymorphism, trade-off.

INTRODUCTION

Insects living in seasonal environments often escape harsh periods by entering a diapause stage. Depending on the length of the reproductive season, many species may also have time to insert a non-diapause, direct-developing summer generation. Thus an individual might, depending on environmental cues (often temperature and/or day length), develop either directly and reproduce or enter diapause and reproduce the next season (Tauber *et al.*, 1986). This ability to diapause or to develop directly is an example of developmental plasticity or

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phenotypic plasticity (*sensu* West-Eberhard, 2003) where each phenotype meets different selective regimes depending on the season (Brakefield *et al.*, 2003). The different phenotypes are not only different in their reproductive mode but also in other traits.

Hence, phenotypic plasticity provides an opportunity to explore internal developmental differences as well as trait differences and their relation to fitness. For instance, in the nymphalid butterfly *Polygonia c-album*, overwintering diapausing adults (dark morph) mate and reproduce in the spring and early summer, whereas the summer form (light morph) develops directly and reproduces in late summer (Frohawk, 1924; Nylin, 1989). Karlsson and Wickman (1989) found that the overwintering morph allocates more resources for survival than the direct-developing morph. However, the direct-developing morph can instead allocate more resources for reproduction, and was also found to have higher fecundity than the overwintering morph (Karlsson *et al.*, 2008).

One of the fundamental trade-offs in life-history theory is between current reproduction versus self-maintenance and survival (Stearns, 1982). Thus, one of the costs of diapause and having a relatively long life is that these animals have to invest in a long-lasting sturdier body with a more effective immune system, i.e. a physiological cost (e.g. Rolff and Siva-Jothy, 2003), leaving fewer resources available for reproductive production after the diapause stage (Wiklund and Tullberg, 2004; Fordyce *et al.*, 2006; Karlsson *et al.*, 2008).

Insects in general have an innate immune system consisting of a cellular mechanism that defends the host from a pathogen or a parasitic infection and provides an immediate response to infection. The immune system in insects is relatively simple compared with that of vertebrates (Hoffmann *et al.*, 1996). The activity of the enzyme phenoloxidase has been used as an indicator of an insect's immune defence, since phenoloxidase is considered the key enzyme for encapsulation, melanization, and wound repair (Söderhall and Cerenius, 1998; Sugumaran, 2002). Insects can encapsulate foreign objects but the mechanisms differ; typical for Dipteran species is humoral (melanotic) encapsulation, whereas Lepidopterans use cellular encapsulation (Gillespie *et al.*, 1997). Both systems provide an efficient defence against parasites (Gotz, 1985; Nappi *et al.*, 1995). A widely used procedure to measure the encapsulation response is insertion of a nylon monofilament (e.g. Rantala and Roff, 2007). This induces both humoral and cellular responses resulting in melanization and encapsulation, and is similar to that observed in response to a parasitoid egg or nematode infection (Vilmos and Kurucz, 1998), and encapsulation helps to prevent infestation (Gupta, 1991).

The immune system is energetically costly and immune defence will thus depend, in part, on the available energy budget (Moret and Schmid-Hempel, 2000; Freitak *et al.*, 2003, 2007; Rolff and Siva-Jothy, 2003; Sadd and Siva-Jothy, 2006; Diamond and Kingsolver, 2011). Resources that are used by the immune system may thus no longer be available for other functions, and this may result in trade-offs with other important life-history traits, such as development time, reproductive output, longevity, and dispersal (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003; Rolff *et al.*, 2004; K.A. Lee *et al.*, 2008; Suhonen *et al.*, 2010). Typically, animals living under benign nutrient conditions have a more effective immune system than animals living in low-quality environments (K.P. Lee *et al.*, 2008). In addition, there may also be sex-specific differences in resource allocation to immune defence due to differences in life-history strategies between males and females (Zuk, 1990; Sheldon and Verhulst, 1996; Schmid-Hempel, 2003; Stoehr, 2007; Lindsey and Altizer, 2009; Nunn *et al.*, 2009).

Living in seasonal environments may lead to time and resource stress (e.g. to fulfil development and to acquire crucial nutrients) during development (e.g. Talloen *et al.*, 2004; Shama and Robinson, 2006). Among many insects, direct-developing (summer) generation animals have a relatively short time for development, since their future offspring must reach the critical,

often species-specific diapause stage before the onset of winter, which reduces the amount of resources that can be acquired during larval growth (Wiklund *et al.*, 1991; Abrams *et al.*, 1996). In contrast, overwintering generation animals have relatively more time, and tend to be larger and to have a longer developmental time during the larval stage to obtain enough resources to survive the winter period (e.g. Wiklund *et al.*, 1991; Teder *et al.*, 2009).

This leads to the hypothesis that because direct-developing insects might be time and resource constrained during development, they may therefore have fewer resources to allocate to their immune defence system than overwintering animals. To test the hypothesis that individuals of the direct-developing generation have a reduced immune defence system compared with individuals of the overwintering generation, we compared the immune response of direct-developing and overwintered individuals of the green-veined white butterfly, *Pieris napi*, L. First, we measured the phenoloxidase activity in the final larval instar of both the direct-developing generation and the overwintering generation. Second, we tested the adults' immune response with nylon monofilaments inserted into their haemocoel. The larvae were reared on one of two different host plant species: *Alliaria petiolata* or *Armoracia rusticana*. This was done to increase the generality of the results and because host plant chemistry can affect an insect's immune function (e.g. Yang *et al.*, 2008; Smilanich *et al.*, 2009).

METHODS AND MATERIALS

Study species and rearing protocol

The green-veined white butterfly, *Pieris napi* (Lepidoptera: Pieridae), is a temperate polyandrous species found in Europe, Asia (including the Indian sub-continent), North Africa, and North America. In Sweden, the first adults appear in the spring (April and May). A second (summer) generation flies in southern and middle Sweden during the end of June and July (Henriksen and Kreutzer, 1982; Eliasson *et al.*, 2005). Females lay their eggs on different species of Brassicae. Diapause and overwintering occur in the pupal stage. Adults from the overwintering generation mate, lay eggs, and their larvae develop directly during the summer (i.e. offspring of the overwintering generation undergo direct development, whereas offspring of the summer generation diapause in the pupal stage).

Ten female butterflies from the spring generation were collected from the vicinity of Stockholm University on 12 June 2009. These females were returned to the laboratory and placed in cages ($0.5 \times 0.7 \times 0.7$ m) for egg laying. At hatching, half of the larvae from each female were reared under a direct development regime (22°C, 22 : 2 light-to-dark ratio, 5 larvae per host plant) to produce summer generation adults. The remaining larvae were reared simultaneously under conditions that induced a pupal diapause (20°C, 12 : 12 light-to-dark ratio). For practical reasons, we could not rear all animals at the same temperature as would have been preferred. The pupae were then stored at 1°C for 6 months. A cohort of these animals was used for measurements of larval phenoloxidase activity. Larvae were reared in 1.5-L plastic containers (five larvae in each container) with fresh field-collected plant cuttings of the host plant. We changed plants often to ensure that the larvae always had an ample supply of food. After 6 months, the pupae were removed from the refrigerator and were kept at 22°C and a 22 : 2 light-to-dark ratio until adult eclosion. For both treatment groups, the larvae were reared on one of two different host plant species: *Alliaria petiolata* or *Armoracia rusticana*. We used plant cuttings collected from the field and

Table 1. Numbers of animals in the two experiments

Sex	Developmental pathway	Host plant	*n*
Encapsulation experiment			
Male	Direct	*Alliaria*	43
Male	Direct	*Armoracia*	50
Male	Overwintering	*Alliaria*	65
Male	Overwintering	*Armoracia*	60
Female	Direct	*Alliaria*	42
Female	Direct	*Armoracia*	56
Female	Overwintering	*Alliaria*	39
Female	Overwintering	*Armoracia*	49
Phenoloxidase activity experiment			
Male	Direct	*Alliaria*	31
Male	Direct	*Armoracia*	46
Male	Overwintering	*Alliaria*	32
Male	Overwintering	*Armoracia*	47
Female	Direct	*Alliaria*	30
Female	Direct	*Armoracia*	44
Female	Overwintering	*Alliaria*	29
Female	Overwintering	*Armoracia*	47

fresh plants were provided regularly to avoid food limitation. At eclosion, adults were separated into treatment groups by sex and the larval host plant species that they were reared on.

The immune defence of the individuals in each developmental pathway (direct or overwintering) was determined by measuring phenoloxidase activity in larvae and the encapsulation response in adults (Table 1).

Phenoloxidase activity

To determine phenoloxidase activity, haemolymph was taken from the fifth larval instar (just before pupation) from both the summer direct-developing and the pupal overwintering generations. Larvae were sexed and weighed to the nearest 0.5 mg on a Sauter electro-balance and capillary tubes were used to pierce the prolegs and collect haemolyph. Phosphate buffer solution was prepared by mixing 0.56 mL of 0.2 mM $Na_2HPO_4 \cdot 12H_2O$ with 0.94 mL of 0.2 mM $NaH_2PO_4 \cdot 2H_2O$ to maintain a buffer of pH 7.0. Five microlitres of haemolymph were diluted in 95 μL of phosphate buffer in an Eppendorf tube and centrifuged at 10,000 *g* for 5 min. Dopa solution was prepared by dissolving 0.0198 g of 3-4-dihydroxy-DL-phenyalanine (Dopa, Sigma-Aldrich, Switzerland) in 5 mL of water. After centrifugation, 20 μL of supernatant from phosphate buffer was transferred into 96-well micro-plates and 80 μL of Dopa solution was added. Then absorbance was measured after 20 min on a micro-plate reader (Anthos Labtec Instruments, GmbH, Austria) at 492 nm and 25°C. Phenoloxidase activity of the sample was measured in phenoloxidase units, where one unit is equal to the amount of enzyme required to increase the absorbance by 0.001 per minute.

Encapsulation

Encapsulation was measured in the adult butterflies via insertion of a nylon monofilament (2 mm long and 0.11 mm in diameter). Adults were weighed after eclosion to the nearest 0.5 mg on a Sauter electrobalance. Before insertion, each nylon filament was scratched with sandpaper (to make a rough surface to which the haemocytes could attach) and sterilized with 95% ethanol (to prevent the implants from causing infection). The sterilized nylon monofilament was inserted laterally in between the 5th and 6th segments of the abdomen so that it was positioned within the haemocoel. After implantation of filaments, butterflies were kept in the dark at 25°C for 6 h to standardize the time available for encapsulation. After 6 h, all butterflies were frozen.

The nylon monofilaments were dissected from the butterfly, and the dry implants were mounted on slides and photographed with constant light intensity in a microscope (Motic Digital Microscope). The average greyscale of the filaments was measured (where 0 = black and 255 = white) using ImageJ software (National Institutes of Health), and used as an indicator of the degree of melanization (Rantala *et al.*, 2002, 2004). The values were transformed to allow a positive connection between blackness and degree of melanization by deducting observed values from the greyscale of non-implanted clear control nylon filaments.

Statistical analysis

Phenoloxidase activity and degree of melanization were analysed using GLMs, with body mass as a continuous predictor and with model terms developmental pathway, sex, and host plant. Before analysis, all the response variables were checked for normality. Phenoloxidase activity values were $\log_{10}$ transformed to improve normality (Statistica v.5.7; StatSoft Inc., Tulsa, OK). Third-order interactions were not significant and thus were excluded from the analyses. In total, 710 animals were tested (Table 1).

RESULTS

Developmental pathways

Overwintering larvae were heavier than direct-developing animals (males: $F_{1,154} = 18.1$, $P < 0.0001$; females: $F_{1,148} = 19.8$, $P < 0.0001$). Similarly, overwintering adults were heavier than direct-developing animals (males: $F_{1,216} = 42.4$, $P < 0.0001$; females: $F_{1,184} = 30.3$, $P < 0.0001$)

There was a highly significant difference in the larval immune response between the summer and the overwintering generations (Table 2), i.e. the phenoloxidase activity of the larvae from the overwintering generation was higher than that of the directly developing (summer) generation (Fig. 1). Furthermore, encapsulation rates were significantly higher in adults from the overwintering generation compared with those of the directly developing summer generation (Table 3; Fig. 2).

Sex differences

There were significant differences in larval phenoloxidase activity and in the adults' encapsulation response between the sexes (Tables 2 and 3; Figs. 1 and 2). Larval

Table 2. Summary of the analyses of phenoloxidase (PO) activity in relation to developmental pathways (summer vs. overwintering), sex (male vs. female), and host plant (*Alliaria petiolata* vs. *Armoracia rusticana*) of *Pieris napi* using GLM

Variable	Effect	*F*-value	*P*
PO activity	Larval body mass	2.2	0.141
	Sex	5.3	0.022
	Developmental pathway	28.1	<0.001
	Host plant	1.2	0.277
	Sex × developmental pathway	0.3	0.571
	Sex × host plant	0.3	0.580
	Developmental pathway × host plant	1.7	0.198

Note: Degrees of freedom = 1,297 for all effects. *N*-values are in Table 1.

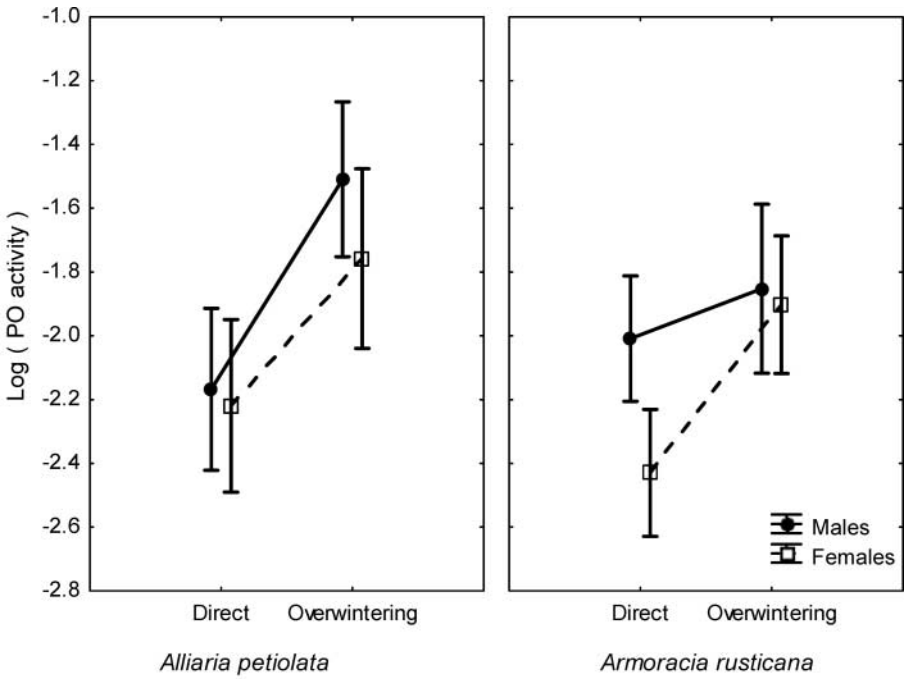

Fig. 1. Phenoloxidase (PO) activity of *Pieris napi* larvae in relation to developmental pathway and host plant species (mean ± S.E.).

phenoloxidase activity was higher in males than females in both developmental pathways (Fig. 1; Table 2). In contrast, adult encapsulation response was higher in females than in males (Fig. 2; Table 3).

Effect of host plant

Encapsulation rate was higher in adults reared on *Armoracia rusticana* than those raised on *Alliaria petiolata* (Fig. 2; Table 3). However, the effect of host plant on phenoloxidase

Table 3. Summary of encapsulation in relation to developmental pathways (summer vs. overwintering), sex (male vs. female), and host plant (*Alliaria petiolata* vs. *Armoracia rusticana*) of *Pieris napi* using GLM

Variable	Effect	*F*-value	*P*
Encapsulation	Adult body mass	4.4	0.037
	Sex	15.7	<0.001
	Developmental pathway	62.2	<0.001
	Host plant	5.1	0.024
	Sex × developmental pathway	2.2	0.134
	Sex × host plant	1.4	0.240
	Developmental pathway × host plant	7.9	0.005

Note: Degrees of freedom = 1,395 for all effects. *N*-values are in Table 1.

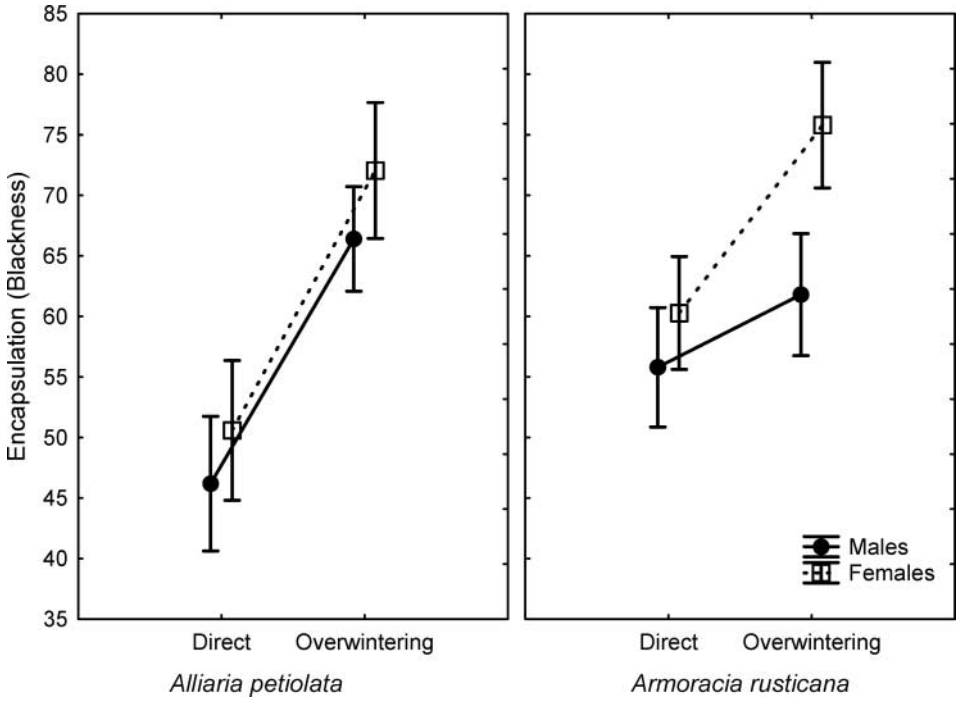

Fig. 2. Encapsulation response of *Pieris napi* adults in relation to developmental pathway and host plant species (mean ± S.E.).

activity was not significant (Table 2). There was a significant interaction between host plant and developmental pathway regarding encapsulation (Table 3); the difference in encapsulation for animals in the two developmental pathways was higher on *Alliaria petiolata* than on *Armoracia rusticana* (Fig. 3). There was no interaction between sex and host plant for either encapsulation response or phenoloxidase activity (Tables 2 and 3).

DISCUSSION

Our aim in this study was to compare two different developmental pathways of *P. napi* for differences in immune defence. In line with our hypothesis, there was a significant difference in immune defence among the animals in the two developmental pathways investigated. The overwintering generation showed significantly higher phenoloxidase activity in the larval stage as well as a higher adult encapsulation response to foreign objects compared with the summer generation. Thus, the results support the idea that resources allocated to immune defence varies depending on the developmental pathway taken. These differences in resource allocation to immune defence may relate to life-history differences between developmental pathways. For example, the summer generation has a shorter life span than the overwintering generation and so might benefit by investing fewer resources to immune defence and more to other fitness traits (Rolff *et al.*, 2004; K.A. Lee *et al.*, 2008). Furthermore, the summer generation of *P. napi* has faster larval development (Wiklund *et al.*, 1991). In contrast, the overwintering generation might increase its fitness by investing more resources in immune defence and building a long-lasting body (cf. Karlsson and Wickman, 1989; Wiklund and Tullberg, 2004; Miller *et al.*, 2007). Karlsson and Johansson (2008) showed that the overwintering generation of *P. napi* had higher fecundity than the summer generation, which implies that the overwintering generation accumulate more resources and have a larger pool of nitrogen resources. In the present study, both males and females of the overwintering generation were heavier than those of the direct-developing generation. In a recent study, Mellström *et al.* (2010) showed that both males and females of the direct-developing generation of *Pieris napi* show signs of both time and nutrient stress compared with overwintering animals. Again, direct-developing females laid fewer eggs and direct-developing males showed lower reproductive performance compared with animals of the overwintering generation.

Temperature could be an important confounding factor in immune defence, since higher resistance might be expected based on the melanization response due to the mobility of haemocytes via a temperature-dependent pro-phenoloxidase cascade (Gillespie *et al.*, 1997). Accordingly, damselflies subjected to higher environmental temperatures showed a higher resistance against mites (Robb and Forbes, 2005), and in the lycaenid butterfly, *Lycaena tityrus*, melanization and encapsulation increased at higher temperatures (Karl *et al.*, 2010). However, in this experiment, animals of the overwintering generation were reared at a lower temperature than animals of the direct-developing generation, but had a higher phenoloxidase activity and higher encapsulation response.

Larval phenoloxidase activity and the adult encapsulation response were significantly different between the sexes in both the overwintering and summer generation. The results showed that adult females had a higher encapsulation response than adult males. However, the phenoloxidase activity of larvae was found to be higher in males. One can only speculate on this difference, especially since the difference between the sexes was not uniform in all treatments, but it could be due to an interaction of sex-specific and larval–adult differences in resource allocation and life-history strategies (Wiklund *et al.*, 2001; Zuk *et al.*, 2004; Stoehr, 2007; Nunn *et al.*, 2009). During mating, *Pieris napi* males provide females with a spermatophore that is about 15% of his body weight (Svärd and Wiklund, 1989). Since spermatophores are costly to produce (Svärd, 1985; Svärd and Wiklund, 1989; Karlsson, 1996), the lower encapsulation ability observed among males in the direct-developing generation could thus be a consequence of a higher investment in costly and intensive spermatophore production (Mellström and Wiklund, 2010). Females incorporate nitrogenous resources from the spermatophore for egg

production (Boggs and Gilbert, 1979; Karlsson, 1998), and it might be that differences in immune defence between the sexes (higher larval phenoloxidase activity/lower adult encapsulation response) are due to a shift in the balance between nitrogen allocation and immune defence during the pupal stage. Yang *et al.* (2007) found a similar pattern in a geometrid moth.

We found that animals reared on *Armoracia rusticana* have similar phenoloxidase activity as larvae grown on *Alliaria petiolata*. However, animals fed on *Alliaria petiolata* have a lower encapsulation response than those fed on *Armoracia rusticana*. Thus, although there were no dramatic differences, our results are in line with the idea that different qualities of host plants with different nutrient properties might have an impact on the strength of the immune defence system (cf. Yang *et al.*, 2007; Diamond and Kingsolver, 2011).

In conclusion, the present results provide support for differential investment in immune defence (larval phenoloxidase activity and adult encapsulation response) between different developmental pathways of the polyphenic green-veined white butterfly, *Pieris napi*. We found that the immune defence of individuals of the overwintering generation was higher than that of individuals of the direct-developing summer generation, suggesting that it may be due to differences in resource allocation during larval development and ultimately due to differences in the adults' life histories.

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