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RESEARCH ARTICLE

Sources of variance in immunological traits: evidence of congruent latitudinal trends across species

Hendrik Meister[1],*, Toomas Tammaru[1], Siiri-Lii Sandre[1] and Dalial Freitak[2]

ABSTRACT

Among-population differences in immunological traits allow assessment of both evolutionary and plastic changes in organisms' resistance to pathogens. Such knowledge also provides information necessary to predict responses of such traits to environmental changes. Studies on latitudinal trends in insect immunity have so far yielded contradictory results, suggesting that multispecies approaches with highly standardised experimental conditions are needed. Here, we studied among-population differences of two parameters reflecting constitutive immunity–phenoloxidase (PO) and lytic activity, using common-garden design on three distantly related moth species represented by populations ranging from northern Finland to Georgia (Caucasus). The larvae were reared at different temperatures and on different host plants under a crossed factors experimental design. Haemolymph samples for measurement of immune status were taken from the larvae strictly synchronously. Clear among-population differences could be shown only for PO activity in one species (elevated activity in the northern populations). There was some indication that the cases of total absence of lytic activity were more common in southern populations. The effects of temperature, host and sex on the immunological traits studied remained highly species specific. Some evidence was found that lytic activity may be involved in mediating trade-offs between immunity and larval growth performance. In contrast, PO activity rarely covaried with fitness-related traits, and neither were the values of PO and lytic activity correlated with each other. The relatively inconsistent nature of the detected patterns suggests that studies on geographic differences in immunological traits should involve multiple species, and rely on several immunological indices if general trends are a point of interest.

KEY WORDS: Parasitoid, Climate change, Geographic variation, Immunology, Lepidoptera, Life-history traits

INTRODUCTION

In the last few decades, there has been increasing interest in research relating to insect ecological immunity. This is largely due to improved practical applications of such knowledge to pest management (Smilanich and Dyer, 2012) and to the control of insect-borne human pathogens (Modiano et al., 1996; Crawford et al., 2012). However, responses of insect immune function to environmental variations remain relatively understudied (Sheldon

[1]Department of Zoology, Institute of Ecology and Earth Sciences, University of Tartu, 51014 Tartu, Estonia. [2]Centre of Excellence in Biological Interactions, Department of Biosciences, University of Helsinki, 00014 Helsinki, Finland.

*Author for correspondence (hmeister@ut.ee)

H.M., 0000-0002-3276-0835

and Verhulst, 1996; Kraaijeveld et al., 2001; Luong and Polak, 2007; Iserbyt et al., 2012; Prokkola et al., 2013). This is despite the undeniable relevance of such knowledge in the context of climate change, for example (Adamo and Lovett, 2011; Seiter and Kingsolver, 2013; Gherlenda et al., 2016). In addition, even though it has been widely accepted that immunity bears costs (Luong and Polak, 2007; Iserbyt et al., 2012; Prokkola et al., 2013), we still do not sufficiently understand the nature of potential trade-offs between immune function and life-history traits. In particular, it is unclear how general such trade-offs are across various species and environmental conditions (for case studies, see Diamond and Kingsolver, 2011; Vogelweith et al., 2013b).

Comparing immunological traits across geographical populations is a way to shed light on evolutionary responses of such traits to environmental factors. Among-population trends in immunity have been studied mostly in birds, crustaceans and fish (Conover et al., 2009). Insects have received less attention, though there is an increasing body of work on the subject, primarily on Lepidoptera, Odonata and Diptera (see Table 1 for references). The results appear to be inconsistent, however, as both an increase and a decrease in the strength of the immune response have been shown with increasing latitude (see Table 1 for an overview).

Populations may have evolved genetic differences, but they also experience different environments. Both can influence immune function (Schmid-Hempel, 2005, 2011). Primarily, as a major factor, temperature can enforce changes in the expression of different immune parameters. The relationships are not straightforward, however. For instance, temperature experienced during larval development has been found to affect resistance to viral infections in adult mosquitos (Samuel et al., 2016), though not in a consistent way. At the same time, no effect of temperature on one of the central immunological parameters – phenoloxidase (PO) activity – was detected in a chrysomelid (Gherlenda et al., 2016). However, lower activity of this enzyme was recorded in *Drosophila* in colder environments (Salehipour-shirazi et al., 2017).

Besides temperature, the parameters of the host plant (nutritional components and defensive compounds, for example) constitute another essential environmental factor affecting life-history traits in insect herbivores (Awmack and Leather, 2002). In particular, polyphagous species, which utilise numerous plant species, are faced with a very variable chemical composition of their food (Schoonhoven et al., 2005). These plant-borne substances may have significant effects on the herbivore itself but can also affect its interactions with other organisms, pathogens included (Sandre et al., 2011; Lampert, 2012). In particular, changes in host plant chemistry which are induced by herbivory can have a direct effect: they may affect the herbivore's immune responses (Bukovinszky et al., 2009). Interestingly, even the evolutionary maintenance of polyphagy in herbivorous insects has been linked to the effects of the alternative host plants on innate immunity (Muller et al., 2015).

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List of abbreviations

AR	*Acronicta rumicis*
CZE	Czechia (Czech Republic)
DGR	differential growth rate
EST	Estonia
FIN	Finland
GEO	Georgia
HP	*Hypomecis punctinalis*
PO	phenoloxidase
PP	*Poecilocampa populi*

Constitutive innate immunity (as opposed to induced immune responses) is determined by a mixture of humoral and cellular components present in the body. Independent from previous encounters with pathogens, it establishes the first line of defence against diseases (Tieleman et al., 2005). Despite the cost of producing and maintaining an immune response (Schmid-Hempel, 2005), upholding some level of immune system activation at any time should give the insect an advantage over its less-prepared conspecifics, should a pathogen attack occur. In studies on ecological immunology of insects, PO activity and lytic activity have been the two primary parameters used to characterise constitutive immunity. PO activity is a general immune response to fight bacteria, fungi and viruses (Cerenius et al., 2008; González-Santoyo and Córdoba-Aguilar, 2012), and it is also effective against multicellular parasite eggs and nematodes (Nappi and Ottaviani, 2000; Marmaras and Lampropoulou, 2009). Total lytic activity, in contrast, indicates an ability to cope with infestation by microorganisms (Bulet et al., 2004), bacteria in particular (McNamara et al., 2013a; Graham et al., 2015).

In addition to its constitutive character, both PO and lytic activity also have inducible elements. This implies that exposure to pathogens or changes in population densities can lead to further upregulation or downregulation of these immunological markers (Cotter et al., 2004; Ruiz-González et al., 2009). At the technical level, this implies that vigorously controlled experimental conditions are needed to maximise the accountability of the data.

It is highly likely that there is synergistic action between PO activity and total lytic activity but, nevertheless, different types of immune responses can also be traded-off against one another (Cotter et al., 2004; Freitak et al., 2007). This is presumably because of the high autoimmune costs associated with PO activity, which may constrain simultaneous upregulation of both these responses (Sadd and Siva-Jothy, 2006; Cerenius et al., 2008). For example, lysozome (a component of lytic activity) can inhibit proPO conversion into PO activity, causing a potential negative relationship between the two (Rao et al., 2010). This leads to some controversy about PO activity as a suitable index to measure immunity (Pauwels et al., 2011).

There is, however, limited evidence about the generality of such trade-offs among immunological parameters across different species and environmental conditions. Indeed, the number of comparable studies across different taxa is not high and inconsistent results are sometimes obtained (Freitak et al., 2009; Gershman et al., 2010; Fedorka et al., 2013). Moreover, caution is needed when interpreting phenotypic correlations as evidence of trade-offs (Van Noordwijk and de Jong, 1986; Reznick et al., 2000). This is because individuals may differ in the amount of resources they are able to allocate to immune function. The simplest way to reduce the effect of variable resource levels is to perform the measurements in highly standardised conditions.

The aim of the present study was to detect possible latitudinal differences in the immune function of Lepidopteran larvae, and to test for consistency of such trends across environmental conditions and study species. Representatives of different geographical populations of three moth species were reared under common-garden conditions (explained in Materials and methods) in the laboratory. PO and lytic activity of the larvae were measured under different temperatures and on different host plants. Rearing larvae on multiple host plants was also necessary to control for possible species-specific host plant adaptations (Thompson, 1994). As immunological indices are known to be highly condition dependent (e.g. Ueda et al., 2002; Lazzaro et al., 2008; Stoepler et al., 2013), special attention was paid to standardisation of the experimental environment and procedures. In particular, haemolymph samples for immunological assays were

Table 1. A review of published studies on latitudinal trends in immunological traits in insects

Order	Indices	Poleward trend	Source
Odonata	PO activity	No trend	Iserbyt et al., 2012; Janssens and Stoks, 2014
Lepidoptera	PO activity	Decreasing	Vogelweith et al., 2013b
Orthoptera	PO activity	Increasing	Fedorka et al., 2013
Odonata	Total PO activity	Decreasing	De Block et al., 2008
Lepidoptera	Melanisation	Decreasing	Karl et al., 2010; Vogelweith et al., 2013b
Orthoptera	Melanisation	Increasing	Fedorka et al., 2013
Diptera	Melanisation	Increasing	Kutch et al., 2014
Hymenoptera	Melanisation	No trend	Doums et al., 2002
Lepidoptera	Haemocyte count	Decreasing	Vogelweith et al., 2013b
Orthoptera	Haemocyte count	Decreasing	Graham et al., 2015
Lepidoptera	Encapsulation	No trend	Karl et al., 2010
Lepidoptera	Encapsulation	Decreasing	Seiter and Kingsolver, 2013
Odonata	Encapsulation	No trend	Kaunisto and Suhonen, 2013; Seiter et al., 2013
Diptera	Encapsulation	Decreasing	Kraaijeveld and Godfray, 1999
Diptera	Expression of immune genes	Increasing	Paparazzo et al., 2015
Hymenoptera	Expression of immune genes	Increasing	Brunner et al., 2013
Lepidoptera	Parasite load	Decreasing	Altizer, 2001
Diptera	Parasite load	Decreasing	Lazzaro et al., 2008
Lepidoptera	Inhibition zone assay	Decreasing	Vogelweith et al., 2013b
Odonata	Fungal susceptibility	Decreasing	Tinsley et al., 2006
Orthoptera	Resistance to infection	Increasing	Fedorka et al., 2013
Diptera	Resistance to infection	Increasing	Corby-Harris and Promislow, 2008; Cory and Myers, 2009; Kutch et al., 2014

PO, phenoloxidase.

taken synchronously in terms of both absolute time and ontogenetic stage of the larvae. Furthermore, to address possible trade-offs of immune status, phenotypic correlations between the two studied immune traits were calculated, as well as those between the immune indices and fitness-related growth parameters.

MATERIALS AND METHODS
Study insects
The species studied formed a subsample of those used in a larger-scale project on latitudinal gradients in insect life histories. The criteria for inclusion of a particular species was a broad distribution covering both northern and southern Europe (Meister et al., 2017), sufficient abundance to allow us to obtain rearing material with reasonable effort, and the absence of known ecological differences across the European range. For the present immunological study, we had to limit ourselves to the subset of larger-bodied species which facilitated obtaining sufficient haemolymph. The three species studied – *Acronicta rumicis* (Linnaeus 1758) (Noctuidae) (hereafter AR), *Poecilocampa populi* (Linnaeus 1758) (Lasiocampidae) (hereafter PP) and *Hypomecis punctinalis* (Scopoli 1763) (Geometridae) (hereafter HP) – represent three distantly related (Mutanen et al., 2010) macrolepidopteran families and are all polyphagous (Table 2).

The extreme points of the sampled latitudinal gradient were in northern Finland (65°N) and in Georgia (Caucasus; 41°N), separated by about 2700 km. Populations from at least one of these extreme areas were compared with populations from Estonia (58°N), dependent on the rearing material available (Table 2).

The laboratory stocks were formed using offspring from field-collected females, with ≥11 founding females per species (Table 2). First generation offspring of the field-collected moths were used for AR and PP, whereas, for technical reasons, the stock of HP had been maintained in the laboratory for two generations prior to the experiment. Both during stock maintenance and the experiments, all insects were reared individually in plastic jars at 20°C, with fresh leaves of host plants being provided in 2 day intervals. Rearing experiments were conducted in 2015 at the University of Tartu, Estonia. Immunological analyses were conducted in the Molecular Ecology and Systematics Laboratory at the University of Helsinki, Finland, in January 2016.

Design of the study
The three species were subjected to an identical experimental protocol (see Table 2 for sample sizes; see Fig. S1 for procedure outline). Individuals representing different geographic populations were reared simultaneously under a common-garden design. Common-garden implies standardising environmental conditions across populations being compared, which facilitates isolation of genetic (as opposed to plastic, i.e. environmentally based) differences. Additionally, simultaneous rearing enabled us to extract haemolymph synchronously in terms of both absolute time and development stage of the larvae. The strict synchronisation of larval development (within species) was achieved by adjusting rearing temperature in a unified manner during the pre-experimental development, mainly through keeping the larvae at +4°C for 1 or 2 days before the experiment (Tammaru and Esperk, 2007; Tammaru et al., 2010) so that the larvae moulted into their final larval instar simultaneously. To minimise any possible error related to the synchronising low-temperature treatments per se, all larvae were kept at +4°C for at least 12 h before the experiment.

During the development in the final instar, the larvae representing all populations and broods (i.e. offspring of a single female) were equally divided between rearing chambers with different temperatures (16, 20 and 24°C), and host plants (Table 2). The position of larvae representing different populations and feeding on different host plants was randomised on rearing trays. Such a crossed-factor design was chosen to reveal potential among-population differences in immunological parameters (consistent differences across all experimental environments) and to separate these from possible population-specific adaptations to different temperatures or host plants (environment-specific among-population differences).

Haemolymph was extracted, after differential growth rate had been measured (see below), from the larvae during the third day of the last larval instar. Haemolymph samples were taken between 09:00 h and 17:00 h in randomised order with respect to brood, population, host plant and temperature treatment. The samples were collected by puncturing larvae (533 in total) with insulin syringes to their dorsal side above the stigma of the antepenultimate segment. Samples of haemolymph (3 µl for HP, 10 µl for AR and 20 µl for PP per larvae) were diluted in ice-cold potassium phosphate buffer (PBS: 10 µl for HP, 30 µl for AR and 60 µl for PP), which was then frozen at −80°C. After haemolymph extraction, the caterpillars in their plastic jars were returned to corresponding environmental chambers. Almost all experimental larvae survived haemolymph sampling, and most of them pupated successfully. Survival status of the overwintering pupae was checked the following spring.

Variables recorded and statistical analyses
Both immunological parameters (PO and lytic activity) were measured from the same individual-specific haemolymph sample,

Table 2. Species used and details of the experiment

Species (abbreviation)	Family	Pupal mass (mg)*	Host plants	Population (abbreviation)‡	No. of broods	No. of larvae§
Acronicta rumicis (AR)	Noctuidae	219	*Cirsium arvense*	Estonia (EST)	7	43
			Melilotus albus	Georgia (GEO)	10	149
Hypomecis punctinalis (HP)	Geometridae	155	*Betula pendula*	Estonia (EST)	4	40
			Tilia cordata	Georgia (GEO)	7	33
Poecilocampa populi (PP)	Lasiocampidae	368	*Alnus incana*	Finland (FIN)	3	41
			Betula pendula	Estonia (EST)	10	190
			Populus tremula	Czechia (CZE)	2	37

*Average pupal mass from the experiment, presented here to characterise the overall body size of the species.

‡Sampled locations, geographical coordinates and number of broods: *A. rumicis* Estonia: Karilatsi (58°7′N, 26°55′E; 7 broods), Georgia: Bakuriani (41°45′N, 43°32′E; 1 brood), Lagodekhi (41°49′N, 46°17′E; 1 brood), Marelisi (41°57′N, 43°16′E; 5 broods) and Sadgeri (41°48′N, 43°25′E; 3 broods); *H. punctinalis* Estonia: Karilatsi (58°7′N, 26°55′E; 4 broods), Georgia: Marelisi (41°57′N, 43°16′E; 4 broods) and Mtirala (41°41′N, 41°53′E; 3 broods); *P. populi* Finland: Oulu (65°1′N, 25°28′E; 3 broods), Estonia: Salinõmme (58°49′N, 22°56′E; 2 broods), Viilupi (58°51′N, 22°54′E; 2 broods), Sõrve (57°54′N, 22°3′E; 2 broods), Tähtvere (58°22′N, 26°42′E; 1 brood) and Tüki (58°22′N, 26°42′E; 3 broods), Czechia (or Czech Republic): Šumava mts (48°49′N, 13°47′E; 2 broods).

§Number of larvae that successfully pupated and were used in the analyses.

allowing us to detect possible correlations between the two indices. PO activity was measured as described in Laughton and Siva-Jothy (2011), with the following modifications. Previously fixed samples were thawed and centrifuged (9000 g) at 4°C for 10 min to obtain clear supernatant. For the PO assay, supernatant (3 µl for HP, 10 µl for AR, 15 µl for PP) was added to 200 µl of 3 mmol l^{-1} L-Dopa (L-3,4-dihydroxyphenylalanine; 333786, Sigma, St Louis, MO, USA). The activity of the enzyme was measured at 30°C, 490 nm for 90 min (1 min intervals) with a spectrophotometer (Enspire, Perkin-Elmer, Waltham, MA, USA). The slope of the absorbance curve from 10 to 60 min (10 to 40 min for AR) was used as an estimate of PO 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 (diameter 9 cm) were filled with 10 ml of sterilised 1× PBS buffer with 1000 µg ml^{-1} *Micrococcus luteus* freeze-dried and lyophilised 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 h at 30°C. Dilution series of chicken egg white lysozyme (Sigma: 2000, 1000, 750, 500, 250, 125, 620 and 310 µg ml^{-1}) were run on two separate plates to create a standard curve. To control between-plate variation, lysozyme controls of 63 and 250 µg ml^{-1} were added to each plate. Lytic activity was determined as the radius of the clear zone around

the sample indicating the equivalent lysozome concentration (µg ml^{-1}). Frequently, no clear zone was formed at all; such cases were scored as 'no lytic activity present'.

Fitness-related growth parameters of the larvae were recorded in the following way. Approximation of differential growth rate (DGR) was based on the mass increment accumulated during the second day of the final larval instar. This is considered the most adequate characteristic of the 'free' growth period in the beginning of an instar; that is, the growth that is not yet affected by physiological preparations for pupation (Ayres and MacLean, 1987; Esperk and Tammaru, 2004; Tammaru et al., 2010). From the mass data, DGR was calculated as (final mass$^{1/3}$ − initial mass$^{1/3}$)/time, where time was in days (Meister et al., 2017), with the cube root transformation having been suggested to linearise larval growth curves of lepidopteran larvae (Tammaru and Esperk, 2007). Development time within the last instar was measured by counting the number of days from the beginning of the final instar to the formation of prepupae. The pupae were weighed no earlier than 5 days after the pupal cuticulae had turned darker in coloration, when sex was also determined from the pupae based on genital scars.

To explore the among-population differences in PO and lytic activity, general linear mixed models (Littell et al., 1996) were constructed with population, temperature treatment, host plant and sex as categorical independent variables. Mass at the time of moulting into final instar was included as a continuous covariate to correct for occasional size dependence (Vogelweith et al., 2013a),

Table 3. Mixed models explaining variation in immunological parameters

Sp.	Effect	PO activity[a]					Lytic activity[a]					Incidence of lytic activity[b]			
		Nd.f.	Dd.f.	F	P	ω^2	Nd.f.	Dd.f.	F	P	ω^2	Nd.f.	Dd.f.	F	P
AR	Population	1	12	12	**0.0048**[d]	0.31[c]	1	16	2.9	0.11	0.040[c]	2	22	0.00	0.96
	Initial mass[e]	1	50	3.6	0.062	0.0069	1	44	0.62	0.44	0	1	137	4.5	0.037
	Host plant	1	43	13	**0.0007**	0.098	1	53	0.00	0.95	0	1	137	0.04	0.83
	Temperature	2	49	8.7	**0.0006**	0.099	2	52	0.21	0.81	0	2	137	0.77	0.47
	Sex	1	45	10	**0.0023**	0.025	1	55	1.6	0.21	0.0084	1	137	1.9	0.17
	Pop.×host[f]	1	44	6.9	**0.012**	0.080	1	52.3	0.08	0.78	0	1	137	4.5	0.037
	Pop.×temp.[g]	2	49	12	**<0.0001**	0.90	2	52.1	1.8	0.17	0.035	2	137	0.65	0.52
HP	Population	1	9	0.19	0.67*	0	1	6	0.68	0.44*	0	1	6	1.7	0.24
	Initial mass	1	22	0.64	0.43*	0	1	20	0.18	0.68*	0	1	22	0.15	0.70
	Host plant	1	11	0.01	0.91*	0	1	12	0.24	0.63*	0	1	22	1.1	0.30
	Temperature	2	12	0.02	0.98*	0	2	24	1.0	0.37*	0.0019	2	22	8.2	**0.0021**
	Sex	1	23	0.01	0.94*	0	1	22	0.02	0.90*	0	1	22	0.48	0.49
	Pop.×host	1	10	0.11	0.74*	0	1	13	0.04	0.84*	0	1	22	2.7	0.11
	Pop.×temp.	2	12	0.20	0.82*	0	2	15	0.80	0.47*	0	2	22	1.4	0.26
PP	Population	2	16	0.01	0.99	0	2	15	1.3	0.29	0.0044	2	12	0.77	0.48
	Initial mass	1	127	1.2	0.28	0	1	146	2.6	0.11	0	1	208	5.1	0.025
	Host plant	2	160	1.8	0.16	0.012	2	158	1.88	**0.42**[h]	0.0024	2	208	1.0	0.36
	Temperature	2	160	2.1	0.13	0.012	2	158	0.88	0.42	0	2	208	0.53	0.59
	Sex	1	160	1.3	0.26	0.0049	1	163	4.1	0.45	0.0006	1	208	3.3	0.069
	Pop.×host	4	159	1.1	0.35	0.0060	4	157	1.9	0.10	0.012	4	208	1.3	0.26
	Pop.×temp.	4	160	0.49	0.74	0	4	158	0.61	0.65	0	4	208	0.59	0.67

[a]Mixed ANOVA models with brood as a random variable.

[b]Mixed generalised linear models with logit link applied to the incidence of lytic activity as a binary trait.

[c]Effect sizes are presented as estimates of the proportions of variance accounted for by respective factors (semi-partial ω^2; some negative estimates have been replaced with zeroes; SAS PROC GLM).

[d]P-values in bold indicate effects that attained significance in reduced models (backward elimination of non-significant effects, α=0.05). Asterisks indicate logarithmically transformed data.

[e]Mass of the larvae at moulting into the last larval instar, included to account for a potential body size effect on immunological traits (Vogelweith et al., 2013a).

[f]Population×host: on *C. arvense*, PO activity values were higher than those on *M. albus* in both Estonia and Georgia (Fig. 2A); population×host: incidence of lytic activity was higher on *M. albus* than on *C. arvense* in Estonia, though the incidence of lytic activity was higher on *C. arvense* in Georgia (Fig. 2C).

[g]Population×temperature: there was an increase in PO activity values with temperature in Estonian populations, and a decrease in PO activity with increasing temperature in Georgian populations (Fig. 2B).

[h]In the reduced model, host plant had a significant effect on lytic activity in PP ($F_{2,178}$=4, P=0.028): PP on *A. incana* attained higher lytic activity than PP on *P. tremula* (post hoc t=3, d.f.=178, P=0.022).

Nd.f., numerator degrees of freedom; Dd.f., denominator degrees of freedom.

and brood was added as a random factor. The cases of zero lytic activity were omitted from this analysis, and were considered separately (see below). Models including all the above variables, as well as population×temperature and population×host plant interactions were fitted first; model simplification was performed thereafter using the backward elimination procedure (α=0.05). Some case-specific transformations of the dependent variables (Table 3) were necessary to meet the assumptions of parametric tests. A few outlier observations were excluded using the modified z-scores as advised by Iglewicz and Hoaglin (1993). The Kenward–Roger method (Littell et al., 1996) for estimating denominator degrees of freedom (Dd.f.) was used. When testing the effect of population, Dd.f. were derived from the number of broods, avoiding pseudoreplication.

Because there was a large number of larvae for which no lytic activity of haemolymph was recorded, we performed additional alternative analyses in which lytic activity was treated as a binary trait (lytic activity present or absent; 'incidence of lytic activity', hereafter). This allowed us to include data on all experimental individuals in the analysis. Generalised linear mixed models (PROC GLIMMIX; Littell et al., 1996) with logit link function but otherwise analogous to those for the continuous traits were fitted. The incidence of lytic activity was also analysed (PROC GLIMMIX) for the data of HP and PP combined, with brood

(nested within species) as a random variable. In this analysis, the incidence of lytic activity was analysed as dependent on latitude (numeric values for populations; see Table 2), host plant (nested within species), temperature and sex.

Spearman correlations were used to study the relationships between the measured immunological indices and growth parameters of the insects (differential growth rate, development time, pupal mass). Different populations were pooled in the analyses. Bivariate correlations were preferred to more complex models for the sake of clarity of presentation and interpretation, and were justified by the prevailing absence of among-population differences in the immunological indices. In the single opposite case (PO activity for AR), the analyses were also performed separately for the two populations.

In all above analyses, only the individuals that survived until pupation were considered, whereas the analyses of survival naturally also included individuals that died during the larval period. Survival of the larvae until pupation and overwintering survival of the pupae to eclosion (as binary traits) were analysed as dependent on the immunological parameters recorded. Generalised linear models (PROC GLIMMIX) with brood as a random factor were applied in these analyses.

Chi-square tests were used to investigate relationships between the incidence of lytic activity and survival. In one case, a
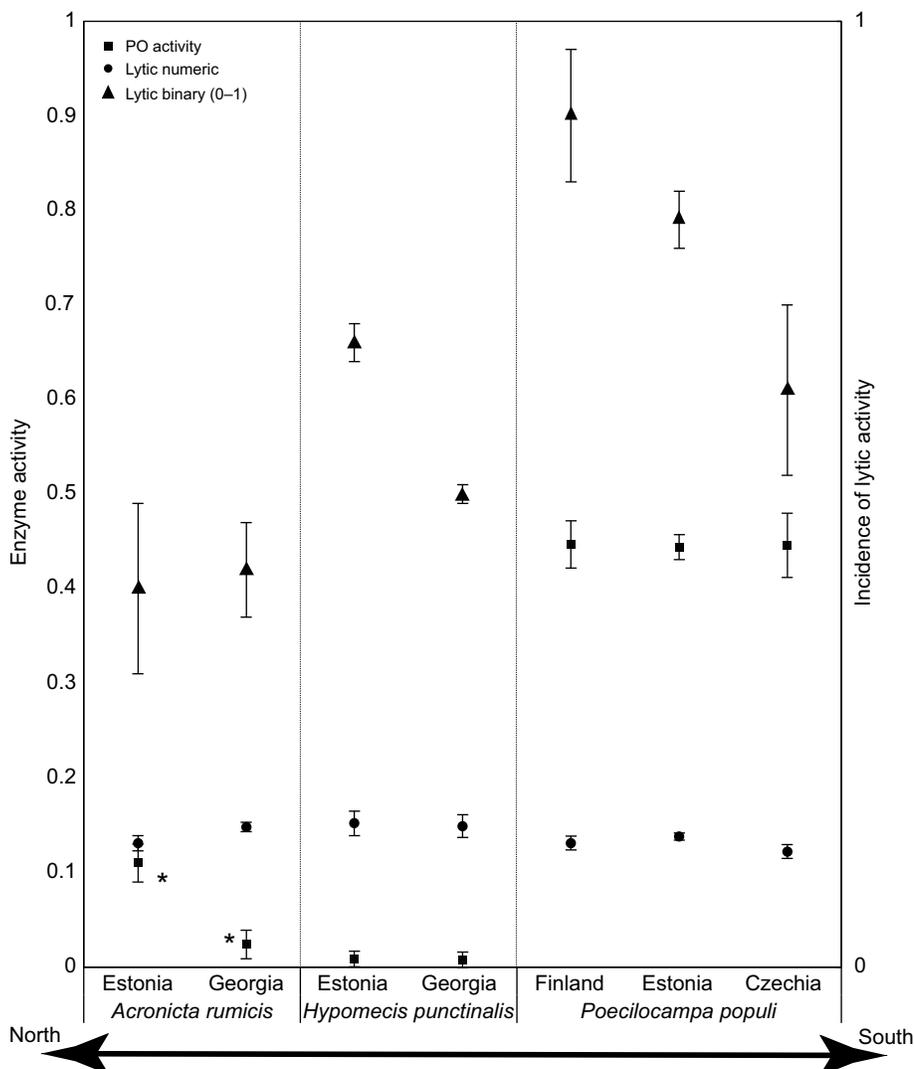

Fig. 1. Enzyme activity by species and population. Least-squares means (±s.e.m.) from the models in Table 3 are presented, with standard errors. Phenoloxidase (PO) activity (absorbance V_{max}) and lytic activity (lysozyme equivalent, µg ml^{-1}) are shown as continuous variables on the left vertical axis; the incidence of lytic activity is shown as a binary trait on the right vertical axis. *Significant pairwise comparison (post hoc for ANOVA in Table 3). PO activity numeric values were multiplied by 10 and lytic numeric values by 0.0001 for better visualisation.

log-likelihood test was used instead as the assumptions of the Chi-square test were not met.

RESULTS

Among-population differences in immunological parameters

There were no significant among-population differences in PO activity in either HP or PP (Table 3, Fig. 1). In contrast, AR showed 4.6 times higher PO activity in the northern population compared with the southern one (Table 3). Similarly, the effects of the other factors in the analysis were not significant for HP and PP, but were so for AR. In this species, PO activity increased with increasing temperature, was higher on *C. arvense* (the better host, in terms of survival) than on *M. albus*, and was higher in females than in males. For AR, there were also significant population×host plant and population×temperature interactions (for interpretations, see Table 3 and Fig. 2A,B); nevertheless, the PO activity level was higher in the northern population under all experimental conditions.

Lytic activity did not show any statistically significant differences among populations in any species investigated (Table 3). Moreover, there was no effect of host plant (with the exception of PP in the reduced model), temperature or sex on lytic activity. Similarly, the incidence of lytic activity (as a binary trait) did not show any statistically significant among-population differences in any species investigated (Table 3), though a trend of increasing towards the north (Fig. 1) could be anticipated for both HP and PP. Indeed, in the analysis of combined data for HP and PP, the effect of latitude on the incidence of lytic activity proved to be significant ($F_{1,29}=5.1$, $P=0.032$), with a higher probability of expressing lytic activity in the north. In the same analysis, host plant nested within species did not affect lytic activity ($F_{4,279}=1.2$, $P=0.33$), nor did sex ($F_{1,279}=0.6$, $P=0.43$).

Increasing temperature led to an increased incidence of lytic activity in one species out of three (HP). Neither host plant nor sex had any effect on lytic activity in any of the studied species. Nevertheless, the population×host plant interaction was statistically significant for the incidence of lytic activity in AR (Table 3, Fig. 2C), though it lost its statistical significance in the reduced model.

Correlations between immunological parameters and life-history traits

No statistically significant ($P≥0.15$; Table 4) correlations were found between the two studied immune parameters – PO and lytic activity – in any of the species.

A negative correlation between larval growth rate and lytic activity was detected in HP (Table 4). In contrast, in PP, faster-growing larvae were more likely to show lytic activity (Table 5). Pupal mass was positively correlated with lytic activity in AR (Table 4). In contrast, negative correlations between pupal mass and lytic activity were found in PP (Table 4). For development time, a negative correlation was shown for PO activity in AR (Table 4), though it lost its statistical significance when analysed separately by population (Estonia $r=-0.055$, $P=0.85$, $N=14$; Georgia $r=0.20$, $P=0.17$, $N=49$); this last analysis was performed because of a significant population effect on PO activity (Table 3). No other analyses on PO activity as a dependent variable yielded qualitatively different results when performed separately for the populations. The incidence of lytic activity was associated with shorter development times in PP (Table 5). A positive correlation was found between development time and lytic activity in HP (Table 4).

Survival

The level of PO activity was associated with larval survival in one species out of three: in PP, survival to pupation increased with increasing PO activity (Table 6). Survival to pupation was more likely in the case of higher lytic activity: in AR, survival rate was higher when lytic activity increased (Table 6); in HP, successful pupation of larvae was more probable with incidence of lytic activity (Table 7).

Survival of pupae to adult emergence was not affected by either PO or lytic activity in any of the species studied ($P≥0.10$, Tables 6 and 7).

DISCUSSION

The primary aim of the present study was to test for latitudinal differences in the immune function of larvae of three moth species, focusing on the consistency of the possible differences across environmental conditions. Based on our results, immunological
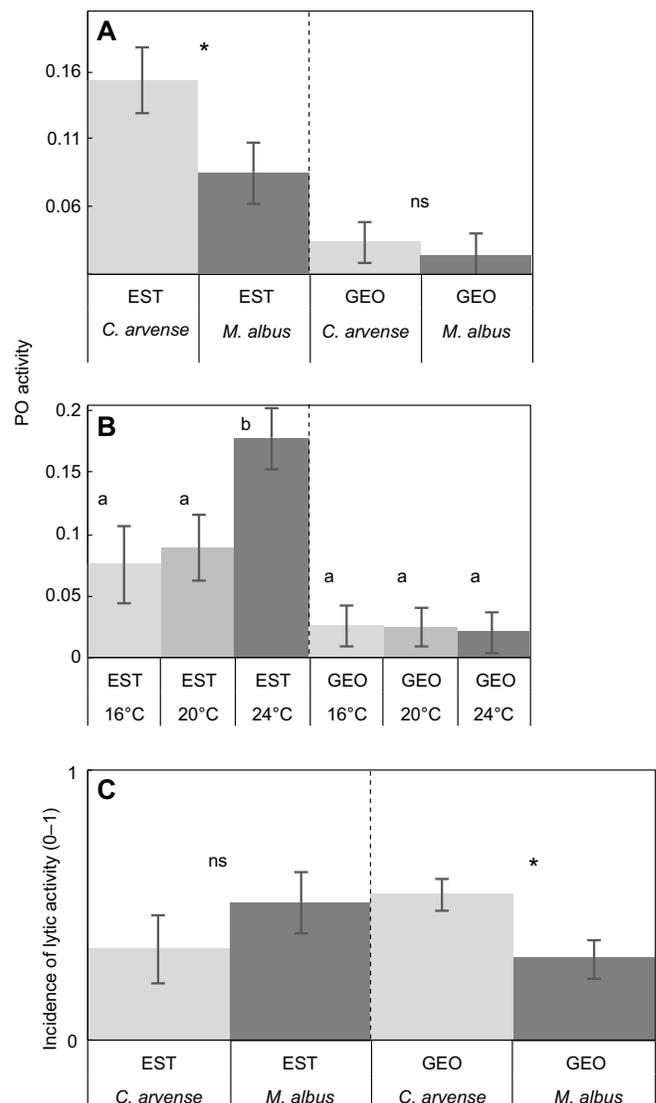

Fig. 2. Immune indices of *Acronicta rumicis* (AR) by population and treatment. (A) PO activity of AR on host plants *Cirsium arvense* and *Melilotus albus* in Estonia (EST) and Georgia (GEO). (B) PO activity of AR in Estonia and Georgia at different temperatures. (C) Lytic activity of AR on host plants *C. arvense* and *M. albus* in Estonia (EST) and Georgia (GEO). Least-squares means (±s.e.m.) from the ANOVA in Table 3 are presented to visualise the interactions. *Significant (marginally) pairwise comparisons (*post hoc* for ANOVA in Table 3); different letters indicate a significant difference; ns indicates non-significant comparisons.

Table 4. Spearman correlations between immunological parameters and indices of growth performance

Sp.	Effect	PO activity			Lytic activity		
		r	P	N	r	P	N
AR	PO activity	–	–	–	–0.0040	0.15	65
	Lytic activity	–0.0040	0.15	65	–	–	–
	Growth rate	0.0053	0.97	64	–0.21	0.10	64
	Development time	–0.27	0.032*	63	0.16	0.21	63
	Pupal mass	–0.22	0.074	65	0.26	0.036	65
HP	PO activity	–	–	–	–0.00034	0.74	37
	Lytic activity	–0.00034	0.74	37	–	–	–
	Growth rate	0.077	0.65	37	–0.45	0.0048	37
	Development time	–0.080	0.65	36	0.42	0.012	36
	Pupal mass	0.22	0.20	36	0.26	0.13	36
PP	PO activity	–	–	–	–0.053	0.51	216
	Lytic activity	–0.053	0.51	216	–	–	–
	Growth rate	0.13	0.092	181	–0.085	0.26	181
	Development time	–0.15	0.053	177	0.11	0.14	177
	Pupal mass	–0.14	0.055	180	–0.15	0.043	180

*This effect was lost when analysed separately by populations (see Results, Correlations between immunological parameters and life-history traits).

traits showed no consistent cross-species latitudinal trends; however, there was some indication of higher immunity at higher latitudes. Immunological parameters showed species-specific relationships with indices of growth performance, which were also dependent on experimental conditions (temperature, host plant). The overall pattern of limited consistency across populations, immune parameters and environmental conditions was despite the highly unified setting used in our common-garden experiments, which should have minimised any environmental noise and yielded comparable results across populations and species. The values of the two immunological indices used were not correlated with each other.

Overall, the values of PO activity were extremely variable: this index showed up to 60-fold differences among the three species (Fig. 1). For one species, the noctuid AR, the values were also variable within the species. In particular, PO activity in AR was the only case in which a clear latitudinal trend was detected: the moths from Estonia had considerably higher values of PO activity than their Georgian (southern) conspecifics. Similar to our work, some previous studies using a damselfly and a ground cricket have found higher PO activity in higher latitude populations (De Block et al., 2008; Fedorka et al., 2013). Conversely, increased PO activity in

Table 5. Generalised linear mixed models for incidence of lytic activity as dependent on PO activity and indices of growth performance

Sp.	Effect	Incidence of lytic activity				Direction of relationship*
		Nd.f.	Dd.f.	F	P	
AR	PO activity	1	145	0.87	0.35	Negative
	Growth rate	1	144	0.51	0.47	Negative
	Development time	1	143	0.44	0.51	Positive
	Pupal mass	1	145	1.77	0.19	Negative
HP	PO activity	1	60	0.06	0.80	Positive
	Growth rate	1	62	2.1	0.15	Positive
	Development time	1	61	2.4	0.13	Negative
	Pupal mass	1	61	3.9	0.050	Negative
PP	PO activity	1	223	3.5	0.064	Positive
	Growth rate	1	223	8.4	0.0042	Positive
	Development time	1	217	6.2	0.014	Negative
	Pupal mass	1	222	0.21	0.65	Positive

*Positive refers to a higher probability of expressing lytic activity at higher values of the independent variable.

the south has been reported in the European grapevine moth (Vogelweith et al., 2013b).

Environmental factors also affected the PO levels in AR: PO activity increased at higher temperatures, and on the better host plant (in terms of survival), *C. arvense*. Females also displayed higher values than males. Somewhat surprisingly, in the other two species, the host plant did not affect the immune parameters. The absence of host plant effect (for opposite examples, see Lampert, 2012; Vogelweith et al., 2011; Muller et al., 2015) may be related to our focus on constitutive immunity: it remains possible that host plant-related differences would have been observed in the case of an induced immune response or actual infection.

Lytic activity had a bimodal distribution in all of the species, and in all their geographical populations: about 40% of the haemolymph samples failed to show any lytic activity at all. When analysed separately, the averages of the positive (=non-zero) values of lytic activity were notably similar across species (Fig. 1), which clearly differs from the situation with PO. Moreover, the rather limited within-population differences in the positive values of lytic activity could not be ascribed to any of the potential predictors (population, host plant, temperature, sex). In contrast to the positive values for lytic activity, the incidence of lytic activity (as a binary trait) showed clear differences among the species. There was also an indication of a latitudinal trend in two of the species (HP and PP): the probability of expressing lytic activity was higher in the northern populations.

Some other predictors occasionally had a species-specific effect on lytic activity as well, though the differences remained much more moderate than those for PO activity. Indeed, lytic activity has previously been shown to vary less than PO activity, with the relative constancy having been primarily attributed to limited phenotypic plasticity in this trait (Triggs and Knell, 2012; McNamara et al., 2013a). Consistent with our observations on PP (but not the other two species), host plant has been shown to affect antimicrobial activity in a moth (*Lobesia botrana*; Vogelweith et al., 2013b) and lytic activity has been found to increase with temperature in *Drosophila* (Lazzaro et al., 2008; Fedorka et al., 2016). We are unaware of any studies showing among-population variation in lytic activity in insects, although a lack of variation has been reported for a crustacean (*Daphnia magna*; Mucklow et al., 2004). Vogelweiht et al. (2013b) found that in warmer geographical regions, the haemolymph of the moth *L. botrana* has a higher ability to inhibit bacterial growth, which, however, differs from lytic

Table 6. GLIMMIX results for survival dependent on the values of immune parameters

Sp.	Effect	Alive/total	Survival to pupation				Direction of relationship	Alive/total	Survival to eclosion				Direction of relationship
			Nd.f.	Dd.f.	F	P			Nd.f.	Dd.f.	F	P	
AR	PO activity	147/192	1	191	0.01	0.92	Positive	9/147	1	143	1.1	0.31	Positive
	Lytic activity		1	86	11	0.0014	Positive		1	61	0.19	0.66	Negative
HP	PO activity	60/73	1	111	0.22	0.64	Negative	51/60	1	62	0.21	0.65	Positive
	Lytic activity		1	52	0.76	0.39	Negative		1	35	0.24	0.63	Positive
PP	PO activity	225/268	1	272	4.4	0.037	Positive	108/225	1	227	0.89	0.35	Negative
	Lytic activity		1	214	0.97	0.33	Positive		1	179	2.7	0.10	Positive

activity in its mode of action (Galdiero et al., 2015). Therefore, we may be the first to document a higher probability of lytic activity in higher latitude populations, even if such a trend could only be shown in an analysis combining two species.

A large share of samples with no lytic activity at all appears noteworthy. Similar to our results, the occasional absence of the clear zone around haemolymph samples has been shown before in a tiger moth (Nokelainen et al., 2013) and in mealworm beetles (Dubuffet et al., 2015). As the most parsimonious explanation for such a qualitative among-individual variation, we propose that lytic activity can be readily downregulated to levels that are beyond detectability limits. Such a high among-individual variability even in highly standardised conditions implies that the expression of lytic activity must be sensitive to subtle variations in the environment, or in individual condition. In turn, the frequently made 'choice' to downregulate lytic activity when not 'needed' is in good agreement with the general idea about the costs associated with immune defences (Schmid-Hempel, 2011).

Costs of high immunocompetence may be expressed as trade-offs between different immunological traits. For lytic activity, Rao et al. (2010) suggested a trade-off with proPO activation in the haemolymph of *Manduca sexta*. ProPO is an inactive form of PO present in the haemolymph and must be activated by specific enzymes (Cerenius and Söderhäll, 2004). At the same time, the activity of PO itself was found not to be affected by lysozyme (Rao et al., 2010). Our results support this view, as we – given our focus on constitutive immunity – also failed to detect any correlations between the values of the two immunological parameters studied. Nevertheless, a positive correlation between PO activity and lytic activity has been found in a cricket (Fedorka et al., 2013) and a noctuid moth (Freitak et al., 2009). It is entirely possible, however, that such a correlation could have been detected if higher lytic activity had been induced by bacterial insult (Freitak et al., 2007).

The reason for some inconsistency in the results may also lie in the fact that lytic activity does not reflect the activity of a single

enzyme. Indeed, the method used for measuring lytic activity does not differentiate between lysozyme and other small peptides able to degrade bacterial cell walls (Rolff and Reynolds, 2009). Different components of the 'cocktail' consisting of various antimicrobial peptides may be differently involved in respective trade-offs. The possibility thus remains that different species exploit slightly different antibacterial warfare for immune defence.

The costs of high immunocompetence may alternatively be expressed as trade-offs between immunological and life-history traits. In insects, adult size is typically a strong predictor of individual fitness: especially in females, reproductive output is often proportional to adult or pupal mass (Tammaru et al., 1996, 2002; Honěk, 1993). Typically, low larval growth rates induced by suboptimal food quality translate into low adult mass and long development periods (Teder et al., 2014). Consequently, high larval growth rates, short development periods and high pupal mass can all be used as correlates of individual fitness. In our data, these indices showed a rather irregular pattern of correlations with immunological indices. For PO activity, there were mostly no relationships at all, though for lytic activity, higher values were still more frequently associated with growth parameters indicating lower growth performance. In contrast, higher values of lytic activity mostly led to higher survival to pupation. Such a pattern was most clear for AR (Table 6) and HP (Table 7), suggesting that the levels of lytic activity may mediate a trade-off between growth performance and resistance to pathogens. Indeed, immunocompetence – the ability or probability to resist and control pathogens and parasites (Koskimäki et al., 2004) – has been shown to correlate with the activity of immunity-related enzymes in the haemolymph (Mucklow et al., 2004). In our case, higher immunocompetence (measured by survival rate) may have come at the cost of slower development (Janssens and Stoks, 2014) and reduced body size (Klemola et al., 2007; McNamara et al., 2013a,b). However, the causal relationship between increased pathogen resistance and the measures of intrinsic immunity needs to be investigated further.

Our evidence indicates that there is no easy way to explain among-population differences in immunological traits by the latitude of origin of the insects. Nor could the differences be explained – in a consistent way – by trade-offs between different immunological traits, or by trade-offs with size-related life-history traits. The reasons for such idiosyncratic patterns (this study, and also Table 1) remain unclear. Nevertheless, we note that such species-specific latitudinal differences are consistent with the frequently proposed crucial role of insect parasitoids as selective agents on immunological traits. Indeed, among-population differences in immunological indices have repeatedly been suggested to reflect adaptations to different rates of parasitism (Tinsley et al., 2006; Seiter and Kingsolver, 2013; Vogelweith et al., 2013b). If a major selective role of insect parasitoids was indeed the case, limited cross-species consistency in latitudinal trends of immunological traits should be the expected result. This is because

Table 7. Associations between the incidence of lytic activity and survival

Sp	Effect	Incidence of lytic activity				Direction of relationship‡
		Test	d.f.	χ^2	P	
AR	Survival to pupation	Chi-square	1	0.72	0.39	Negative
	Survival to eclosion	Log-likelihood*	1	0.64	0.42	Negative
HP	Survival to pupation	Chi-square	1	6.7	0.0098	Positive
	Survival to eclosion	Chi-square	1	1.8	0.18	Positive
PP	Survival to pupation	Chi-square	1	0.59	0.44	Positive
	Survival to eclosion	Chi-square	1	0.64	0.42	Positive

*Log-likelihood analysis was applied because the assumptions of the Chi-square test were violated.

‡Positive refers to a higher probability of expressing lytic activity at higher values of the independent variable.

lepidopteran larvae are attacked by highly species-specific assemblages of parasitoids (Waage and Greathead, 1989; Quicke, 1997). Different (groups of) parasitoids necessarily differ in their ecological requirements and geographic distributions, which should lead to unique species-specific patterns in parasitism rates, and adaptive responses to these.

In turn, plastic responses of immunological traits to temperature were weak and inconsistent. This may suggest that whenever such a plastic effect of temperature is detected, it is more likely to be an adaptive response (based on anticipatory plasticity) rather than a purely physiological phenomenon (responsive plasticity; see Whitman and Agrawal, 2009, Esperk et al., 2013, for a general discussion of this distinction). Indeed, we should perhaps expect more cross-species similarity in physiological phenomena, while ecologically based adaptive responses may well differ. As a possible example of anticipatory plasticity, higher temperature could potentially serve as a cue of calendar date (Tauber et al., 1986), theoretically predictive of the abundance of natural enemies in the environment. The insects could then adjust their level of immune defence accordingly. We see no reason why responses to host plant-related traits could not be explained in the same way.

As a methodological summary, the results of the present study strongly caution against relying on a single species and a single immunological marker when one aims at detecting general trends in geographic differences in immune function. Similarly, regarding trade-offs, general conclusions can only be reached when numerous species and/or infection assays with relevant pathogens are involved in studies under highly standardised experimental conditions.

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Supplement Figure S1. Experimental procedures