RESEARCH PAPER

WILEY | JOURNAL OF Evolutionary Biology ⟨⟨eseb

Longer life span is associated with elevated immune activity in a seasonally polyphenic butterfly

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Funding information
Suomen Akatemia, Grant/Award Number: 284666; Eesti Teadusfondi grant, Grant/Award Number: 9273; Estonian Ministry of Education and Research, Grant/Award Number: IUT20-33

Abstract

Seasonal polyphenism constitutes a specific type of phenotypic plasticity in which short-lived organisms produce different phenotypes in different times of the year. Seasonal generations of such species frequently differ in their overall lifespan and in the values of traits closely related to fitness. Seasonal polyphenisms provide thus excellent, albeit underused model systems for studying trade-offs between life-history traits. Here, we compare immunological parameters between the two generations of the European map butterfly (*Araschnia levana*), a well-known example of a seasonally polyphenic species. To reveal possible costs of immune defence, we also examine the concurrent differences in several life-history traits. Both in laboratory experiments and in the field, last instar larvae heading towards the diapause (overwintering) had higher levels of both phenoloxidase (PO) activity and lytic activity than directly developing individuals. These results suggest that individuals from the diapausing generation with much longer juvenile (pupal) period invest more in their immune system than those from the short-living directly developing generation. The revealed negative correlation between pupal mass and PO activity may be one of the reasons why, in this species, the diapausing generation has a smaller body size than the directly developing generation. Immunological parameters may thus well mediate trade-offs between body size-related traits.

KEYWORDS
Araschnia levana, immunity, insect, life history, longevity, seasonality, trade-offs

1 | INTRODUCTION

Phenotypic plasticity is the ability of a single genotype to express different phenotypes in response to environmental stimuli. Polyphenisms constitute special cases of plasticity being defined by the discrete character of the environmentally induced phenotypes (Evans & Wheeler, 2001; Nijhout, 2003; Prendergast, Kriegsfeld, & Nelson, 2001; Simpson, Sword, & Lo, 2011). Polyphenic traits range from colouration (Drury, Anderson, & Grether, 2015; Nylin, 2013; Simpson, McCaffery, & Hagele, 1999) and life-history characteristics (e.g., Karlsson & Johansson, 2008; Teder, Esperk, Remmel, Sang, & Tammaru, 2010) to morphology (Greene, 1989; Laforsch & Tollrian, 2004), castes (Wheeler, Buck, & Evans, 2014), (Crews, 2003; Moczek & Kijimoto, 2014) and secondary sexual traits (Moczek, 2009).

Polyphenisms are conventionally assumed, and sometimes explicitly shown (see e.g., Simpson et al. (2011) for insect examples), to represent adaptive responses to environmental changes (Beldade, Mateus, & Keller, 2011; Nijhout, 2003). The adaptive character of such responses is particularly intuitive when the phenotypic change is triggered prior to the onset of the conditions it is assumed to have evolved for (=anticipatory plasticity; Esperk et al., 2013; Whitman & Agrawal, 2009). Phenotypes are, however, integrated (Pigliucci, 2003; Whitman & Agrawal, 2009), and therefore, any major changes in the traits directly subjected to natural selection should be associated with modifications in a number of other traits across the phenotype. Our knowledge of such multivariate responses related to the induction of polyphenic morphs is, however, limited (Nylin, 2013).

One of the most remarkable polyphenisms is the induction of seasonal morphs in insects, triggered by photoperiodic cues and/or temperature (e.g., Shapiro, 1976, Brakefield, 1996; Brakefield & Frankino, 2009; Nylin, 2013). Different seasonal morphs develop coherent sets of morphological and physiological adaptations, which have evolved to match environmental conditions specific to different seasons. Colour polyphenisms in some lepidopteran species constitute perhaps the best understood examples of such seasonal changes (reviewed in Brakefield, 1996; Brakefield & Frankino 2009, Nylin, 2013). Nevertheless, even for such well-studied polyphenic systems, we know little about adaptive significance of each individual element of these multitrait responses, and physiologically based trade-offs between the traits (Esperk et al., 2013; Nylin, 2013; Parker, Barribeau, Laughton, Griffin, & Gerardo, 2017). Yet, seasonally polyphenic insects of temperate regions are ideal models for integrative studies on phenotypic plasticity, due to the sharply contrasting environments the different generations experience, and due to the high feasibility of laboratory studies on these animals.

In temperate regions, some generations of multivoltine (i.e., having more than one generation per year) species enter diapause in certain (species-specific) developmental stage, whereas some do not (Schmidt, 2011; Tauber, Tauber, & Masaki, 1986). As the (winter) diapause typically lasts for months, the individuals from *directly developing* and *diapausing* (overwintering) generations are not only exposed to different environmental conditions, but also have different life expectancies. The long-lived individuals are under selection for physiological and morphological adaptations to varying environmental conditions, as well as for resistance to ageing-related internal stressors (Eleftherianos & Castillo, 2012; Münch, Amdam, & Wolschin, 2008).

In particular, a longer life span demands higher resistance to withstand the impact of pathogens (Lee, Wikelski, Robinson, Robinson, & Klasing, 2008). We can thus expect that the considerable differences in life expectancy between seasonal generations will be reflected in different resource allocation patterns between diverse bodily functions, including the immune function (Schmid-Hempel, 2011). This should specifically apply to the activity of innate (constitutive) immune system which is measured as the activity of humoral (various antimicrobial molecules) and cellular (hemocytes) immune components, and is independent of previous encounters

with pathogens (Tieleman, Williams, Ricklefs, & Klasing, 2005). The presumably higher level of innate immunity in the diapausing generation may, however, bear costs in terms of affecting values of other fitness-related traits, as the costs in activating the immune systems are especially high in small long-living organisms (Brace et al., 2017). On the other hand, all immunological differences we may see among seasonal generations are not necessarily linked to different individual life spans. This is because the different generations face different environments, and immunity is known to respond to environmental variation. For example, temperature has been shown to affect the immunity in an ant, a cricket and a fruitfly (Fedorka, Kutch, Collins, & Musto, 2016; Ferguson, Heinrichs, & Sinclair, 2016; Pamminger, Steier, & Tragust, 2016). Immune function has also been found to depend on the presence of predators (Fincke, 2011) and population density (Cotter, Hails, Cory, & Wilson, 2004; Wang, Yang, Cui, & Kang, 2013), as well as to vary among host plants (Meister, Tammaru, Sandre, & Freitak, 2017; Muller, Vogelweith, Thiéry, Moret, & Moreau, 2015; Vogelweith, Thiéry, Quaglietti, Moret, & Moreau, 2011).

The aim of the present study was to link immunological parameters of a seasonally polyphenic insect to seasonal differences in its environment, as well as to associated differences in life-history traits. In particular, we predicted that the longer lifespan of the overwintering individuals would also lead to higher investment into immunity and set up experiments to evaluate the magnitude of the difference. As the study species, we chose European map butterfly (*Araschnia levana*), an insect with two distinct seasonal morphs differing in several traits such as wing colouration, body size and duration of pupal period. Although the proximate physiological mechanisms leading to different developmental pathways in *A. levana* have been known for some time (Koch, 1992; Reinhardt, 1984), the nature of the possible trade-offs between the generation-specific traits remains surprisingly poorly understood (Ihalainen & Lindstedt, 2012; Joiris, Korati, & Van Dyck, 2010; Morehouse et al., 2013).

Between-generation differences in the immunological traits have been nearly neglected in insect studies. However, two earlier studies have found some evidence of better immune capacity in the overwintering generation (Baudach, Lee, Vogel, & Vilcinskas, 2018; Prasai & Karlsson, 2011). Unfortunately, both were relying on artificial laboratory settings only, so that the results cannot necessarily be extrapolated to the field conditions (see van Zweden, Heinze, Boomsma, & d'Ettorre, 2009; Rivera, Rodriguez-Saona, Alborn, & Koppenhöfer, 2016; Gall, Scoles, Magori, Mason, & Brayton, 2017, for cautionary examples). Moreover, in the experiments using *Pieris napi* as a model (Prasai & Karlsson, 2011), the larvae representing different generations were reared under different temperature regimes which does not facilitate unequivocal separation of the generation-specific effects.

To contribute to the field from a different methodological and conceptual angle, we compared the levels of constitutive (innate) immunity between the two seasonal generations of *A. levana* in a combination of field and laboratory studies. We measured the values of two immunological parameters—general lytic (antibacterial)
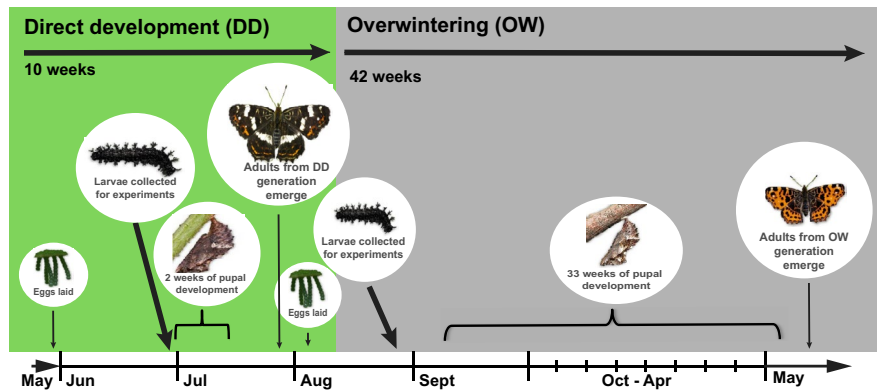
FIGURE 1 Schematic presentation of the life cycle of *Araschnia levana* in the study region (Estonia, Northern Europe). The range covered by images of different life stages (eggs, caterpillar, pupa and adult) represents the approximate occurrence time of the particular stage in the field during an average year. The individuals of the directly developing generation (marked with green-coloured background and "DD") are typically present from the end of May until the beginning of August, whereas diapausing/overwintering generation (marked with grey background and "OW") occurs from August to May. The arrows indicate the sampling times of field-collected caterpillars in 2015 (27–29 June for directly developing generation and 25–27 August for overwintering generation). Note the markedly long duration of the pupal stage in overwintering generation and the larger size of the directly developing individuals.

activity and phenoloxidase activity—from the haemolymph of last instar larvae. Both of these immunological indices are well-studied markers in insect ecological immunity, which gives us good grounds to relate our results to those of other studies. Focussing on constitutive rather than induced defences (compare Baudach et al., 2018, for a recent work on the same species) was motivated by our intention to interpret the results in the context of putative trade-offs between immunity and larval growth. In addition to controlled laboratory trials, in which the different developmental pathways were artificially induced, we sampled the larvae representing either direct (short) or diapause (long) developmental pathway also in the field. Such a design allowed us to combine the rigour of a manipulative study with the realism of monitoring natural variation.

2 | MATERIALS AND METHODS

2.1 | Study species

The European map butterfly, *A. levana* L (Lepidoptera: Nymphalidae), is a temperate Palaearctic (Tolman & Lewington, 2008) butterfly species characterized by several episodes of recent range expansions (Betzholtz, Pettersson, Ryrholm, & Franzen, 2012; Mitikka et al., 2008; Parmesan, 2001). The species is bivoltine in most parts of its range (including the study area). Females lay their eggs in string-like clusters (mostly 20–70 eggs in the clutch, Figure 1) on the leaves of the host plant (*Urtica* spp.). Caterpillars hatching from a particular egg clutch aggregate and stay in groups during most part of their larval life, whereas the size of the groups decreases with progressing larval ontogeny (see Ruf, 2002, for a discussion of the phenomenon).

The map butterfly is a well-known example of a species with seasonal polyphenism. The diapausing generation (form *levana*, sometimes also called "overwintering generation," "first generation" or "spring form") and directly developing generation (form

prorsa, known also as "second generation" or "summer form") differ remarkably in wing colouration and patterning (Figure 1). In addition, the individuals of the diapausing generation also have considerably longer pupal periods than the directly developing individuals (~8 months in the study area compared to ~2 weeks in directly developing generation), due to overwintering in the pupal stage (Figure 1). Moreover, the diapausing form is also characterized by smaller adult size (Friberg & Karlsson, 2010; Morehouse et al., 2013, this study), longer development in the last (5th) larval instar (Windig & Lammar, 1999), lower water content in the pupal stage (Morehouse et al., 2013), different body design in the adult stage resulting in lower flight performance and dispersal ability (Friberg & Karlsson, 2010; Fric & Konvička, 2002) and lower adult abundance (Viidalepp & Remm, 1996) compared to the directly developing generation. Like in most other bi- and multivoltine temperate insects, seasonal polyphenism in *A. levana* is controlled primarily by photoperiod: long day conditions during larval development induce the directly developing pathway, whereas short day lengths lead to the diapausing form. Although the pupa is the hibernating stage in this species, the decision about developmental pathway is already made during the two last (i.e., 4th and 5th) larval instars (Friberg, Haugen, Dahlerus, Gotthard, & Wiklund, 2011).

2.2 | Field trials

To investigate the between-generation differences in immunological traits in the field population, *A. levana* larvae were collected in two rounds, in the end of June (N = 114) and in the end of August 2015 (N = 120; see Figure 1) from six sites in central and southern Estonia, all situated within 60 km from the city of Tartu (Table 1). All caterpillars collected in June developed directly, whereas those collected in August gave rise to diapausing pupae. Most of the larvae were in their last or penultimate instar at the time of collecting, but

TABLE 1 Collection sites and sample sizes in the experiment. Maximum distance between the collection sites was ~70 km (Arisvere and Reolasoo) in the field trial and ~100 km (Laeva and Vastseliina) in the laboratory trial

Trial	Collection site	Coordinates (°)	Developmental pathway	Number collected[b]	Number surviving until pupation[c]	
					Females	Males
Field[a]	Arisvere	N: 58.77; E: 26.03	Direct	78	33	34
			Diapausing	45	14	12
	Ilmatsalu	N: 58.40; E: 26.52	Diapausing	18	10	7
	Laeva	N: 58.48; E: 26.49	Diapausing	23	9	10
	Pangodi	N: 58.20; E: 26.57	Direct	16	7	9
	Reolasoo	N: 58.26; E: 26.75	Direct	10	5	5
	Tartu	N: 58.39; E: 26.70	Direct	10	4	6
		N: 58.36; E: 26.69	Diapausing	34	8	9
Laboratory	Laeva	N: 58.48; E: 26.49	Direct	3	9	23
			Diapausing	3	5	14
	Tartu	N: 58.39; E: 26.70	Direct	2	21	37
			Diapausing	2	28	25
	Tsooru	N: 57.74; E: 26.65	Direct	1	5	5
			Diapausing	1	4	5
	Vastseliina	N: 57.73; E: 27.33	Direct	3	5	9
			Diapausing	3	21	11

[a]Average temperature during the larval period of the directly developing generation (presumed to last from 1st of June until 10th of July) in Tartu in 2015 was 15.5°C (maximum 26.7°C, minimum 5.5°C). For the larval period of the overwintering generation (from 1st of August until 10th of September), the average temperature was 16.8° (maximum 30.5°C, minimum 8.1°C). Data from https://meteo.physic.ut.ee. [b]Number of collected larvae (in the field trial) and number of female adults that laid eggs (i.e., number of broods, in the laboratory trial). [c]Number of individuals that survived until pupation, different broods from the same collection site are pooled in the laboratory trial.

younger larvae were also collected if spotted to avoid a potential bias towards individuals hatching from early-laid eggs. The median time from collecting to pupation was 8 days for the directly developing and 11 days for the diapausing individuals while the minimum was 4 days and the maximum 20 days.

After capturing, the larvae were kept in groups of *ca.* 30 individuals from the same collection site, and the groups were housed in 1 L plastic containers each. Alternatively, there were 10–15 individuals in 0.5 L containers when the number of larvae from the particular site was low. The boxes were kept at outdoor temperature, and the ontogenetic stage of the larvae was recorded by daily inspection. The boxes were cleaned and freshly cut free-growing nettle plants were provided as food every other day. Approximately at the time when the caterpillars attained the maximal body mass in their last (5th) larval instar (determined by visual inspection of head capsule width to body width ratio), the larvae were punctured with insulin syringe from the dorsal side of their antepenultimate segment and 3 μl of haemolymph was sampled and diluted into 10 μl of ice-cold potassium phosphate buffer. Following this procedure, the caterpillars were weighed and transferred individually to 50-ml plastic vials. The vials were kept at room temperature (22°C) until pupation of the larvae, and the larvae were inspected daily for survival and pupation.

The median time from the haemolymph sampling until pupation was 3 days for the directly developing and 5 days for the diapausing individuals and ranged from 1 to 12 days. We preferred to measure immunological indices from the larvae rather than pupae for two reasons. First, as our study focusses on trade-offs between immunity and larval growth, the immunological traits of the actually growing insects were of primary interest. Second, as the length of the pupal period is markedly different between the two generations, it would be hard to know which timing of the sampling would yield comparable results, due to the physiological changes related to pupal development.

The pupae were weighed and sexed (based on Reinhardt, 1984) 3 days after pupation and kept at 22°C until the emergence of adults (directly developing individuals) or were transferred to outdoor temperature conditions for overwintering 2 weeks after pupation (diapausing individuals). Six pupae from which a dipteran parasitoid emerged were discarded from the analyses. The diapausing pupae were brought to the laboratory at 22°C in the second half of April to ensure their natural timing of emergence in May. Adults were weighed 2 days after emergence.

2.3 | Laboratory trial

A laboratory rearing experiment was performed to examine the difference in immunity between directly developing and diapausing individuals in controlled conditions. Wild female adults of *A. levana* were collected in August 2015 from four locations situating within 80 km radius from Tartu (N = 9, Table 1) and allowed to lay eggs in

the laboratory. The newly hatched larvae (N = 600) were placed, in groups of 10 sibling individuals, to 50-ml plastic vials for rearing. The vials were equally distributed in respect to brood and hatching date between the two rearing chambers in which different photoperiodic treatments were applied. Specifically, long day conditions (18L: 6D; inducing direct development) and short day conditions (12L: 12D; giving rise to the diapausing form) were created. Even if the temperature was set to 17°C in both chambers, the photoperiodic treatments (and respective larvae) were rotated between the rearing chambers in every 3 days to decrease the potential impact of any microclimatic differences between the chambers on larval growth.

As there was about 30%–40% mortality in the course of the first two larval instars, the number of larvae per vial decreased to typically 6–7 in the later instars. Therefore, from the 3rd instar onwards, the larvae were switched between the vials within the same brood and hatching date to ensure that the number of caterpillars per vial was never lower than 5 and never higher than 8. Fresh nettle leaves were provided every other day, or more frequently when needed to avoid food depletion. Approximately at the time when the larvae achieved their maximal mass, 3 µl of haemolymph was taken from each individual following the procedure described above. Thereafter, the caterpillars were weighed, transferred individually to 50-ml vials and kept at 22°C, being inspected daily for survival and pupation. The median time from haemolymph sampling until pupation was 5 days for the directly developing and 7 days for the diapausing individuals and reached from 1 to 13 days. The pupae and adults were handled as described in the "field trials" section.

2.4 | Immunological assays

Phenoloxidase activity was measured as described in Laughton & Siva-Jothy (2011), with the following modifications. The earlier fixed samples were thawed and centrifuged (9,000 g) at 4°C for 10 min to obtain clear supernatant. For the phenoloxidase assay, 8 µl of supernatant was added to 200 µl of 3 mM L-Dopa (L-3,4-dihydroxyphenylalanine; Sigma, #333786). The activity of the enzyme was measured at 30°C, 490 nm for 90 min (1-min intervals) with a spectrophotometer (Enspire, Berkin-Elmer). The slope of the absorbance curve from 10 to 60 min (alternatively, 10–40 min for AR) was used as an estimate of phenoloxidase activity.

For estimation of the total lytic activity of the haemolymph (the ability to degrade bacterial cell walls), a lytic zone assay was performed. Petri dishes (ø 9 cm) were filled with 10 ml of sterilized 1× PBS buffer with 1 mg/ml *Micrococcus luteus* freeze-dried and lyophilized cells (Sigma) with final concentration of 1.5% agar. Wells within plates (2 mm diameter) were made by puncturing the agar with a plastic pipette and removing the agar plug by suction. Haemolymph samples (4 µl) were pipetted directly into the wells, and the plates were incubated for 38 hr at 30°C. Dilution series of chicken egg white lysozyme (Sigma; 2, 1, 0.750, 0.500, 0.250, 0.125, 0.62, and 0.31 mg/ml) was run on two separate plates to create a standard curve. To control in between plate variation in lytic activity standards with 0.063 mg/ml and 0.250 mg/ml of lysozyme and were

added to each plate. Lytic activity was determined as the radius of the clear zone around the sample indicating to lysozyme equivalent mg/ml. If no clear zone was visible, the sample was scored as "no lytic activity present" or "0."

2.5 | Data analysis

When analysing the dependence of immunological traits on developmental pathway (direct/diapause), trial (field/lab) and sex (male/female), general linear mixed models (PROC MIXED, Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2006) were applied. If not stated otherwise, the data from field trials and laboratory experiments were analysed jointly in a common setting. Collection site was included as a random factor in all analyses except when laboratory trial individuals were analysed separately. In the latter case, brood nested within collection site was included as a random factor. To meet the assumptions of parametric tests, the values of lytic activity were $\log_{10}$ transformed, whereas PO values were square-root transformed. Denominator degrees of freedom were estimated using the Satterthwaite option. The cases with zero lytic activity or PO activity were excluded from the analyses described above. However, additional analyses with a binary response variable (presence or absence of lytic activity and PO activity) were run using generalized linear mixed models (PROC GLIMMIX; Littell et al., 2006; Stroup, 2013) with logit link function. The same approach was used when analysing the dependence of larval and pupal survival on immunological traits. Body mass after haemolymph sampling and time from sampling until pupation were included as covariates in the analyses of immunological traits. Minimum adequate models were constructed by sequentially removing nonsignificant interaction terms. All analyses were conducted in SAS 9.2 (SAS Institute Inc., Cary, NC).

3 | RESULTS

3.1 | Lytic activity

From the larvae that successfully pupated, 30 individuals (out of 418) did not have any measurable lytic activity at the end of their last instar. The proportion of such individuals was higher within the directly developing than in the diapause pathway and in the field trial compared to the laboratory trial (Table 2). However, when the trials were analysed separately (justified by significant interaction term, Table 2), the difference between developmental pathways remained significant only in the field trial (Table 2).

From the individuals that showed at least some lytic activity, those that developed through the diapause pathway had higher values of lytic activity compared to the directly developing individuals (Figure 2a, Table 3). The mean lytic activity between the developmental pathways differed as much as 5.8 times (estimate based on untransformed data). The difference between developmental pathways was larger in the field trial than in the laboratory trial (Figure 2a), as indicated by the significant interaction term (Table 3). However, diapausing individuals had significantly higher lytic activities also if

Effect	Lytic activity			PO activity		
	df	F	p	df	F	p
(D)evelopmental pathway[a]	1, 394	6.9	0.0089	1, 395	7.9	0.0051
(T)rial[b]	1, 394	9.4	0.0024	1, 395	3.2	0.076
Sex[c]	1, 394	6.4	0.012	1, 395	0.0	0.99
Mass at haemolymph sampling[d]	1, 394	6.6	0.011	1, 395	0.1	0.71
Time to pupation[e]	1, 394	2.5	0.12	1, 395	2.9	0.087
D*T[f]	1, 394	9.0	0.0029			

TABLE 2 Results of the generalized linear mixed model (SAS, PROC GLIMMIX, type 3 sums of squares, logit link function) on the binary lytic activity (individuals with no lytic activity compared to those showing at least some lytic activity) and binary PO activity approximately at the time of attainment of maximum body mass in the last instar *Araschnia levana* larvae. Collection site was incorporated as a random effect. Individuals that died before pupation were excluded. Only the interactions that were statistically significant at 0.05 level were included in the model

[a]Directly developing versus diapausing individuals, non-zero lytic activity and PO activity were more frequent in diapausing ones. [b]Field-collected (field trial) versus laboratory-reared (lab-trial) individuals. Non-zero lytic activity was more frequent in the field than in the lab trial. [c]More frequent in females than in males. [d]Mass approximately at the time of achieving maximum body mass in the last instar. Larvae that showed some lytic activity were heavier than those with zero lytic activity. [e]Since haemolymph sampling in the last instar. [f]High proportion of individuals that showed zero lytic activity among field collected larvae that developed directly.
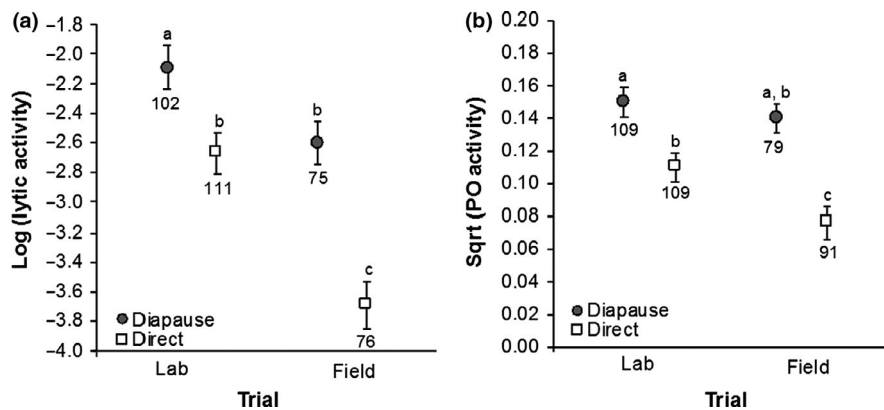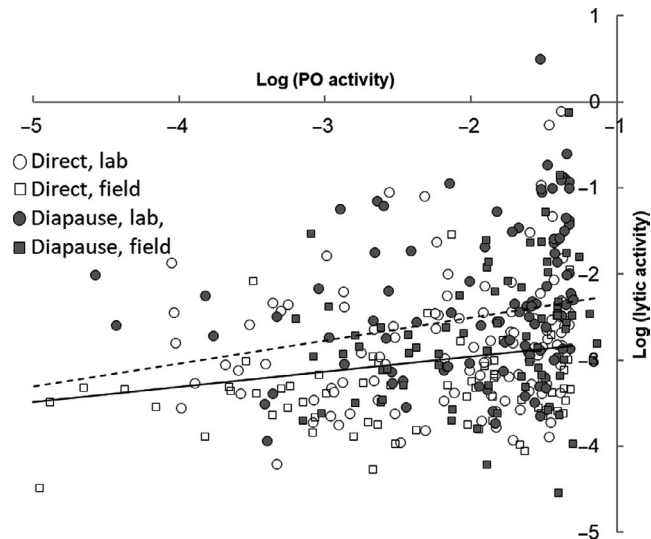

FIGURE 2 Lytic activities ($\log_{10}$ transformed, (a)) and PO activities (square-root transformed, (b)) of the last instar *Araschnia levana* larvae as dependent on developmental pathway (directly developing vs. diapausing) and trial (field-collected vs. lab-reared individuals). Symbols indicate means ± 1 *SE*. The values are corrected for the effects of sex, body mass at the haemolymph sampling in the last instar and time from haemolymph sampling until pupation by the SAS, PROC MIXED, least square mean option. Letters represent significant differences based on Tukey–Kramer post hoc multiple comparisons: groups marked with the same letter are not significantly different at the *p* = 0.05 level. Numbers are sample sizes

Effect	Lytic activity			PO activity		
	df	F	p	df	F	p
(D)ev. pathway[a]	1, 304	89.0	<0.0001	1, 225	42.8	<0.0001
(T)rial[b]	1, 92.3	31.0	<0.0001	1, 16	6.4	0.022
Sex	1, 345	0.8	0.39	1, 370	1.8	0.18
Mass at haemolymph sampling[c]	1, 347	1.6	0.2	1, 365	5.6	0.019
Time to pupation[d]	1, 346	9.3	0.0024	1, 362	31.3	<0.0001
D*T[e]	1, 282	9.3	0.0025	1, 154	2.8	0.094

TABLE 3 Results of the general linear mixed model (SAS, PROC MIXED, type 3 sums of squares) on lytic activity ($\log_{10}$ transformed) and PO activity (square-root transformed) of last instar *Araschnia levana* larvae. Individuals that died before pupation were excluded from both analysis and those that had zero lytic activity or zero PO activity were omitted from the respective analysis. Collection site was incorporated as a random effect. Degrees of freedom were estimated by the Satterthwaite method. See Table 2 for further details

[a]Higher lytic activity and PO activity in diapausing than in directly developing individuals. [b]Higher lytic and PO activity in the laboratory than in the field trial. [c]Larvae with lower body mass at haemolymph sampling showed higher PO activities. [d]Lytic activity correlated positively but PO activity correlated negatively with time from sampling to pupation. [e]The difference between developmental pathways was larger in the field trial than in the laboratory trial.

FIGURE 3 Relationship between lytic activity and PO activity (both log$_{10}$ transformed) in directly developing (solid line) and diapausing (dashed line) *Araschnia levana* in the last larval instar

both trials were analysed separately (laboratory trial: $F_{1,203} = 32.3$, $p < 0.0001$; field trial: $F_{1,126} = 64.0$, $p < 0.0001$).

3.2 | Phenoloxidase activity

Of larvae that successfully pupated ($N = 418$, Table 1), the haemolymph samples of 18 individuals did not have any measurable PO activity at the end of the last larval instar. The frequency of individuals with zero PO activity was higher in the directly developing than in the diapausing generation (Table 2).

From the individuals that showed at least some PO activity in the last instar, the PO activity was higher in the diapausing than in the directly developing individuals (54% higher in the former group, estimate based on untransformed data). Lytic activity and PO activity were positively correlated in the data set pooled over trials and developmental pathways ($r = 0.27$, $p < 0.0001$, $N = 349$; Figure 3). PO activity was negatively correlated with pupal mass (Table 4). Interestingly, the

larvae that died before the pupation had had 21% higher PO activity than those that reached the pupal stage (significant difference, Table 6, Figure 4). At the same time, pupal survival was not dependent on lytic activity or PO activity measured during the larval stage (Table 6).

3.3 | Body mass

Directly developing individuals had significantly (12%) higher pupal mass than diapausing ones (Table 4). Adults from the directly developing generation were significantly heavier than those from the diapausing generation and females were significantly heavier than males (Table 4). Relative mass loss during the pupal stage (calculated as (pupal mass – adult mass)/pupal mass) differed significantly between developmental pathways but did not depend on the levels of lytic or PO activity in the last larval instar (Table 5).

3.4 | Survival

A total of 114 larvae from 532 died during the few days between haemolymph sampling and pupation (parasitized larvae excluded). Survival during that period differed significantly between developmental pathways as 87% of the directly developing individuals and 71% of the diapausing individuals pupated successfully (Table 6). Survival through the pupal stage differed between developmental pathways (Table 6): 193 from 221 (87%) and 121 from 197 (61%) pupae from the directly developing and overwintering generation, respectively, produced adults.

4 | DISCUSSION

Our results demonstrate a clear difference in the immunity between different developmental pathways in the polyphenic butterfly *A. levana*. In particular, the diapause-destined last instar caterpillars had considerably higher activities of immunity-related enzymes in the haemolymph compared to the directly developing ones. Importantly, the results were consistent between laboratory-reared

TABLE 4 Results of the general linear mixed model (SAS, PROC MIXED, type 3 sums of squares) on *Araschnia levana* pupal and adult mass. Collection site was incorporated as a random effect. Degrees of freedom were estimated by the Satterthwaite method. See Table 2 for further details

	Pupal mass			Adult mass		
	df	F	p	df	F	p
(D)ev. pathway[a]	1, 252	53.5	<0.0001	1, 199	37.9	<0.0001
(T)rial[b]	1, 47.8	13.3	0.0007	1, 59.6	6.7	0.012
(S)ex[c]	1, 366	193.4	<0.0001	1, 251	505.7	<0.0001
Lytic activity	1, 364	3.1	0.08	1, 251	3.0	0.086
PO activity[d]	1, 368	12.3	0.0005	1, 256	1.9	0.17
D*T[e]	1, 238	17.3	<0.0001	1, 196	21.9	<0.0001
D*S[f]	1, 365	9.6	0.0021	1, 251	3.5	0.063

[a]Pupal mass and adult mass higher in directly developing than in diapausing individuals. [b]Masses higher in the field than in the laboratory trial. [c]Masses higher in females than in males. [d]PO activity correlated negatively with pupal mass. [e]Larger size difference between directly developing and diapausing individuals in the field trial compared to the laboratory trial. [f]Sexual size dimorphism in pupal mass was larger in directly developing than in diapausing generation.
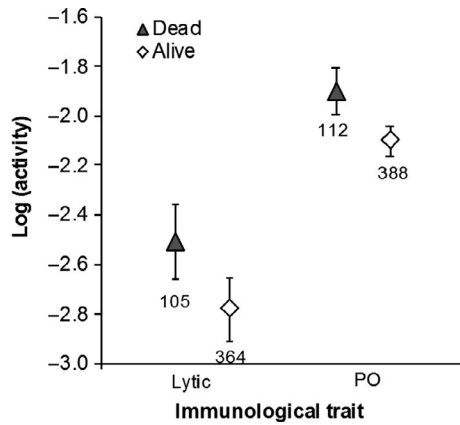
FIGURE 4 Log$_{10}$ transformed lytic activities (±1 *SE*) and PO activities (±1 *SE*) of *Araschnia levana* larvae that either survived or died during the period from haemolymph sampling until pupation in the last larval instar. Values are corrected for the effects of sex, body mass at the haemolymph sampling in the last instar and time from haemolymph sampling until pupation by the SAS, PROC MIXED, least square mean option. Numbers indicate sample sizes

TABLE 5 Results of the general linear mixed model (SAS, PROC MIXED, type 3 sums of squares) on *Araschnia levana* relative mass loss during the pupal stage (calculated as (pupal mass − adult mass)/pupal mass). Collection site was incorporated as a random effect. Degrees of freedom were estimated by the Satterthwaite method. See Table 2 for further details

	df	*F*	*p*
(D)evelopmental pathway[a]	1, 180	18.6	<0.0001
(T)rial	1, 36.8	1.2	0.28
Sex[b]	1, 252	703.2	<0.0001
Lytic activity	1, 253	2.1	0.15
PO activity	1, 257	1.4	0.23
D*T[c]	1, 177	24.3	<0.0001

[a]Higher mass loss in diapausing than in directly developing individuals. [b]Higher in males than in females. [c]Difference between the mass loss of diapausing and directly developing individuals was larger in the field trial than in the laboratory trial.

and field-collected individuals. The two immune parameters studied were positively correlated with each other. This suggests that the detected difference between the developmental pathways is not specific to a particular trait and may also indicate the ability of the insect to up-regulate different parts of its immunity simultaneously.

Diapausing *A. levana* individuals exhibited higher levels of both PO activity and general lytic activity in the haemolymph. In contrast, individuals with direct development not only had lower expression of both immune parameters but also a considerably higher proportion of individuals lacked any measurable activity of respective enzymes. There are at least two ways how natural selection may have caused such plastic differences. First, the large (more than 4-fold) difference in life span between the individual representing different developmental pathways is an obvious candidate of the selective factor. These differences in life span should imply that the probability of

encountering any pathogens must be much higher for the diapausing generation. This is in good consistence with the suggestion that organisms with short lifespan may not need to invest into defence mechanisms, but can instead avoid being infected by faster development (Schmid-Hempel, 2011).

Second, a nonexclusive explanation is that the larvae of the two *A. levana* generations may face different pathogen assemblages, and different overall abundances of such organisms. Such environmental differences should select for season-specific activity levels of the immune system. There appears to be no overall understanding of seasonal variation in the abundance of insect pathogens (see, however, Antúnez et al., 2015; Małagocka, Jensen, & Eilenberg, 2017, for case studies). Indeed, abundance patterns of insects pathogens in natural environments have only recently started to receive research attention (Małagocka et al., 2017), being facilitated by the development of respective methodology. Nevertheless, it appears reasonable to assume that pathogen abundance increases towards the end of the season, a pattern which has been documented at least for a fungal pathogen of ants (Małagocka et al., 2017). If so, pathogen pressure may well be higher for the larvae of the overwintering generation which should have selected for allocating of more resources into defence mechanisms, perhaps even at the cost of lower body mass.

Indeed, we found that the directly developing individuals were heavier upon pupation compared to the diapausing ones. It is not clear why *A. levana* shows such an among-generation size difference while the opposite pattern prevails in Lepidoptera (Teder et al., 2010, but see Wiklund & Forsberg, 1991 and Esperk et al., 2013 for patterns consistent with those in *A. levana*). The results of the present study suggest that one potential reason might lie in the higher costs of the up-regulated immunity in diapausing individuals of *A. levana*. For example, it appears possible that the directly developing *A. levana* maximizes the investment into body size at the cost of reducing investment into immunity. Under the likely limited pathogen pressure of early summer, it should indeed be adaptive to invest into large body size, just to take the advantage of the strong fecundity advantage of size, typical of insects (Honěk, 1993; Tammaru, Esperk, & Castellanos, 2002; Tammaru, Kaitaniemi, & Ruohomaki, 1996). Earlier studies that have investigated the relationship between body size and immune response in insects have yielded conflicting results (reviewed and discussed in Brace et al., 2017; Krams, Daukšte, Kivleniece, Krama, & Rantala, 2011; Vogelweith, Thiery, Moret, & Moreau, 2013). In accordance with some of these studies (Krams et al., 2015; Rantala & Roff, 2005; Rantala, Roff, & Rantala, 2007), we found that smaller rather than larger individuals had stronger immune response in terms of higher PO activity. This further supports the idea that a trade-off between immunity and development may exist in *A. levana* (*cf.* Brace et al., 2017). A potentially related observation is that the relative mass loss during the pupal stage was linked to the developmental pathway. This result could be seen as a mechanistic consequence of the much longer pupal period of the overwintering individuals. Alternatively, or at least additionally, the overwintering pupae

TABLE 6 Results of the generalized linear mixed model (SAS, PROC GLIMMIX, type 3 sums of squares, logit link function) on survival of *Araschnia levana* larvae and pupae. Collection site was incorporated as a random effect

Effect	Larval survival[a]			Pupal survival		
	df	F	p	df	F	p
Developmental pathway[b]	1, 485	8.3	0.0041	1, 359	6.3	0.012
Trial	1, 485	3.5	0.061	1, 359	0.2	0.65
Lytic activity	1, 485	0.1	0.8	1, 359	1.2	0.28
PO activity[c]	1, 485	5.4	0.021	1, 359	0.3	0.58
Mass at haemolymph sampling[d]	1, 485	6.6	0.01	1, 359	0.4	0.55
Pupal mass[e]				1, 359	22.6	<0.0001
Time to pupation[f]				1, 359	3.9	0.049
Sex				1, 359	0.5	0.47

[a]After haemolymph sampling in the last instar. [b]Larval and pupal survival were both higher in directly developing than in diapausing individuals. [c]The larvae that survived to pupation had lower PO activity than larvae that died. [d]The larvae that survived were heavier at the time of haemolymph sampling. [e]The pupae which produced adults were heavier than those that died before adult emergence. [f]Individuals that were closer to pupation at the time of haemolymph sampling had higher survival during the pupal stage.

might have kept investing into immunity in order to keep the infections under control.

A further reason for the seasonal difference in immune parameters may result from phenological changes in the host plant. The biochemical profile of *Urtica dioica*, the host plant of *A. levana*, has been shown to change considerably across the season, with measurable effects on its insect herbivores (Pullin, 1987). *Urtica dioica* has high levels of phenolics (Farag, Weigend, Luebert, Brokamp, & Wessjohann, 2013) known to cause oxidative stress in herbivores (War et al., 2012). As a host specialist, *A. levana* must be adapted to high levels of defensive compounds, such as flavonoids, which have been shown to have antimicrobial effects against variety of pathogens (Cushnie & Lamb, 2005; Gülçin, Küfrevioğlu, Oktay, & Büyükokuroğlu, 2004). If the younger leaves eaten by the directly developing larvae are high on defensive compounds (see Ben Ahmed et al., 2017; Lee, Cory, Wilson, Raubenheimer, & Simpson, 2006; Palo, Sunnerheim, & Theander, 1985), the direct developers may well rely on plant secondary metabolites as antiparasite defence (Abbott, 2014; Sandre, Tammaru, & Hokkanen, 2011). Naturally, this possibility needs to be empirically verified but could eventually provide an example of how multivariate life-histories are shaped in multitrophic systems.

In the present study, parallel measurements were performed on laboratory-reared and wild-caught animals. Typically, immunological studies on insects are limited to laboratory grown animals in highly controlled conditions (Lemaitre & Hoffmann, 2007). This raises the question about how well can we extrapolate the laboratory-based results to natural conditions. This aspect is also relevant for interpreting the studies on *P. napi* (Prasai & Karlsson, 2011) and *A. levana* (Baudach et al., 2018) that reported differences in the immunity between two different developmental pathways relying on laboratory measurements. One of the most significant results in the present study is the good correlation in measured traits between the

laboratory and wild grown individuals. As the field study supports the data obtained in the laboratory, we can claim the effects to be "real," that is there is strong indication that our laboratory trials adequately reflected the situation under natural conditions.

In turn, importantly, the manipulative study allowed us to circumvent a major ambiguity inherent to correlative studies in the field of ecological immunology. In particular, relying on the results of the field study, we could not exclude the possibility that the elevated immunity in diapausing larvae was fully explainable by encounters with actual pathogens, which are likely to be more abundant in the second half of summer (see above). In our manipulative laboratory study, different developmental pathways were induced photoperiodically in otherwise standardized conditions, and the differences in immunological traits were nevertheless observed. This allows us to exclude a direct response to pathogens or host plant traits as a sole explanation. Indeed, for the differences detected between the photoperiodic treatments it is hard to propose explanations other than those based on anticipatory plasticity, that is adaptive allocation decisions causally related to the developmental pathway.

We found that PO activity and lytic activity can be simultaneously expressed at high levels in the diapausing generation. This adds to the contradictory evidence about the proposed trade-off between these two central immunity-reflecting parameters (Cotter et al., 2004; Fedorka, Copeland, & Winterhalter, 2013; Freitak, Heckel, & Vogel, 2009; Freitak, Wheat, Heckel, & Vogel, 2007; Meister et al., 2017). The reason why it has been thought that PO and lytic activity should be expressed at different time points is linked to high autoimmune costs, associated with PO activity (Sadd & Siva-Jothy, 2006). These costs are related to the release of free radicals during the synthesis of phenoloxidase which causes oxidative stress and tissue damage (Cerenius, Lee, & Söderhäll, 2008), leading to the need to allocate resources to counteract the damage to tissues and DNA (Jena, 2012).

Urtica dioica, the host plant of *A. levana*, has been reported to have high anti-oxidative capacity (Gülçin et al., 2004; Khare et al., 2012). It is possible that stinging nettle supplies the larvae with high levels of anti-oxidative compounds (Upton, 2013), and allows the insects to express high levels of PO activity without suffering autoimmune effects. In any case, our results suggest that species-specific physiological and ecological factors can well affect the severity of immunological trade-offs. This implies that instead of asking *if* there are such trade-offs, we should perhaps ask *when* are we expected to see them. In this context, it might be interesting to see how the activities of these enzymes would be influenced when the animal gets infected. It may well be the case that, for example, bacterial infection would down-regulate PO activity on the cost of up-regulating the lytic activity (Cotter et al., 2004; Freitak et al., 2007). A recent study investigating the differences in induced immunity in different developmental pathways of *A. levana* is in good correlation with our predictions. It showed that also in the case of artificial immune challenge, the larvae entering the diapause had higher survival and higher induced antibacterial activity than directly developing ones (Baudach et al., 2018).

Summing up, our results suggest that immunological traits may well be integrated into the structure of trade-offs which underlies the life-history differences between different morphs of a polyphenic insect. By using both laboratory and field grown individuals, we examine various life-history traits potentially related to innate immunity. In consistence with our expectations, we see higher level of immune system activation in the generation with diapause development and longer life span. In particular, our results suggest that immune status might be compromised in order to achieve faster development and higher body mass under some but not all conditions. Such a difference in allocation decisions may stem from the differences in expected longevity of the individuals, or it may constitute an anticipatory response to temporal differences in the pathogen prevalence in the environment. Our results also suggest that the widely stated trade-off between phenoloxidase activity and lytic activity may not be universal, but may well depend on physiological and ecological context of the expression of these traits.

AUTHORS' CONTRIBUTIONS

TE, TT, SLS and DF conceived the ideas and designed the experiments. TE and HM performed the experiments. TE analysed the data. DF and TE led the writing of the manuscript, and other authors provided editorial advice.

ACKNOWLEDGMENTS

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